Protein and Phosphatidylinositol Kinases in Yeast Protein Sorting

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Dedication

This thesis is dedicated to the memory of my mother, Phyllis V. Stack, whose constant encouragement and support made this work possible. A day doesn't go by that I don't miss her.

This thesis is also dedicated to the memory of my grandmother, Ruth P. Fraser. Her love of life and courage will always be an inspiration to me.

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Abstract

The yeast *vps* mutants are defective in the delivery of proteins to the vacuole. The products of the *VPS15* and *VPS34* genes encode homologs of a serine/threonine protein kinase and a phosphatidylinositol 3-kinase (PtdIns 3-kinase), respectively, that are required for the sorting of soluble vacuolar proteins. Mutations altering highly conserved residues in the catalytic domain of either protein result in the missorting and secretion of vacuolar hydrolases such as carboxypeptidase Y, suggesting that protein and lipid phosphorylation reactions are required for the vesicular transport of vacuolar proteins. Biochemical characterization of Vps34p has shown that, in addition to possessing PtdIns 3-kinase activity, Vps34p undergoes an autophosphorylation reaction, indicating that it is a novel multiple specificity kinase able to phosphorylate both lipid and protein substrates.

The Vps15 protein kinase both functionally and physically interacts with the Vps34 PtdIns 3-kinase and the two proteins form a complex associated with the cytoplasmic face of an intracellular membrane fraction most likely corresponding to a late Golgi compartment. In addition to recruiting Vps34p to the membrane site of its phospholipid substrate, we have found that Vps15p is also required for the activation of Vps34p as Vps34p PtdIns 3-kinase activity is extremely defective in *vps15* mutant strains. Vps15p protein kinase activity appears to be responsible for the association with and subsequent activation of Vps34p because *vps15* kinase domain mutations result in defects in PtdIns 3-kinase activity and the mutant Vps15 proteins are unable to associate with Vps34p. Together,

these results have demonstrated that a functional and stable complex between Vps15p and Vps34p is absolutely required for vacuolar protein sorting.

Use of a temperature-conditional allele of *VPS34* that is *ts* for both protein sorting and PtdIns 3-kinase activity has allowed us to demonstrate the direct involvement of PtdIns 3-kinase in vacuolar protein sorting. Our findings with Vps34p suggest that the functions of mammalian phosphoinositide 3-kinase may include the regulation of membrane trafficking and have led us to propose that PtdIns(3)P is involved in regulating intracellular protein sorting reactions in all eukaryotic cells.

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Contributions to manuscripts of which I am not the first author:

Chapter 2. I was responsible for the *in vivo* phosphorylation analysis of both wild-type and mutant Vps15 proteins.

Chapter 3. I was involved with almost all aspects of the analyses of the Vps15p mutants, especially the phosphorylation experiments, and contributed to interpretation of most experiments. The manuscripts presented in Chapters 2 and 3 were the result of a collaboration between me and another graduate student, Paul Herman, on a project initiated by Paul.

Chapter 4. I had a significant contribution to the data presented in this paper, in addition to a major role in writing the manuscript. Essentially, I was responsible for the fractionation experiments of Vps34p, generation of fractionated yeast extracts for the PI kinase assays, performed the *in vivo* labeling of wild-type and Δ*vps34* strains with [^{3}H]-inositol, and did a complete rewrite of the manuscript. I was also involved in all aspects of the intellectual development of the project.

Chapter 1:

Introduction

Eukaryotic cells are characterized by the presence of numerous functionally distinct membrane-enclosed organelles. The functional identity of intracellular organelles is defined in large part by the unique set of proteins residing within them. Therefore, the accurate and efficient delivery of proteins to a given organelle is of fundamental importance in establishing and maintaining their structure and function. The secretory pathway is utilized in eukaryotic cells to sort and transport proteins from their cytoplasmic site of synthesis to destinations ranging from intracellular organelles to secretion from the cell. Therefore, mechanisms must exist within the secretory pathway that recognize and divert proteins destined for other organelles in addition to delivering proteins to the cell surface and retaining resident endoplasmic reticulum (ER) and Golgi proteins. The lysosome/vacuole represents one of the major diversions of protein and membrane flow in the secretory pathway. While extensive study of the transport of proteins to the mammalian lysosome in both normal and mutant cell types has defined the predominant mechanism of lysosomal protein recognition, very little is known about the molecules and biochemical mechanisms regulating this pathway. Genetic selections in *Saccharomyces cerevisiae* have resulted in the isolation of a large number of genes required for the delivery of proteins to the lysosome-like vacuole. Analysis of the products of these *VPS* (for vacuolar protein sorting) genes has revealed numerous molecules whose biochemical activities have suggested that they may represent factors regulating vacuolar protein localization. Some of these proteins have homologs which are known to act in membrane trafficking reactions: *VPS10* encodes

a transmembrane receptor for the vacuolar hydrolase carboxypeptidase Y; *VPS21* and *VPS1* encode GTP-binding proteins of the rab and dynamin families, respectively; *VPS6* and *VPS45* encode homologs of proteins involved in synaptic vesicle targeting/fusion. These analyses have also uncovered unexpected, novel molecules which define roles for these activities in regulating intracellular membrane traffic: *VPS15* encodes a serine/threonine protein kinase which is to date the only characterized protein kinase known to be involved in a protein sorting pathway; *VPS34* encodes a phosphatidylinositol 3-kinase which suggests a role for phospholipid phosphorylation in membrane trafficking. These studies have also characterized genetic and biochemical interactions between several of these components which has indicated regulatory relationships involved in vacuolar protein localization. This thesis describes characterization of the biochemical activities of the Vps15 protein kinase and the Vps34 PI 3-kinase and the regulatory interactions between the two proteins which demonstrate that a complex of the Vps15 protein kinase and the Vps34 PI 3-kinase is required for the efficient delivery of soluble proteins to the yeast vacuole. These analyses establish that protein and lipid phosphorylation events are involved in the regulation of membrane trafficking to the vacuole and suggest that these activities may act at multiple points in the cell to regulate membrane flow in all eukaryotic cells.

1. Lysosomal Protein Sorting in Mammalian Cells

Soluble and membrane lysosomal proteins are synthesized on ER-bound ribosomes and translocated into the lumen of the ER due to the presence of an N-terminal hydrophobic signal sequence. Once in the ER, the signal sequence is cleaved off and carbohydrate modifications are added. Lysosomal proteins are then delivered via transport vesicles to the Golgi complex where they undergo further post-translational modifications before being sorted in a late Golgi compartment away from proteins to be secreted (Kornfeld and Mellman, 1989). Soluble (lumenal) enzymes are delivered to the lysosome due to the acquisition of a specific carbohydrate moiety, mannose-6-phosphate, which is recognized by specific transmembrane receptors (Sly and Fischer, 1982). Ligand-receptor complexes are transported by clathrin-coated vesicles to an acidic endosomal compartment where ligand dissociates from receptor. The soluble ligand continues on to the lysosome while the mannose-6-phosphate receptor recycles back to the Golgi for additional rounds of transport or is delivered to the cell surface, where it functions to internalize extracellular lysosomal enzymes (Kornfeld and Mellman, 1989). A variety of signals utilized for sorting of mannose-6-phosphate receptors (MPR) from the Golgi and plasma membrane and for the sorting of lysosomal membrane proteins have been identified and components which interact with these signals have been characterized.

A. Mannose-6-Phosphate Receptors

The importance of the mannose-6-phosphate modification of soluble lysosomal proteins is illustrated by the human disorder, I-cell disease (ICD). ICD eliminates the phosphotransferase activity responsible for mannose-6-phosphate modification and results in a cellular deficiency of lysosomal enzymes due to their secretion from the cell (Kornfeld and Mellman, 1989). The mannose-6-phosphate modification of soluble lysosomal proteins is due to the combined actions of a phosphotransferase and a glucosaminidase. The recognition of lysosomal proteins by the phosphotransferase provides the first level of regulation of the sorting process. The signal recognized by phosphotransferase appears to be a conformational feature created by two non-contiguous sequences in lysosomal proteins that are close in the apparent final folded structure (Baranski *et al.*, 1992). Lysosomal proteins containing phosphomannosyl residues bind to mannose-6-phosphate receptors in a late Golgi compartment. Ligand-receptor complexes are concentrated in coated pits and transported to an endosomal compartment by clathrin-coated vesicles (Kornfeld, 1992). The endosome represents the intersection of the endocytic and biosynthetic pathways from which endocytosed and lysosomal proteins traffic to the lysosome.

There are two mannose-6-phosphate receptors in mammalian cells. Both are type I integral membrane proteins and the cytoplasmic tail of each contains signals for delivery from the Golgi to the endosome and for endocytic internalization from the cell surface. MPR300 has an apparent molecular weight mass of 275-300 kD and consists of a lumenal domain

containing 15 contiguous repeats of 147 resides and a 163 amino acid cytoplasmic tail. MPR46 has an apparent molecular weight of 46,000 and consists of a 159 amino acid lumenal domain which is similar to a single repeating unit of the MPR300 lumenal domain, and a 67 residue cytoplasmic tail (Kornfeld, 1992). MPR300 is also referred to as the cation-independent MPR (CI-MPR) and MPR46 as cation-dependent MPR (CD-MPR) due to their requirement for ligand-binding. MPR300 is thought to be the major carrier of lysosomal enzymes because tumor cell lines lacking it secrete ~70% of newly synthesized lysosomal enzymes and overexpression of MPR46 in these cells results in only a modest decrease in secretion of newly synthesized lysosomal enzymes (Kornfeld and Mellman, 1989). The observation that overproduction of MPR46 in cells containing wild-type MPR300 induces the secretion of 50% of newly synthesized lysosomal enzymes led to the suggestion that the two receptors may release ligand at different points in the endosomal system. It has been proposed that MPR46 may release ligand in a proximal endosomal compartment from which ligand is able to traffic to the cell surface or to the lysosome and MPR300 may release ligand in a more distal endosomal compartment whose only destination is the lysosome (Chao *et al.*, 1990). The different requirements for ligand binding (cation, oligomeric structure, etc.) of the two mannose-6-phosphate receptors may suggest a biochemical basis for this hypothesis. While it is clear that MPR300 is capable of delivering lysosomal enzymes to the lysosome with high efficiency, a recent study in which the gene for MPR46 was deleted points to a role for MPR46 in the sorting of lysosomal enzymes. Cell lines derived from mice homozygous for a disruption of the gene encoding

MPR46 secreted a significant fraction of newly synthesized lysosomal enzymes (Koster *et al.*, 1993; Ludwig *et al.*, 1993). These data indicate that MPR46 performs an essential function in lysosomal protein binding which cannot be completely compensated for by MPR300. It has been demonstrated that MPR will bind ligand at neutral pH and ligand dissociates from receptor at acidic pH (Dahms *et al.*, 1989). The fact that the pH of the Golgi is near-neutral and endosomal compartments are acidic, combined with immunochemical localization of MPR300 in acidic endosomes (Kornfeld, 1992), supports the notion that MPRs bind lysosomal enzymes in the Golgi and discharge them in the acidic endosomes. Ligand-receptor complexes formed in the Golgi are concentrated in clathrin-coated pits and exit the Golgi in coated vesicles that transport ligand-receptor complexes to the endosome (Kornfeld and Mellman, 1989). The interaction between clathrin coat proteins and receptor-ligand complexes is mediated by the cytoplasmic tail of MPRs and adaptin proteins which link clathrin to the receptor tail. The signals for delivery to the endosome have been mapped for both mannose-6-phosphate receptors to their cytoplasmic tails. Deletion and mutagenesis analyses have identified a short sequence at the C-terminus of MPR46 consisting of His-Leu-Leu which is required for the sorting of cathepsin D to the lysosome (Johnson and Kornfeld, 1992b). Mutant receptors are able to bind ligand and recycle from the cell surface with wild-type kinetics indicating that the sorting defect occurs in exiting the Golgi. Similar studies with MPR300 have indicated that it may contain two independent signals for enzyme sorting in the Golgi (Johnson and Kornfeld, 1992a). The first is remarkably similar to the sorting signal in MPR46; deletion of

the C-terminal Leu-Leu-His-Val sequence partially impaired MPR300-mediated sorting. Combining mutations corresponding to the C-terminal motif with those in a tyr-containing motif in the cytoplasmic tail led to a complete loss of sorting function. The tyr-containing signal appears to function as a plasma membrane internalization signal also. Di-leucine and tyr-containing motifs in the cytoplasmic tails of the T-cell receptor γ and δ chains have also been found to direct chimeric T-cell receptors to the lysosome without first appearing at the cell surface (Letourneur and Klausner, 1992). The molecular basis for interaction between clathrin coat proteins and receptor-ligand complexes is due to the interaction of adaptor complexes with the cytoplasmic tail of MPRs. It has been shown that Golgi-specific adaptins (AP I) will associate with a fusion protein containing the MPR300 cytoplasmic tail (Glickman *et al.*, 1989). Recycling of mannose-6-phosphate receptors from the endosome back to the trans Golgi network (TGN) does not appear to require clathrin. Draper et al. found that antibodies directed against clathrin which disrupt clathrin assembly will inhibit endocytosis from the plasma membrane but not recycling of MPR300 to the TGN (Draper *et al.*, 1990). Interpretation of this result is complicated by a recent report that recycling to the TGN is inhibited by antibodies raised against a region of the cytoplasmic tail of MPR46 containing tyrosine residues which are implicated in internalization of the receptor from the cell surface (Schulze-Garg *et al.*, 1993). Endocytic internalization of MPRs is mediated by a clathrin-dependent process whereby plasma membrane-specific adaptin proteins link clathrin to MPR cytoplasmic tails by binding to tyr-containing motifs (Kornfeld, 1992). It is unclear whether the tyr internalization signal of

MPR46 acts in a clathrin-dependent manner in both internalization from the plasma membrane and recycling to the TGN or in a clathrin-dependent manner at the plasma membrane and a clathrin-independent manner in recycling. The possible multifunctional nature of this signal will require further investigation.

MPR300 has been reported to be phosphorylated on two serines in the cytoplasmic tail by a casein kinase activity associated with the AP I adaptor complex. One of the phosphorylated serines is found very near the carboxy terminal Leu-Leu-His-Val signal involved in sorting of MPR300 from the Golgi to the endosome (Chen *et al.*, 1993; Meresse *et al.*, 1990). This raises the possibility that phosphorylation of the cytoplasmic tail of MPR300 upon adaptor complex binding may stabilize receptor-adaptor complexes. Indeed, it was found that the binding of AP I adaptors to membranes requires a phosphorylated cytoplasmic domain of MPR300 (Le Borgne *et al.*, 1993). Meresse and Hoflack have further found that phosphorylation of the MPR300 cytoplasmic tail on these two serines is closely coupled with exit of the receptor from the TGN but not with internalization from the cell surface (Meresse and Hoflack, 1993). These results suggest an intriguing correlation between phosphorylation and sorting of MPR300 and the consequence of mutagenesis of these residues will be of interest. MPR46 also contains a serine in the cytoplasmic tail which is in the context of a consensus casein kinase II phosphorylation site. Similar to MPR300, this serine is very near the carboxy terminal His-Leu-Leu Golgi sorting signal and has been shown to be phosphorylated *in vivo* (Hemer *et al.*, 1993). However, site-directed mutagenesis of this serine showed that it had no effect on sorting or internalization of MPR46

(Hemer *et al.*, 1993; Johnson and Kornfeld, 1992b). The recent development of an *in vitro* assay for the binding of the AP I adaptor complex to Golgi membranes should allow the dissection of the biochemical requirements for clathrin coat assembly and possibly for clathrin coated pit and vesicle formation. The initial description of this assay has found a requirement for GTP, cytosol, and the small GTP-binding protein ADP-ribosylation factor (ARF) (Stamnes and Rothman, 1993; Traub *et al.*, 1993).

As mentioned earlier, both MPR46 and MPR300 cycle between the plasma membrane, Golgi complex and endosome. The function of MPR300 at the cell surface appears to be in the internalization of secreted mannose-6-phosphate-containing lysosomal proteins. The inability of MPR46 to internalize ligand at the cell surface reflects its poor binding of extracellular ligand rather than its failure to cycle to the plasma membrane (Kornfeld and Mellman, 1989). In a manner similar to exit from the Golgi, endocytosis of MPR300 and MPR46 occurs by a clathrin-mediated process. MPRs are internalized at clathrin-coated pits and transported to the endosome in clathrin-coated transport vesicles. The signals for internalization of MPR46 and MPR300 consist mainly of tyr-containing motifs in their cytoplasmic tails. While the cytoplasmic tail of MPR300 will bind to both Golgi (AP I) and plasma membrane-specific (AP II) adaptins, mutation of a critical tyr in the cytoplasmic tail eliminates AP I binding without affecting AP II binding (Glickman *et al.*, 1989). Tyr-containing motifs are common in endocytosed plasma membrane proteins and consist of short motifs (4-6 amino acids) that appear to have the ability to fold into a tight turn independent of adjacent residues

(Collawn *et al.*, 1990). Indeed, it has been demonstrated that the tyr-containing internalization signal from MPR300 will substitute for the tyr-containing internalization signal in the transferrin receptor and vice versa (Collawn *et al.*, 1991). Collectively, the work on Golgi sorting and plasma membrane internalization signals indicate that plasma membrane adaptins associate with tyr-containing motifs in the MPRs to facilitate clathrin-dependent endocytosis while Golgi adaptins associate with other determinants in the cytoplasmic tail to affect transport to the Golgi also in a clathrin-dependent process.

B. Mannose-6-Phosphate Independent Sorting

In I-cell disease (ICD), the phosphotransferase activity responsible for the mannose-6-phosphate modification is eliminated and lysosomal enzymes are missorted and secreted (Kornfeld and Mellman, 1989). Some cell types in I-cell patients exhibit normal levels of cellular lysosomal enzymes, suggesting the existence of alternative mechanisms for targeting lysosomal enzymes. Cathepsin D is a soluble lysosomal enzyme normally delivered to the lysosome by the mannose-6-phosphate receptor system. In lymphoblasts derived from an I-cell disease patient, >50% of newly synthesized cathepsin D was retained intracellularly and had undergone proteolytic cleavages indicative of arrival in the lysosome (Glickman and Kornfeld, 1993). Percoll density fractionation demonstrated that 80% of intracellular cathepsin D was in dense lysosomes and biochemical analyses showed that all cellular cathepsin D in ICD lymphoblasts lacked the mannose-6-phosphate moiety. Indeed, a mutant form of cathepsin D lacking both its N-linked glycosylation sites was transported to the

lysosome in ICD lymphoblasts in a manner nearly identical to wild-type cathepsin D in the same cells. These data indicate that the sorting of cathepsin D to the lysosome in ICD lymphoblasts occurs by a pathway independent of mannose-6-phosphate or carbohydrate recognition. Glickman and Kornfeld were able to identify an ~80 amino acid domain in cathepsin D required for mannose-6-phosphate independent sorting in ICD lymphoblasts (Glickman and Kornfeld, 1993). This MPR-independent pathway is not restricted to cathepsin D as the lysosomal hydrolase a-L-frucosidase is retained inside the cell in ICD lymphoblasts in the absence of mannose-6-phosphate modification. The efficiency of cathepsin D sorting in ICD lymphoblasts decreases by ~15% if cathepsin D is overproduced, suggesting a saturable delivery system, possibly including a receptor which recognizes a peptide determinant in lysosomal enzymes. Characterization of this mannose-6-phosphate independent sorting system will be of great interest and may reveal the existence of sorting receptors which recognize peptide determinants and are analogous to the yeast Vps10p CPY sorting receptor (see Vacuolar Protein Sorting is a Receptor-Mediated Process).

C. Lysosomal Membrane Proteins

The delivery of membrane proteins to the lysosome appears to be independent of the mannose-6-phosphate recognition system. Lysosomal membrane proteins do not contain mannose-6-phosphate modification and are found at normal levels in the lysosomes of cells from ICD patients. Lysosomal membrane glycoproteins (lgp) share common structural features: they contain a single transmembrane domain, a short (10-11 residues) cytoplasmic tail and the lumenal domain contains a large

number of N-linked carbohydrates (Fukuda, 1991). The cytoplasmic tail of all lgps characterized thus far contain a conserved motif consisting of Gly-Tyr. The presence of tyr-containing motifs in many plasma membrane proteins, which may function as a signal for endocytosis, suggested the possibility that lysosomal membrane proteins may be transported initially from the Golgi to the cell surface and then internalized to the lysosome through endocytosis. This does not appear to be the case for lgp120 (known as lamp I in human cells). Both kinetic and surface labeling techniques support the view that the great majority of lgp120 is transported directly to the lysosome without appearing at the cell surface (Harter and Mellman, 1992). Significant levels of lgp120 at the cell surface could be achieved in several ways; increasing the expression of lgp120 or mutation of conserved Gly-Tyr residues in the cytoplasmic tail led to significant cell surface appearance. Mutant proteins with alterations in the conserved glycine were able to be endocytosed at the same rate as wild-type lgp120, suggesting a role for Gly-Tyr in Golgi to lysosome sorting. Overexpression of lgp120 resulted in significant cell surface appearance, suggesting a saturable Golgi to lysosome sorting system. Interestingly, overexpression of lgp120 also led to the mislocalization to the plasma membrane of another lysosomal membrane protein, lgp-B, suggesting that related lgp molecules may utilize the same lysosomal sorting components (Harter and Mellman, 1992). The presence of a Gly-Tyr motif in lgp-B molecules is consistent with this notion.

Lysosomal acid phosphatase (LAP) is found as a soluble protein in lysosomes; however, it is synthesized and transported to the lysosome as a membrane protein precursor. LAP appears to be distinct from the lgp

family of lysosomal glycoproteins in that the half-time of delivery of LAP to lysosomes is five to six hours compared to 30 minutes for lgps (Braun *et al.*, 1989). While conflicting reports exist (Tanaka *et al.*, 1990), delivery of LAP to lysosomes appears to involve appearance at the cell surface (Braun *et al.*, 1989). LAP is rapidly transported to the plasma membrane where it is endocytosed and recycled back to the cell surface many times before delivery to the lysosome. The short cytoplasmic tail of LAP contains a tyrosine residue that is essential for clathrin-mediated internalization. Deletion of the cytoplasmic tail or mutation of the critical tyr residue results in cell surface accumulation of mutant LAP (Peters *et al.*, 1990). Further evidence for endocytosis-mediated delivery of LAP to lysosomes comes from studies of the binding of adaptor complexes to the cytoplasmic tail of LAP. A peptide corresponding to the cytoplasmic tail of LAP efficiently bound plasma membrane-specific AP II adaptins whereas Golgi-specific AP I adaptins bound poorly (Sosa *et al.*, 1993). In addition, mutation of the tyr critical for LAP internalization resulted in a significant decrease in bound AP II adaptins. These data indicate that there may be multiple routes for delivery of membrane proteins to the lysosome; membrane proteins may be transported directly from the Golgi to the endosome and then to the lysosome or may be transported to the plasma membrane through the constitutive secretory pathway and then may be endocytosed into the lysosome.

2. Vacuolar Protein Sorting in Yeast

A. Biosynthesis of Soluble Vacuolar Proteins

The class of soluble (lumenal) vacuolar proteins include the hydrolases carboxypeptidase Y (CPY), proteinase A (PrA) and proteinase B (PrB). These soluble vacuolar proteins transit the early stages of the secretory pathway before being sorted in a late Golgi compartment away from proteins destined for the cell surface (Graham and Emr, 1991). Cloning and sequencing of the genes encoding CPY (*PRC1*), PrA (*PEP4*) and PrB (*PRB1*) found that the predicted coding sequence of each contains an N-terminal hydrophobic region which serves as a signal sequence to direct translocation into the ER (Klionsky *et al.*, 1990; Raymond *et al.*, 1992). Entry into and progression through the secretory pathway result in compartment-specific modifications of these vacuolar proteins which allow determination of their relative localization in the pathway. The post-translational modifications of CPY serve as particularly good indicators of transport through the secretory pathway and delivery to the vacuole; therefore, CPY maturation will be described as a paradigm for the biosynthesis of soluble vacuolar proteins. CPY is synthesized as a prepro precursor molecule and translocated into the lumen of the ER, where its signal sequence is cleaved off to form proCPY (Johnson *et al.*, 1987; Stevens *et al.*, 1982). Upon translocation into the ER, proCPY receives N-linked core glycosylation at four asparagine residues which results in a 67 kD form known as p1CPY. Therefore, the presence of p1CPY is indicative of translocation into the ER and serves as a marker for ER localization. p1CPY is delivered to the Golgi complex via transport vesicles together with proteins destined for the cell surface. This intercompartmental

transfer requires the action of *SEC* gene products acting in the ER to Golgi step of the secretory pathway (Stevens *et al.*, 1982). After arrival in the Golgi, the core oligosaccharides of p1CPY are extended by the addition of mannose-linked carbohydrates. This results in an increase in the apparent molecular weight of CPY to 69 kd; this form is known as p2CPY. Use of a novel CPY-α factor-invertase fusion protein has indicated that p2CPY is sorted away from proteins destined for the cell surface in a late Golgi compartment that corresponds to or is distal to the compartment containing the Kex2 protease (Graham and Emr, 1991). p2CPY is proteolytically processed in the vacuole in a *PEP4*-dependent manner to generate the 61 kd enzymatically active mature form, referred to as mCPY. The requirement for early *SEC* gene products, the presence of a signal sequence and the N-linked modifications of CPY indicate that it transits the early stages of the secretory pathway. The delivery of CPY to the vacuole is not affected by mutations in *SEC* genes whose products facilitate vesicular traffic between the Golgi and cell surface, indicating that CPY is not transported to the cell surface and subsequently endocytosed into the vacuole (Stevens *et al.*, 1982). In addition, studies with a temperature-sensitive allele of *SEC18* have elucidated the requirement for Sec18p in the delivery of CPY to the vacuole. Sec18p is the yeast homolog of N-ethylmaleimide sensitive factor (NSF), which is required for membrane fusion events in the mammalian secretory pathway (Rothman and Orci, 1992). While *sec18* mutants were initially characterized as blocking ER to Golgi transport, Graham and Emr demonstrated a requirement for Sec18p in ER to Golgi, intra-Golgi, and Golgi to cell surface steps (Graham and Emr, 1991). Surprisingly, *sec18* mutants did not block Golgi to vacuole

delivery of CPY, suggesting the possibility of a Sec18p homolog functioning between Golgi and vacuole.

Biochemical fractionation studies have demonstrated that, like mammalian lysosomal hydrolases, p2CPY is transported to the vacuole via an endosomal intermediate acting between the Golgi and vacuole. Pulse-chase labeling and fractionation of membranes found that a significant proportion of p2CPY fractionated with a post-Golgi, prevacuolar compartment (Vida *et al.*, 1993). A mutant form of CPY that lacks a functional sorting signal was found in fractions corresponding to the Golgi but not the prevacuolar compartment, indicating that the prevacuolar compartment is distal to the compartment where vacuolar proteins are sorted. These biochemical characteristics are very similar to those of the mammalian endosome. Finally, morphological analyses of mutants defective in Golgi to vacuole transport of soluble hydrolases have identified a class of mutants which accumulate vacuolar protein precursors in a novel, prevacuolar compartment (see Vacuole Morphology). This compartment most likely represents the yeast analog of the endosome suggested by the biochemical studies of CPY maturation. The movement of CPY through the secretory pathway and delivery to the vacuole is fairly rapid; the conversion of CPY to the mature, vacuolar form occurs with a half-time of approximately six minutes (Stevens *et al.*, 1982).

Proteinase A and proteinase B are also synthesized as inactive precursors which are processed to the active enzymes in the vacuole. Like CPY, PrA and PrB exhibit multiple carbohydrate-modified forms and transit the secretory pathway before sorting to the vacuole (Klionsky *et al.*,

1990; Raymond *et al.*, 1992). While the respective precursor forms are not quite as easily interpretable as CPY, they represent valuable markers to test for the sorting of multiple soluble vacuolar proteins in a given mutant.

B. Sorting Signals for Soluble Vacuolar Hydrolases

As is the case for the well-characterized mannose-6-phosphate recognition system for mammalian lysosomal proteins, there is compelling evidence for the existence of positive sorting signals directing soluble hydrolases to the vacuole in yeast. However, glycosyl modifications do not appear to be involved in the targeting of hydrolases to the yeast vacuole. CPY and PrA are efficiently delivered to the vacuole in the presence of tunicamycin, which blocks N-linked carbohydrate addition (Klionsky *et al.*, 1990; Raymond *et al.*, 1992). In addition, a mutant form of CPY in which all the N-linked glycosylation acceptor sites have been altered is also efficiently sorted to the vacuole (Winther *et al.*, 1991). Rather than resulting from the post-translational modification of a carbohydrate moiety, the sorting signal of vacuolar hydrolases in yeast residues in their primary amino acid sequence.

Studies of fusion proteins formed between CPY and the normally secreted enzyme invertase (Inv) have mapped residues in CPY that are sufficient to direct a CPY-Inv fusion protein to the vacuole. The *SUC2* gene encoding secreted invertase was engineered by removing portions corresponding to the signal sequence and then fused to the *PRC1* gene

encoding CPY. This produced a fusion protein which contains the N-terminus of CPY fused in-frame with invertase. This CPY-Inv molecule retains invertase enzyme activity and thus serves as an excellent marker for determining cellular localization of the fusion. Preliminary work found that the N-terminal 433 amino acids of CPY fused to invertase could target the CPY-Inv fusion to the vacuole (Bankaitis *et al.*, 1986). Further mapping studies narrowed the CPY sorting signal to the N-terminal 50 amino acids. These 50 residues include a 20 amino acid signal sequence which is cleaved upon translocation into the ER to expose a vacuolar targeting signal within the remaining 30 residues of pro CPY. Deletion of residues 21-50 in wild-type CPY caused the secretion of the Golgi-modified precursor form of CPY, confirming the presence of a vacuolar sorting signal in these residues (Johnson *et al.*, 1987). Extensive deletion and site-directed mutagenesis studies of CPY also demonstrated the existence of a vacuolar sorting signal in the propeptide of CPY (Valls *et al.*, 1987). It was found that a stretch of four amino acids, $Q_{24}RPL_{27}$, constitutes the core of the vacuolar targeting signal of CPY. Mutations altering this sorting signal resulted in the missorting and secretion of the Golgi-modified p2 precursor form of CPY, indicating that these residues are necessary for delivery of CPY to the vacuole (Valls *et al.*, 1990). The secretion of p2CPY requires the action of *SEC* gene products functioning between the Golgi and cell surface (Stevens *et al.*, 1982), indicating that secreted mutant precursor CPY has progressed through the ER and Golgi compartments in a manner similar to wild-type CPY. Yeast cells expressing both sorting-defective and wild-type forms of CPY effectively localize wild-type CPY to the vacuole and missort and secrete the mutant form of CPY (Valls *et al.*,

1990). This suggests that there is no interaction between the various forms of CPY during transit through the secretory pathway and does not support a role for CPY oligomerization during the sorting process. Taken together, these studies have demonstrated that a positive sorting signal for delivery to the vacuole exists in the propeptide of CPY and alteration of this signal leads to the missorting and secretion of precursor CPY. Therefore, secretion represents the default pathway that CPY follows in the absence of *cis*-linked signals directing it to the vacuole. This is further supported by the observation that p2CPY is secreted from the cell in mutants lacking Golgi to vacuole transport function (see Mutants Defective in Vacuolar Protein Sorting).

Similar invertase fusion protein studies have established that a vacuolar protein sorting signal is also present in the pro region of proteinase A (PrA). PrA-Inv fusions containing the N-terminal 76 amino acids of PrA were efficiently delivered to the vacuole (Klionsky *et al.*, 1988). In a manner identical to CPY-Inv fusion proteins, vacuolar localization of PrA-Inv required *SEC* gene function and secretion of mutant PrA-Inv fusion proteins is blocked in *sec* mutants defective in Golgi to cell surface transport. Unfortunately, deletions in wild-type PrA corresponding to the sorting signal mapped using PrA-Inv fusions were unstable. This may indicate an important function for the propeptide of PrA in the folding of the protein into a protease-resistant form upon translocation into the ER. Small deletions in the propeptide of PrA, while stable, did not result in the secretion of precursor PrA. Despite these difficulties, the analyses of PrA-Inv fusion proteins have demonstrated that a region of the propeptide of PrA is sufficient to direct the PrA-Inv

fusion to the vacuole. Sequence comparisons of the pro regions of CPY and PrA have not detected any significant similarities which suggests that they may not be recognized by the same receptor (see Receptor-Mediated Sorting of Vacuolar Hydrolases).

C. Vacuolar Protein Sorting Mutants

Studies with CPY and PrA clearly demonstrate the existence of sequence information within the propeptide of each protein which serves in targeting to the vacuole. Alteration of the vacuolar sorting signal in the wild-type proteins or in fusions with invertase results in their appearance at the cell surface. The mislocalization of vacuolar proteins to the cell surface is the basis of several genetic selections that have resulted in the isolation of a large number of mutants specifically defective in delivery of proteins to the vacuole. Genetic selections using a CPY-Invertase fusion which is efficiently targeted to the vacuole in wild-type cells led to the isolation of *vpt* (vacuolar protein targeting defective) mutants. A selection scheme based on detection of secreted CPY enzyme activity identified numerous *vpl* (vacuolar protein localization defective) mutants. These mutants are collectively referred to as vacuolar protein sorting defective, or *vps*, mutants. Screens for yeast mutants defective in protease activity in the vacuole (*pep* mutants) have also identified mutants in the vacuolar protein sorting pathway. Complementation analyses of the *vpt*, *vpl* and *pep* mutant collections determined that there

was extensive genetic overlap between them; they collectively define >40 complementation groups (Rothman *et al.*, 1989). This large number of genes indicates that protein sorting to the vacuole is a complex process. Possible molecular activities involved in vacuolar protein sorting include: 1) receptor(s) for vacuolar protein precursors, 2) protein complexes for the recognition/segregation of receptor-ligand complexes and packaging into vesicular carriers, 3) factors responsible for vesicle budding, formation and membrane fusion, 4) vesicle coat proteins (clathrin and non-clathrin coat proteins), 5) proteins involved in targeting to and recognition of target organelles such as the endosome and vacuole, 6) receptor recycling mechanisms, 7) proteins involved in the biogenesis and maintenance of the vacuole.

vpt Mutants

Extracellular invertase activity is required for yeast to grow on media containing sucrose as their sole fermentable carbon source. Invertase cleaves sucrose to fructose and glucose which can then be taken up and utilized by the cell. As described earlier, a fusion protein containing the N-terminal domain of CPY, including its vacuolar targeting signal, and normally secreted invertase is efficiently targeted to the vacuole. Yeast cells containing this CPY-Inv fusion as their only form of invertase are therefore unable to grow on sucrose due to the sequestration of invertase activity in the vacuole. The fact that mutations in the sorting signal for wild-type CPY or the CPY-Inv fusion result in its appearance at the cell surface led to a positive selection for mutants defective in the delivery of the CPY-Inv fusion to the vacuole. Disruption of the Golgi-to-vacuole

step in the pathway result in the mislocalization of the CPY-Inv fusion to the cell surface and this provides the extracellular invertase activity required for growth on sucrose-containing media. Selection for Suc^+ mutants in strains containing the CPY-Inv fusion allowed the isolation of >500 mutants (Bankaitis *et al.*, 1986; Robinson *et al.*, 1988). Enzyme assays confirmed that these mutants were indeed secreting invertase activity. In addition, assays for a cytoplasmic marker enzyme demonstrated that extracellular invertase activity in the mutants was not the result of cell lysis. The recessive *vpt* mutants were subjected to complementation analysis and were found to define 33 complementation groups (Robinson *et al.*, 1988). Analysis of representatives of each of the complementation groups confirmed that they were also defective in the delivery of wild-type CPY to the vacuole. Pulse-chase labeling experiments followed by immunoprecipitation of CPY demonstrated that the *vpt* mutants missort and secrete CPY as the Golgi-modified p2 precursor form (Robinson *et al.*, 1988). These data confirmed that the CPY-Inv fusion protein and wild-type CPY were sorted in an identical fashion in *vpt* mutant cells. Examination of two other soluble vacuolar hydrolases, proteinase A (PrA) and proteinase B (PrB), showed that they were missorted and secreted in *vpt* mutant strains in a similar manner to CPY. These data indicate that *vpt* mutants exhibit pleiotropic defects in the delivery of multiple soluble vacuolar hydrolases.

Analysis of vacuolar membrane proteins suggested that they may not be transported to the vacuole in a *VPT* dependent manner. Mislocalization of the vacuolar membrane protein α-mannosidase to the cell surface would result in extracellular enzyme activity. The majority of

vpt mutants do not exhibit a significant increase in external α-mannosidase activity compared to a wild-type strain. The *vpt* strains that do show an increase in external α-mannosidase activity include those mutants that exhibit severe morphological changes in the vacuole (see below), which may suggest that an intact vacuole is required for vacuolar α-mannosidase localization. As it has also been recently suggested that α-mannosidase may enter the vacuole directly from the cytoplasm (Yoshihisa and Anraku, 1990), the lack of *VPT*-dependent delivery may not be surprising. The vacuolar membrane protein alkaline phosphatase (ALP) has been shown to transit the secretory pathway together with soluble vacuolar proteins (Klionsky and Emr, 1989). Analysis of the maturation of ALP in *vpt* mutants also indicated that mislocalization of ALP is much less sensitive to mutations in the *VPT* genes than are soluble vacuolar hydrolases (Robinson *et al.*, 1988). Recent studies on the cellular localization of ALP has also indicated that it is not mislocalized to the cell surface in *vps* mutants (Raymond *et al.*, 1992). Collectively, these data suggest that the mechanisms of delivery of soluble and membrane vacuolar proteins are distinct and further implicate the majority of the *VPS* gene products specifically in the delivery of soluble vacuolar proteins.

vpl Mutants

Overproduction of CPY results in secretion of a portion of p2CPY into the periplasm, where it is converted to an enzymatically active form by an unknown periplasmic protease. Selection for external CPY activity allowed the isolation of *vpl* mutants. CPY will cleave the peptide bond of the dipeptide N-carbobenzoxy-L-phenylalanine-L-leucine (CBZ-PheLeu) to

release leucine. Strains auxotrophic for leucine are able to grow on media containing CBZ-PheLeu as the sole source of leucine in the presence of secreted CPY activity. This selection scheme isolated ~600 *vpl* mutants that were categorized into 19 complementation groups (Rothman and Stevens, 1986). Confirming the selection scheme, pulse-chase immunoprecipitation experiments found that the *vpl* mutants mislocalized significant amounts of CPY to the cell surface. The mislocalized form of CPY was found to be the Golgi-mediated p2 precursor. All *vpl* mutants secreted multiple soluble vacuolar hydrolases, demonstrating the pleiotropic nature of these mutants. Secretion of p2CPY in *vpl* mutants was also shown to require the action of *SEC* gene products which act between the Golgi and cell surface (Rothman and Stevens, 1986). In *vpl sec1* double mutants incubated at the non-permissive temperature, p2CPY accumulated inside the cell, presumably within secretory vesicles known to accumulate in *sec1* mutants. These data indicate that missorted CPY utilizes the secretory pathway in a manner identical to secreted proteins. In addition, it was shown that the secretion of invertase in *vpl* mutants was very similar to wild-type cells indicating that the *vpl* mutants do not significantly affect portions of the secretory pathway prior to the diversion of vacuolar proteins.

pep Mutants

Yeast mutants defective in carboxypeptidase Y enzyme activity were isolated and designated as *pep* mutants (Jones, 1977). Genetic analysis of recessive *pep* mutants demonstrated that they define 17 complementation groups. These mutants showed pleiotropic defects in several vacuolar

proteolytic activities, including CPY, PrA and PrB. There appear to be two distinct subclasses of pep mutants. One class is represented by the *pep4* mutant, which contains mutations in the structural gene for PrA (Rothman *et al.*, 1986). The fact that PrA is involved in the proteolytic maturation of several other vacuolar hydrolases, including CPY and PrB, accounts for the pleiotropic nature of *pep4* mutants. Analysis of the other *pep* mutants showed that they had characteristics very similar to *vpl* and *vpt* mutants; all *pep* mutants, with the exception of *pep4*, secrete p2CPY and proPrA from the cell. Complementation analysis between the *pep*, *vpt* and *vpl* mutants revealed substantial genetic overlap (Rothman *et al.*, 1989). Therefore, it is likely that a large number of *pep* mutants are defective in vacuolar hydrolase activity as the result of missorting and secretion of vacuolar protein precursors in a manner identical to the *vps* mutants.

Other *vps* Mutants

The yeast vacuole appear to play a cellular homeostatic role under a variety of growth conditions. The multiple functions of the vacuole are reflected in the pleiotropic defects observed in mutants lacking vacuolar function. Mutants defective in the delivery of proteins to the vacuole have been isolated in screens for maturation of yeast mating pheromone (*lam*; Wilsbach and Payne, 1993b), yeast genes required for sporulation (*spo*; Yeh *et al.*, 1991), endocytosis (*end*, *ren*; Chvatchko *et al.*, 1986; Davis *et al.*, 1993), normal vacuolar morphology (*vam*; Anraku *et al.*, 1992), and resistance to high concentrations of calcium (*cls*; Kitamoto *et al.*, 1988) or lysine (*slp*; Ohya *et al.*, 1986). A number of *vps* mutants are unable to grow

at elevated temperature. Such strains are *ts* for growth at 37°C and presumably reflect a requirement for vacuolar functions at high temperature, as these strains missort vacuolar proteins at both the permissive and non-permissive growth temperatures. Several *vps* strains also exhibit sensitivity to osmotic stress; they are unable to grow on 1.5 M NaCl plates (Banta *et al.*, 1988). Osmotically sensitive *vps* strains are among the most defective mutants for vacuolar protein sorting and underscore the importance of the vacuole in responding to environmental stress.

D. Vacuole Morphology and Biogenesis

The morphological aspects of the yeast vacuole have been the subject of a number of studies. These include analyses of vacuolar morphology under a variety of growth conditions and in a number of mutant strains at both the light and electron microscopic levels. Vacuole inheritance, the segregation of vacuolar material during cell division, has also received attention recently. Morphological issues are relevant to vacuolar protein sorting because efficient delivery to the vacuole requires the presence of an organelle which is competent to serve as a target for vesicular traffic. Indeed, a number of mutants isolated as being defective in vacuolar protein sorting have been found to be defective in aspects of vacuole morphology and biogenesis and vice versa. It is likely that factors regulating the formation and maintenance of a vacuolar structure would be required for efficient delivery of enzymes to the vacuole.

Vacuole Morphology

A number of techniques, including use of vital dyes which accumulate in the vacuole, have revealed that in wild-type yeast cells the vacuole is a large, prominent structure consisting of one to three organelles per cell (Raymond *et al.*, 1992; Roberts *et al.*, 1991). Time-lapse video microscopy using differential interference contrast optics to visualize organelles showed that there is interconversion between one large vacuole and several small vacuoles (Jones *et al.*, 1993). Banta *et al.* performed an initial morphological characterization of the *vps* mutants using light and

electron microscopy (Banta *et al.*, 1988). It was found that the *vps* mutants could be categorized into three general classes based on vacuole morphology. Class A mutants contained a wild-type or near wild-type appearing vacuole. Most of the *vps* mutants fell into this category. The vacuoles of most class A *vps* mutants could also be stained with quinacrine which indicates that the vacuole has been acidified. The phenotype of class A *vps* mutants suggest that they are competent for assembly and maintenance of a morphologically normal vacuole. The fact that many of the class A mutants are highly defective for the sorting of multiple soluble vacuolar hydrolases further suggests that soluble and membrane vacuolar constituents may utilize different transport pathways to the vacuole. The class A mutants may therefore represent mutants defective in specific functions required for the delivery of soluble vacuolar proteins rather than components required for vacuole biogenesis. Mutants in the three complementation groups comprising the class B category exhibited gross alterations in vacuolar structure. In contrast to the 1-3 large vacuoles found in wild-type cells and class A mutants, class B *vps* mutants contained highly fragmented vacuoles with each cell containing ~35-40 small vacuoles. These fragmented vacuoles accumulated both vital dyes and quinacrine, suggesting that they may retain some vacuolar function (Banta *et al.*, 1988). The class B vacuoles are not recognized as functional targets for the delivery of soluble vacuolar proteins as class B mutants are severely defective for the sorting of CPY and other soluble hydrolases (Robinson *et al.*, 1988). The small vacuoles observed in class B mutants may represent intermediates in the assembly of a wild-type vacuole, possibly analogous to the proposed maturation of

endosomes into lysosomes in mammalian cells. Alternatively, the class B mutants may lack a factor(s) required for maintaining vacuolar structure and this results in fragmentation of the vacuole. The class C *vps* mutants lack any identifiable vacuolar structure. In addition to the lack of accumulation of dyes in the vacuole, electron microscopic analysis of class C mutants demonstrated the absence of a vacuole. These mutants also accumulated abnormal membrane-enclosed structures, including vesicles, multilamellar structures, and Berkeley body structures which have been observed to accumulate in certain *sec* mutants (Banta *et al.*, 1988). These abnormal intracellular structures are most likely the consequence of abnormal membrane flow to the vacuole that has affected the secretory pathway combined with lack of ability to assemble a vacuole. Indeed, one of the class C mutants, *vps18*, is defective for late Golgi functions in addition to vacuolar functions (Robinson *et al.*, 1991). Consistent with the complete absence of a vacuole, the class C mutants are among the most severely affected mutants in the *vps* collection. In addition to being extremely defective for sorting of vacuolar proteins, the class C mutants also exhibit growth defects under conditions of environmental stress; they are temperature-sensitive for growth, sensitive to osmotic stress and grow much more slowly than wild-type strains under optimal nutrient conditions. As these mutants almost certainly lack any vacuolar function, these phenotypes may reflect a need for a functional vacuole under conditions of environmental stress. The fact that no *VPS* gene yet characterized is essential when deleted is consistent with this notion and underscores the non-essential nature of the vacuole. The class C mutants are good candidates for factors involved in vacuolar biogenesis. As such,

the vacuolar protein sorting defect observed in class C mutants may be the consequence of the lack of a functional acceptor organelle. Alternatively, the class C (and class B) mutants may indeed be defective in the sorting of a vacuolar component critical for organelle integrity. A more detailed analysis of the role of the gene products affected in these mutants is needed to differentiate between these general models.

Raymond et al. have conducted a detailed morphological re-examination of the *vps* mutants using antibodies raised against the vacuolar integral membrane protein ALP and the peripheral membrane 60 kD subunit of the vacuolar H^+-ATPase (Raymond *et al.*, 1992). They confirmed many of the classifications of Banta et al. and further subdivided the original class A mutants into several categories based on morphological criteria. The analyses using antisera to ALP and the H^+-ATPase revealed that many of the original class A mutants characterized by Banta et al. as having wild-type appearing vacuoles are actually defective to some degree in aspects of vacuolar acidification, ATPase assembly, vacuole segregation at mitosis or some vacuolar morphological feature (Raymond *et al.*, 1992). The revised classification scheme consists of six categories: classes A-F. The characteristics of mutants in classes A, B and C are unchanged from those of Banta et al.; class A *vps* mutants possess a morphologically wild-type vacuole, class B mutants have fragmented vacuoles and class C mutants have no readily identifiable vacuole. The class D mutants have a normal appearing vacuole; however, they are defective in mother to daughter vacuole inheritance and do not properly assemble the vacuolar H^+-ATPase. Class F *vps* mutants have a large central vacuole surrounded by smaller class B-like fragmented

vacuolar structures. The vacuole in class F mutant stains with ALP and ATPase antisera and accumulates quinacrine; however, mutants in this class are severely defective for the sorting of soluble vacuolar hydrolases. The most interesting observation by Raymond et al. is that class E *vps* mutants appear to accumulate a novel organelle distinct from the vacuole (Raymond *et al.*, 1992). This structure is stained by antibodies specific for ATPase but not ALP and accumulates quinacrine. In addition, the novel class E compartment also contains significant amounts of soluble vacuolar hydrolases as indicated by its staining with antibodies to CPY, PrA and PrB. These characteristics suggest that the class E compartment may represent an exaggerated prevacuolar endosome-like organelle similar to that identified by Vida et al. using biochemical techniques (Vida *et al.*, 1993). Interestingly, this novel compartment also stains with antibodies specific for a late Golgi membrane protein (Raymond *et al.*, 1992). This suggests that resident late Golgi integral membrane proteins may normally traffic to this prevacuolar compartment and would presumably be recycled back to the Golgi in wild-type cells. Further evidence for an endosomal-like function for the class E compartment comes from the observation that the cell surface α-factor receptor, Ste3p, which is normally endocytosed and degraded in the vacuole, accumulates in this compartment in a class E *vps* mutant (Davis *et al.*, 1993). This is consistent with the view of an endosomal pathway described for mammalian cells where endocytosed material from the plasma membrane is initially delivered to an early endosome and then to a late endosome where it meets traffic from the biosynthetic route. Proteins destined for the vacuole from both the biosynthetic and endocytic pathways then move from the late endosome

to the vacuole. In such a pathway, the class E prevacuolar organelle would correspond to a late endosomal compartment. The class E category contains 13 *vps* complementation groups (Raymond *et al.*, 1992), indicating that delivery from this putative prevacuolar intermediate to the vacuole may be a complicated process.

Vacuole Biogenesis

The organelles in a cell must be faithfully segregated between mother and daughter cells during cell division. The yeast vacuole is segregated in an active process that occurs very early following bud emergence. Vacuoles can be detected in buds that are as small as one-tenth the size of the mother cell (Weisman *et al.*, 1987). Fluorescence microscopy using endogenous vacuolar dyes demonstrated that vacuolar material from the mother cell is transferred to the daughter cell in the form of small membranous vesicles or tubules (Weisman and Wickner, 1988). These "segregation structures" form prior to nuclear migration upon bud emergence; time lapse photomicrography determined that within 15 minutes of bud emergence, 80% of the cells contained vacuolar segregation structures (Gomes de Mesquita *et al.*, 1991). The concept of mother to daughter transfer of vacuolar material (vacuole inheritance) is well illustrated by the phenotypic lag observed when *PEP4/pep4* heterozygotes are sporulated. Haploid progeny that are genotypically *pep4* exhibit a wild-type phenotype with respect to activity of the *PEP4* gene product, proteinase A (Jones *et al.*, 1982). The wild-type *PEP4* phenotype persists for several generations before a combination of dilution and

turnover result in loss of proteinase A activity and acquisition of the *pep4* mutant phenotype. Mutants defective in vacuole inheritance have been isolated and described (Weisman *et al.*, 1990). Despite a defect in mother to daughter vacuole inheritance in these *vac* mutants, most of the daughter cells do contain a vacuole by the time of cell division. This suggests that a mechanism for *de novo* biosynthesis of a vacuole must exist that functions in concert with or in the absence of vacuolar inheritance from the mother cell. The concept of *de novo* vacuole biosynthesis was also demonstrated using class C *vps* mutants, which contain no observable vacuolar structure. If two class C mutants from different complementation groups are mated, vacuoles are observed in each of the conjugating cells of the zygote (Banta *et al.*, 1988). These data indicate that the formation of a vacuole can occur in the absence of a normal template organelle.

Unlike other *vac* mutants, which missort vacuolar proteins and are allelic to known *vps* mutants, the *vac2* mutant appears to be selectively defective in vacuole segregation (Shaw and Wickner, 1991). The *vac2-1* strain was isolated from a collection of yeast *ts* mutants and has a temperature-conditional vacuole segregation defect; *vac2-1* cells grown at the permissive temperature have wild-type vacuole morphology whereas a significant fraction of *vac2-1* cells grown at the non-permissive temperature for three hours do not contain a vacuole in unbudded cells or in large buds. CPY and PrA are matured normally in *vac2-1* cells at the permissive and non-permissive growth temperatures. These data suggest the existence of a vacuole inheritance/segregation pathway which has components distinct from those involved in the proper localization of

vacuolar proteins. The vacuolar segregation defect observed in many *vps* mutants may reflect the need for crucial component(s) to be delivered to the vacuole for efficient vacuole inheritance.

As many of the *vps* mutants affect vacuolar morphology, it is not surprising that some are also defective in vacuole segregation at mitosis. As indicated earlier, one of the criteria for characterization of the class D *vps* mutants is a defect in vacuolar inheritance. The eight complementation groups in this class suggest that the sorting of vacuolar proteins may affect this process. Indeed, the pleiotropic nature of *vps* mutants is emphasized by the finding that nearly all of the *vac* mutants are allelic to existing *vps* mutants and again suggests that the sorting of a critical component(s) is important for mother to daughter vacuolar inheritance. The recent development of an *in vitro* reconstitution of vacuole inheritance may allow for the biochemical characterization of factors required for this process (Conradt *et al.*, 1992).

E. The Sorting of Soluble Vacuolar Proteins is a Receptor-Mediated Process

In contrast to the mannose-6-phosphate recognition system for soluble lysosomal proteins, the receptor-mediated sorting of soluble vacuolar proteins appears to be independent of carbohydrate modification and instead is based on sorting signals found in the polypeptide sequence of vacuolar proteins (see Sorting Signals for Vacuolar Proteins). Numerous

early observations indicated that a sorting receptor is utilized in the delivery of soluble hydrolases to the vacuole in yeast. The existence of a sorting receptor for vacuolar proteins was initially suggested by the observation that overproduction of a CPY-invertase fusion protein or CPY itself resulted in the missorting and secretion of a substantial proportion of these proteins (Bankaitis *et al.*, 1986; Stevens *et al.*, 1986). One explanation for these results is that overproduction of CPY leads to saturation of a limiting component, possibly a receptor protein, required for the recognition and/or sorting of CPY. A similar result was obtained when wild-type PrA was overexpressed (Rothman *et al.*, 1986). Interestingly, overproduction of PrA does not lead to the missorting and secretion of CPY (Rothman *et al.*, 1986), and overproduction of CPY does not result in secretion of PrA (Stevens *et al.*, 1986). These data suggest that CPY and PrA may utilize different receptors, which is consistent with the lack of significant homology among their sorting signals.

The apparent difference in sorting mechanisms between CPY and PrA was utilized to isolate a receptor protein for CPY. Mutations in the *VPS10* gene result in a CPY-specific sorting defect; PrA, PrB and the membrane protein ALP are delivered normally to the vacuole while CPY is missorted and secreted in a Δ*vps10* strain (Marcusson *et al.*, 1994). Characterization of the Vps10 protein revealed that it has the structural and functional features expected of a transmembrane receptor protein. It is a ~180 kD type I integral membrane protein localized by differential centrifugation primarily to a late Golgi compartment (Marcusson *et al.*, 1994), the site where sorting of vacuolar proteins appears to take place (Graham and Emr, 1991). Immunoprecipitation with carbohydrate-specific antisera

showed that Vps10p is modified by mannosyl residue added by glycosyltransferases present in the Golgi complex, further indication of a Golgi localization for Vps10p. Vps10p was shown by chemical cross-linking to specifically interact with the Golgi-modified p2 form of CPY; Vps10p could not be cross-linked to the ER (p1) or vacuolar (mCPY) forms of CPY. As an important control, Vps10p was unable to interact with a mutant form of CPY that is defective for sorting to the vacuole (Marcusson *et al.*, 1994). This mutant CPY protein is derived from a single point mutation in the gene that encodes CPY which alters a single amino acid residue within the previously characterized sorting signal found in proCPY (Valls *et al.*, 1990). In addition, Vps10p could also be cross-linked to CPY-invertase fusion proteins that contain the N-terminal 50 amino acids of CPY (the 20 residue signal sequence and 30 residues of the prosegment) and are correctly delivered to the vacuole (Marcusson *et al.*, 1994). These data indicate that Vps10p specifically binds the Golgi-modified p2 form of CPY and the interaction is dependent of the presence of an intact CPY vacuolar protein sorting signal.

It is likely that vacuolar protein receptors in yeast function in a manner analogous to the mannose-6-phosphate receptors in mammalian cells. Vacuolar proteins are presumably bound to receptors in the late Golgi sorting compartment and delivered via vesicular carriers to a prevacuolar endosomal compartment. In the endosome, ligand would dissociate from the receptor due to the pH characteristics of the compartment, ligand would continue on to the vacuole and the receptor would recycle back to the Golgi for additional rounds of sorting. In such a scenario, multiple components would be required for the efficient

function of the receptor. Interestingly, Δ*vps35* and Δ*vps29* strains exhibit a CPY-specific sorting defect indistinguishable from a Δ*vps10* strain and may represent components that specifically interact with Vps10p to facilitate CPY sorting (Paravicini *et al.*, 1992; E. Marcusson, unpublished). Other components involved in the trafficking of receptor molecules may interact with the cytoplasmic tail of receptors such as Vps10p. Indeed, deletion of the cytoplasmic tail of Vps10p results in the missorting and secretion of CPY (E. Marcusson, unpublished). Candidates for factors binding to the tail of receptors include coat proteins such as clathrin and clathrin-associated adaptin proteins. Clathrin has recently been shown to be required for the sorting of soluble vacuolar proteins including CPY (Seeger and Payne, 1992a). The cytoplasmic tail of Vps10p contains several tyrosine residues which may interact with clathrin adaptin proteins to facilitate the assembly of a clathrin coat in a manner similar to the tyr-based signals present in the mammalian mannose-6-phosphate receptor and resident lysosomal membrane proteins (see Lysosomal Protein Sorting). In addition to clarifying the issue of vesicle coats, analysis of tail mutants of Vps10p may also reveal factors involved in segregation and packaging of receptor-ligand complexes.

F. Rab-like Small GTP-binding Proteins in the Sorting of Vacuolar Proteins

GTP-binding proteins of the rab family are known to be involved in the regulation of intracellular vesicular trafficking in the secretory and endocytic pathways of yeast and mammalian cells (Novick and Brennwald, 1993). It has been proposed that GTP hydrolysis by rab proteins provides directionality to the pathway and interactions between a rab protein and its regulator(s) and effector(s) provide specificity in the vesicle targeting step (Bourne, 1988; Bourne *et al.*, 1991). Interacting/regulatory proteins identified for rab proteins include GTPase activating proteins (GAP), guanine nucleotide exchange factors (GNEF) and GDP dissociation inhibitors (GDI) (Novick and Brennwald, 1993).

YPT7

Ypt7p is a yeast homolog of mammalian rab7. Deletion of the *YPT7* gene led to defects in vacuolar morphology and vacuolar protein maturation while protein secretion was unaffected (Wichmann *et al.*, 1992; B. Horazdovsky, unpub.). Endocytic uptake of the mating pheromone α-factor was also severely inhibited in *ypt7* mutant cells. On this basis, it was concluded that Ypt7p acts in the endocytic pathway leading to the vacuole (Wichmann *et al.*, 1992). Analysis of internalized α-factor using sucrose gradients suggested that α-factor accumulates in late endosomes in Δ*ypt7* cells (Schimmoller and Riezman, 1994). Collectively, these data indicate that Ypt7p most likely acts at the endosome to vacuole step, and therefore, would affect both biosynthetic traffic from the Golgi and endocytic traffic from the cell surface.

VPS21

Cloning and sequencing of the *VPS21* gene revealed that it encodes a rab5-like small GTP-binding protein (Horazdovsky *et al.*, 1994). *vps21* mutants exhibit severe vacuolar protein sorting defects; both CPY and PrA are missorted and secreted in Δ*vps21* strains. Subcellular fractionation experiments showed that Vps21p is associated with multiple membrane compartments, possibly corresponding to Golgi, vesicular and vacuolar membranes. A role for Vps21p in the targeting of transport vesicles was suggested by the observation that Δ*vps21* cells accumulate 40-50 nm vesicles (Horazdovsky *et al.*, 1994). This result is similar to that obtained with mutants in the rab homolog Sec4p, which acts at the Golgi to plasma membrane step in the yeast secretory pathway (Salminen and Novick, 1987). While the transport vesicles that accumulate in *sec4* mutant strains are distinct from those in Δ*vps21* strains (80-100 nm vs. 40-50 nm) and Δ*vps21* cells are clearly competent for secretion as they efficiently secrete missorted p2CPY, the accumulation of vesicles suggests that Sec4p and Vps21p may execute similar functions in their respective pathways.

Many small GTP-binding proteins are associated with intracellular membranes through isoprenyl modification of a C-terminal cysteine; in many cases, isoprenylation is essential for membrane association (Glomset *et al.*, 1990; Powers, 1991). Vps21p appears to acquire geranylgeranyl isoprenyl modification at its C-terminus. In addition to containing a consensus sequence for C-terminal geranylgeranylation, the membrane association of Vps21p is abolished if its acceptor cysteines are mutated or if the yeast geranylgeranyl transferase β subunit, Bet2p, is mutated (Horazdovsky *et al.*, 1994). These data indicate that geranylgeranylation of Vps21p is required for membrane association.

Site-directed mutagenesis of the *VPS21* gene has further established that domains corresponding to GTP-binding domain I and the putative effector domain are required for vacuolar protein sorting (Horazdovsky *et al.*, 1994). Mutation of residues within GTP-binding motif I result in the inactivation of Vps21p as these mutant strains missort and secrete p2CPY. In addition, these mutant proteins are unable to bind GTP in a blot binding assay. Mutation of residues corresponding to the putative effector domain also result in severe defects in CPY sorting. As mentioned earlier, strains containing a mutation of the C-terminal geranylgeranyl addition site in Vps21p are very defective for CPY sorting; however, these mutant Vps21 proteins are able to bind GTP at wild-type levels. Collectively, the mutagenesis data indicate that multiple functional domains in Vps21p are required for the efficient sorting of vacuolar proteins. In addition, these defined point mutants will serve as valuable reagents in genetic and biochemical screens for upstream regulators and downstream effectors of Vps21p.

A role for Vps21p in early stages of endocytosis is unlikely as α-factor is internalized in Δ*vps21* strains at a rate nearly identical to a wild-type strain (Horazdovsky *et al.*, 1994). The data presented lead to two possible sites of action for Vps21p: it may be involved in Golgi to endosome traffic or in endosome to vacuole transport. The accumulation of vesicles in Δ*vps21* cells further suggests that Vps21p is specifically required for the targeting and/or fusion of transport vesicles with their target membrane. Further biochemical characterization is necessary to precisely define the site and mechanism of action of Vps21p in vacuolar protein sorting.

G. *VPS1* Encodes a Dynamin-like GTPase

The cloning and sequencing of the *VPS1* gene revealed that it encodes a protein containing a tripartite GTP-binding domain conserved in a number of known GTP-binding proteins. In addition, Vps1p was shown to be homologous to the Mx family of antiviral proteins (Rothman *et al.*, 1990) and subsequent isolation and characterization of the mechanochemical enzyme dynamin found that it is homologous to Vps1p (Obar *et al.*, 1990). The homology between Vps1p, the Mx proteins and dynamin is most pronounced in their N-terminal regions where the GTP-binding domain is present. The Mx proteins were identified in a number of vertebrate species as interferon-inducible proteins which appear to interfere with viral infection. The mechanisms responsible for the antiviral properties of the Mx proteins are not known and the significance of its homology to Vps1p is unclear. Dynamin was isolated as a microtubule-associated mechanochemical enzyme postulated to be involved in microtubule-based motility (Obar *et al.*, 1990; Shpetner and Vallee, 1992). Although subsequent studies have cast doubt on this activity (Maeda *et al.*, 1992), a role for dynamin in endocytosis is suggested by its homology to the Drosophila *shibire* gene product (Chen *et al.*, 1991; van der Bliek and Meyerowitz, 1991). Flies with a *shibire* mutation appear to exhibit a generalized defect in endocytosis which is manifested as a paralytic phenotype in mutant flies due to defects in vesicle-mediated events at nerve terminals. A direct role for dynamin in endocytosis was

demonstrated by the expression in mammalian cells of dominant mutant forms of dynamin containing alterations in the GTP-binding domain that led to a block in receptor-mediated endocytosis (Herskovits *et al.*, 1993; van der Bliek *et al.*, 1993). In addition to a role for dynamin GTPase activity in endocytosis, these studies also suggested that the basic C-terminus of dynamin may be responsible for interaction with other components of the endocytic pathway. The C-terminus of dynamin has been shown to be involved in several interactions which may regulate its GTPase activity and thereby affect endocytosis. Dynamin was shown to bind to and be activated by a subset of SH3 domains, including those found in receptor tyrosine kinases and proteins associated with these receptors (Gout *et al.*, 1993; Scaife *et al.*, 1994). These findings suggest that the binding of dynamin to SH3 domain-containing proteins may be important for the formation of a protein complex required for the endocytic internalization of activated cell surface receptor tyrosine kinases. In addition, the C-terminus of dynamin has been found to be phosphorylated by protein kinase C and this phosphorylation results in a tenfold activation of the GTPase activity of dynamin (Robinson *et al.*, 1993). Finally, a recent report showed that dynamin GTPase activity is also stimulated by acidic phospholipids and membrane vesicles, possibly due to interaction with the basic C-terminus of dynamin (Tuma *et al.*, 1993). Collectively, these observations indicate that dynamin is involved in the endocytic trafficking of cell surface molecules and is subject to multiple levels of post-translational regulation and further suggest that it is a key regulator of endocytosis.

Mutations in the *VPS1* gene result in a severe defect in the sorting of soluble vacuolar proteins (Rothman *et al.*, 1990). In addition, *VPS1* was found to be identical to the *S. cerevisiae SPO15* gene; mutations in this gene lead to a defect in meiotic cell division required for sporulation (Yeh *et al.*, 1991). The sporulation defect observed in *vps1* cells is reminiscent of that observed in *pep4* mutants. *PEP4* encodes proteinase A which is required for the proteolytic activation of several important vacuolar proteolytic enzymes; strains containing mutations in the *PEP4* gene are defective in vacuolar proteolytic activity and sporulate extremely poorly, implicating vacuolar proteases in the sporulation process. The severe protein sorting defect in *vps1* cells is likely to lead to a gross deficiency in vacuolar protease activity and result in a sporulation defect similar to *pep4* cells. Indeed, mutations in several *VPS* genes, which lead to severe vacuolar protein sorting defects, also result in sporulation-defective strains. As dynamin originally was reported to be a microtubule-dependent mechanoenzyme and Spo15p could associate irreversibly with microtubules *in vitro*, Vater et al. tested whether microtubules are required for vacuolar protein sorting in yeast. While cells treated with the microtubule-depolymerizing drug nocodazole or containing mutations in the yeast β-tubulin gene lacked any visible microtubules by immunofluorescence, they did not exhibit any vacuolar protein sorting defect; CPY was processed to the mature form with kinetics identical to wild-type in strains lacking microtubules (Vater *et al.*, 1992). In addition, immunofluorescence experiments detected no colocalization of Vps1p and microtubules in control cells and treatment with nocodazole did not lead to a redistribution of Vps1p within the cell. These data indicate that Vps1p

most likely does not associate with microtubules *in vivo* and microtubules do not appear to be essential for vacuolar protein sorting.

Vps1p expressed in *E. coli* has been demonstrated to bind GTP *in vitro*, and Vps1p immunoprecipitated under non-denaturing conditions from yeast catalyzed the hydrolysis of GTP (Vater *et al.*, 1992). Therefore, Vps1p appears to be able to bind GTP and possesses an intrinsic GTPase activity. Mutational analysis of Vps1p led to the proposal that the protein is composed of two functionally distinct domains. Mutations in *VPS1* generated by hydroxylamine mutagenesis fell into two categories: 1) dominant-negative mutations which cause a protein sorting defect in wild-type cells and 2) recessive loss of function mutations. Mapping of the mutation responsible for these phenotypes yielded a surprising result. Mutations resulting in a dominant-negative phenotype all mapped to the N-terminal region of Vps1p while recessive loss of function mutations gave rise to unstable or truncated protein products. To test whether the dominant-negative phenotype required an intact Vps1p C-terminus, a point mutation which on its own results in a dominant-negative phenotype was combined with a frameshift mutation that causes a C-terminal truncation. Transformation of the double mutant into wild-type cells did not result in a vacuolar protein sorting defect, indicating that deletion of the C-terminus of dominant-negative mutants eliminates dominant interference (Vater *et al.*, 1992). This mutational analysis of Vps1p led to the proposal that any mutation in the N-terminus which results in a non-functional protein, presumably by interfering with GTP binding or hydrolysis, will give rise to a dominant-negative phenotype. The requirement of the C-terminal region for a dominant-negative

phenotype suggests that Vps1p may interact with other components of the sorting machinery through its C-terminal domain. In such a model, the dominant-negative phenotype of catalytically impaired Vps1p is due to sequestration of factors required for sorting by non-functional Vps1 proteins. Consistent with this hypothesis, overproduction of wild-type Vps1p will suppress the dominant-negative vacuolar protein sorting defect seen in cells expressing N-terminal Vps1p mutants (Vater *et al.*, 1992). Identification of the putative component titrated by dominant-negative Vps1p mutants may be possible by transforming a dominant mutant strain with a multicopy genomic library and screening for suppression of the sorting defect.

A role for Vps1p in the retention of Golgi membrane proteins has been recently described. Wilsbach and Payne performed a genetic screen to isolate yeast mutants defective in the retention of the Golgi membrane protein, Kex2p. Kex2p resides in a late Golgi compartment and is involved in the proteolytic processing of the mating pheromone α-factor. In mutants exhibiting defective α-factor processing, Kex2p was found to be delivered to the vacuole and degraded in a *PEP4*-dependent manner (Wilsbach and Payne, 1993b). The *lam1* mutant (for low α-factor maturation) was also defective for the sorting of CPY to the vacuole and was found to be allelic to *VPS1*. A model for Vps1p action was presented whereby Vps1p is involved in the recycling of membrane proteins from a post-Golgi prevacuolar endosome-like intermediate compartment. Loss of Vps1p function would then lead to delivery of normally recycled membrane proteins from the intermediate compartment to the vacuole (see Membrane Proteins). Included in the mislocalized membrane

proteins would be the putative transmembrane receptor protein(s) for soluble vacuolar proteins (see Vacuolar Protein Sorting is a Receptor-Mediated Process). Blockage of sorting receptor recycling back to the Golgi would then result in the vacuolar protein sorting defect observed in *vps1* cells (Wilsbach and Payne, 1993a; Wilsbach and Payne, 1993b). An alternative explanation is that Vps1p may act at the late Golgi to facilitate the sorting of vacuolar proteins and loss of Vps1p function may compromise the integrity of the Golgi compartment such that resident membrane proteins are not retained and are delivered to the vacuole by default (see Vacuolar Membrane Proteins for a discussion on the default pathway for membrane proteins). Such a model is consistent with immunofluorescent localization experiments of Vps1p which suggest an association with the Golgi (Rothman *et al.*, 1990). Use of a temperature-sensitive allele of *VPS1*, in which the protein is immediately inactivated upon shift to the non-permissive temperature, should allow resolution of direct vs. indirect models for the role of Vps1p in membrane protein trafficking.

H. Clathrin and Coat Proteins

Most, if not all, vesicle-mediated intracellular transport events involve vesicle-associated coat proteins (Pryer *et al.*, 1992). In general, components of the coat associate with the cytoplasmic tails of transmembrane proteins and this serves to concentrate membrane

proteins prior to packaging into transport vesicles. In addition, self-assembly of coat proteins onto the donor membrane has been proposed to provide the energy to deform the membrane bilayer to allow vesiculation. Classical clathrin-coated vesicles play a role in receptor-mediated endocytosis of proteins from the plasma membrane to the early endosome and in delivery of proteins from the trans Golgi network (TGN) to the late endosome in mammalian cells. Non-clathrin coated vesicles appear to mediate intra-Golgi transport in mammalian cells and ER to Golgi transport in yeast (Barlowe *et al.*, 1994; Pryer *et al.*, 1992; Rothman and Orci, 1992).

Transport events involving clathrin coats provide the best understood vesicular delivery system. Clathrin-coated vesicles are formed by the association of clathrin and associated adaptin proteins at coated pits (Keen, 1990; Pearse and Robinson, 1990). The specificity of the coat assembly reaction is mediated by adaptor complexes (APs) composed of distinct sets of adaptin proteins. The adaptins associate with the cytoplasmic tails of transmembrane proteins and promote the assembly of clathrin heavy and light chains into the coat. There are two classes of adaptor complexes in mammalian cells which correspond to the requirement for clathrin coated transport at the plasma membrane and the TGN. Adaptor complex I (AP I) mediates the delivery of proteins from the TGN to the endosome and adaptor complex II (AP II) from the plasma membrane to the endosome. It appears that there may be distinct signals present in the cytoplasmic tails of transmembrane cargo proteins that specify which set of adaptor proteins are utilized (see Lysosomal Protein Sorting). Once coated vesicles are

formed, the clathrin coat is disassembled to allow fusion of the vesicle with the target membrane.

Clathrin and Vacuolar Protein Sorting in Yeast

Initial characterization of the yeast clathrin heavy chain gene *CHC1* indicated that clathrin was not required for cell growth, protein secretion, or delivery of proteins to the vacuole (Payne *et al.*, 1988; Payne *et al.*, 1987). The latter observation was quite surprising given the well-characterized role of clathrin in the delivery of lysosomal proteins from the TGN to the lysosome. Biochemical characterization of yeast clathrin showed that it is capable of assembling into triskelions, suggesting that it is capable of forming a coat complex. Resolution of this apparent paradox came with the generation of a temperature-conditional allele of the yeast clathrin heavy chain gene (*chc1-ts*). In contrast to strains deleted for the *CHC1* gene which show no defect in maturation of CPY, one of the immediate consequences of shifting *chc1-ts* cells to the non-permissive temperature is the missorting of the soluble vacuolar hydrolases CPY and PrA (Seeger and Payne, 1992a). These same experiments also demonstrated that clathrin is not required for the delivery of resident vacuolar membrane proteins as ALP is matured normally in *chc1-ts* cells incubated at the non-permissive temperature. These data indicate that clathrin is required for the sorting of soluble vacuolar proteins. Remarkably, it was found that extended incubation at the non-permissive temperature prior to labeling allowed *chc1-ts* cells to recover the ability to sort CPY. After several hours of pre-incubation at non-permissive temperature, *chc1-ts* cells showed essentially no defect in CPY sorting and exhibited the same vacuolar

protein sorting phenotype as Δ*chc1* cells. This unusual adaptation phenomenon suggests that a non-clathrin mechanism may functionally substitute for clathrin in the sorting of soluble vacuolar proteins. Delivery of CPY to the vacuole in Δ*chc1* cells does not appear to involve transport of CPY to the cell surface and subsequent endocytosis into the vacuole. *sec1* mutants block Golgi to plasma membrane transport and a *sec1* Δ*chc1* double mutant does not block CPY maturation, indicating that CPY is not delivered to the vacuole via the cell surface (Payne *et al.*, 1988). The extremely rapid onset of the sorting defect in *chc1-ts* cells shifted to the non-permissive temperature argues for a direct role for clathrin in vacuolar protein sorting. By analogy to lysosomal protein sorting in mammalian cells, it has been suggested that clathrin functions in yeast to concentrate receptors for soluble vacuolar proteins (e.g., Vps10p) into coated pits. A model can be proposed whereby clathrin-coated vesicles are budded off the late Golgi sorting compartment and receptor-ligand complexes are delivered to an endosomal compartment where ligand dissociates from receptors. Vacuolar proteins then continue on to the vacuole and receptors recycle back to the late Golgi for additional rounds of transport. Inactivation of clathrin function due to shifting *chc1-ts* cells to the non-permissive temperature would result in a blockage in the formation of coated pits and vesicles. Newly synthesized vacuolar protein precursors would then rapidly saturate the blocked receptors and be efficiently secreted from the cell.

Clathrin in yeast cells also appears to be involved in the retention of some resident Golgi membrane proteins. Payne and colleagues found that *chc1* mutant strains showed a defect in mating efficiency (Payne *et al.*,

1988). In Δ*chc1* cells or in *chc1-ts* cells incubated at the non-permissive temperature, the mating pheromone α-factor was secreted from the cells as the highly glycosylated unprocessed precursor (Payne *et al.*, 1988; Seeger and Payne, 1992b). The molecular basis for this observation was found to be the mislocalization in clathrin-deficient strains of Kex2p, the enzyme responsible for the processing of α-factor in the Golgi. Incubation of *chc1-ts* cells at the non-permissive temperature led to the appearance of Kex2p and another Golgi membrane protein, diaminopeptidyl amino peptidase A (DPAP A), at the cell surface (Seeger and Payne, 1992b). The fact that the vacuolar membrane protein ALP is processed normally in *chc1-ts* cells at the non-permissive temperature demonstrates the selective nature of the action of clathrin on membrane proteins in the late Golgi.

Receptor-mediated endocytosis in yeast also appears to involve clathrin. Internalization of mating pheromone involves transmembrane receptors which bind and endocytose ligand and deliver it to the vacuole for degradation. Receptor-mediated internalization of α-factor is partially defective in Δ*chc1* cells (Payne *et al.*, 1988) or *chc1-ts* cells incubated at the non-permissive temperature (Tan *et al.*, 1993). The fact that some endocytic function persists in the absence of clathrin function suggests that clathrin may be involved in increasing the efficiency/fidelity of endocytosis or that clathrin-independent mechanisms of endocytosis may exist.

Collectively, these data indicate that clathrin acts in multiple membrane trafficking pathways at several different intracellular location. This is consistent with the view of clathrin function from mammalian

cells and suggests the conservation of clathrin function in eukaryotic vesicular trafficking pathways.

I. Synaptic Vesicle Proteins and SNAREs in Vacuolar Protein Sorting

Studies of synaptic vesicle exocytosis and intra-Golgi vesicular transport in mammalian cells and vesicular traffic in the yeast secretory pathway have made significant progress in determining the biochemical mechanisms regulating the formation and docking/fusion of vesicles with their target membrane. Identification of molecules involved in these processes has revealed remarkable similarities that point to conserved mechanisms regulating vesicular movement throughout the secretory pathway in all eukaryotic cells. Rothman and co-workers have extensively characterized molecules involved in intra Golgi vesicular trafficking: these molecules include N-ethylmaleimide sensitive factor (NSF), soluble NSF attachment proteins (SNAPs), ADP ribosylation factor (ARF), and COP non-clathrin coat proteins (Rothman and Orci, 1992). Characterization of proteins associated with NSF-SNAP complexes revealed that they included components previously identified as being involved in synaptic vesicle docking/fusion with the plasma membrane (Bennett *et al.*, 1993; Sollner *et al.*, 1993a; Sollner *et al.*, 1993b). This merging of mechanisms and molecules initially characterized from different processes led Rothman and colleagues to propose a generalized hypothesis for the regulation of transport vesicle docking and fusion with

the target membrane (Rothman and Warren, 1994). In this model, soluble SNAP proteins bind to their receptors (SNAREs) on both the transport vesicle (v-SNARE) and the target membrane (t-SNARE). Specificity in the targeting step would result from unique SNAREs present on distinct membranes.

In addition to functional homologs of NSF, SNAPs, ARF and COPs, structural homologs of v- and t-SNAREs have been identified that act in the secretory pathway in yeast (Aalto *et al.*, 1992; Aalto *et al.*, 1993; Bennett and Scheller, 1993). These homologs have been implicated at multiple steps in the secretory pathway, including ER to Golgi and Golgi to plasma membrane transport. The conservation of these molecules between different organisms and in multiple vesicle targeting reactions suggests that a combination of unique and common molecules act at individual vesicle transport steps throughout the cell. Bennett and Scheller have proposed that several unique classes of molecules are utilized at specific steps. These components include: a small GTP-binding protein of the rab/ypt family, v-SNARE, t-SNARE and a component analogous to Sec1p (which acts at the Golgi to plasma membrane step in yeast) (Bennett and Scheller, 1993). Several of these classes of molecules have been identified as being required for Golgi to vacuole transport. Vps21p and Ypt7p are small GTP-binding proteins most homologous to mammalian rabs (Horazdovsky *et al.*, 1994; Wichmann *et al.*, 1992), Pep12p (Vps6p) (E. Jones, unpublished) is structurally homologous to t-SNARE and Vps33p (Banta *et al.*, 1990) and Vps45p (C. Cowles, B. Horazdovsky, and S. Emr, unpublished) are homologous to Sec1p. A corresponding v-SNARE acting in this pathway has not been identified. While it is not clear whether

distinct components will be required for Golgi to endosome and endosome to vacuole steps, it is apparent that Golgi to vacuole transport involves conserved mechanisms regulating vesicular traffic throughout the cell. Therefore, insights gained from genetic and biochemical analyses of these components should prove generally applicable in a variety of systems and may help illuminate mechanistic aspects of the SNARE hypothesis.

J. Vacuolar Membrane Proteins

In addition to soluble hydrolases, the yeast vacuole contains a variety of membrane proteins which function in macromolecular degradation and maintenance of an acidic environment (Klionsky *et al.*, 1990). Therefore, the accurate and efficient delivery of soluble and membrane vacuolar proteins is essential for proper vacuolar function. In mammalian cells, the lysosomal membrane proteins characterized to date appear to contain a sorting signal which directs their delivery to the lysosome (see Lysosomal Protein Sorting). The sorting of lysosomal membrane proteins also does not appear to involve the mannose-6-phosphate receptor system as lysosomal membrane proteins do not receive the mannose-6-phosphate modification and many lysosomal membrane proteins are correctly localized in cells from individuals afflicted with I-cell disease (Kornfeld, 1992; Kornfeld and Mellman, 1989). Thus, in mammalian cells, the delivery of membrane proteins to the

lysosome appears to involve a positive sorting signal and in the absence of a lysosomal sorting signal, membrane proteins appear at the cell surface. This suggests that the default pathway for lysosomal membrane proteins is to the cell surface. This idea is supported by the observation that disruption of signals for retention in several Golgi membrane proteins results in their appearance at the cell surface (Machamer, 1993; Pelham and Munro, 1993). Thus, it appears that in the absence of specific signals for retention or sorting to an organelle, membrane and soluble proteins in the mammalian secretory pathway are delivered to the cell surface.

Alkaline phosphatase (ALP) is a well-characterized vacuolar integral membrane protein. It is synthesized as an inactive precursor that is proteolytically processed to the active enzyme in the vacuole in a *PEP4*-dependent manner (Klionsky *et al.*, 1990; Raymond *et al.*, 1992). Biochemical and immunolocalization studies have found that ALP delivery to the vacuole is relatively insensitive to lesions in *VPS* genes (Herman *et al.*, 1991; Klionsky and Emr, 1989; Paravicini *et al.*, 1992; Raymond *et al.*, 1992). These data suggest that soluble and membrane proteins may be sorted to the vacuole by different mechanisms. Initial studies of ALP using ALP-Invertase fusion proteins suggested that a signal for sorting to the vacuole may exist in the transmembrane domain or cytoplasmic tail (Klionsky and Emr, 1990). Recent studies with another vacuolar membrane protein and several resident Golgi membrane proteins, however, have cast doubt on the requirement of a sorting signal for localization of vacuolar membrane proteins.

Yeast contains two major organellar dipeptidylaminopeptidase (DPAP) activities: DPAP A is localized to the Golgi and is involved in processing

of the mating pheromone, α-factor and DPAP B is found in the vacuole (Klionsky *et al.*, 1990; Raymond *et al.*, 1992). The genes encoding DPAP A and B have been cloned and sequenced (Roberts *et al.*, 1989; J. Thorner, unpublished). DPAP A and DPAP B are both predicted to be type II integral membrane proteins and share extensive sequence similarity in their catalytic lumenal domains while their transmembrane and cytoplasmic domains are not significantly homologous. Attempts to map a vacuolar localization signal in DPAP B examined the lumenal, transmembrane, and cytoplasmic domains separately (Roberts *et al.*, 1992). Fusion of the signal sequence of prepro-α-factor to the lumenal domain of DPAP B resulted in secretion of the protein. Replacement of the lumenal domain of DPAP B with the coding sequence of the normally secreted enzyme invertase resulted in a DPAP B-Inv fusion protein that was delivered to the vacuole. These data indicate that the lumenal domain of DPAP B is neither necessary nor sufficient to target the protein to the vacuole. Replacement of the transmembrane domain of DPAP B with the transmembrane domain of DPAP A resulted in a hybrid protein that was efficiently localized to the vacuole. Finally, deletion of essentially the entire cytoplasmic tail of DPAP B still led to delivery to the vacuole. Delivery of these chimeric and mutant proteins to the vacuole does not appear to require their appearance at the cell surface and subsequent endocytosis. Use of a *sec1* mutant, which blocks Golgi to plasma membrane traffic, demonstrated that these forms of DPAP B are targeted directly from the Golgi to the vacuole (Roberts *et al.*, 1992). These unexpected data led Stevens and co-workers to conclude that a positive vacuolar sorting signal is not present in the lumenal, transmembrane, or

cytoplasmic domains of DPAP B and led to the proposal that delivery of membrane proteins to the vacuole does not require specific sorting information, i.e., that the default destination for membrane proteins in yeast is the vacuole (Nothwehr and Stevens, 1994).

Studies defining the retention signals in the yeast Golgi membrane proteins DPAP A, Kex2p, and Kex1p have also lended support to the notion that the vacuole is the default destination for membrane proteins. Fusion protein, deletion and site-directed mutagenesis analyses have demonstrated that DPAP A is retained in the Golgi complex due to a retention signal that has been narrowed down to an eight residue stretch in the cytoplasmic tail that contains several critical phenylalanine residues (Nothwehr *et al.*, 1993). Deletion or mutation of this Golgi retention signal in DPAP A results in appearance of the mutant protein in the vacuole. Similar results have been obtained for Kex2p (Wilcox *et al.*, 1992), which possesses a tyrosine-containing retention signal in its cytoplasmic tail, and Kex1p (Cooper and Bussey, 1992), where the retention signal also appears to reside in its cytoplasmic domain. Interestingly, overproduction of DPAP A, Kex2p, or Kex1p also causes their delivery to the vacuole, suggesting that the Golgi retention system may be saturable (Cooper and Bussey, 1992; Nothwehr *et al.*, 1993; Wilcox *et al.*, 1992). A common mechanism of retention in the Golgi for Kex2p and DPAP A is suggested by the observation that overproduction of wild-type, but not retention-defective, Kex2p results in the delivery to the vacuole of a DPAP A fusion protein normally retained in the Golgi (Nothwehr *et al.*, 1993).

Results with vacuolar and Golgi membrane proteins have challenged the view that transport of membrane proteins to the vacuole requires a

specific sorting signal. One strong prediction of this model is that delivery of membrane proteins to the plasma membrane requires a targeting signal. As this does not appear to be the case for mammalian cell surface integral membrane proteins, demonstration of such a sorting signal would lend considerable support to the notion that divergent mechanisms of sorting membrane proteins exist in the eukaryotic secretory pathway. The study of fusion proteins between DPAP B and cell surface membrane proteins may prove particularly useful. Complicating the determination of mechanism(s) of Golgi retention and delivery to the vacuole is the observation that while clathrin is involved in the Golgi retention of Kex2p, loss of clathrin function results in delivery of Kex2p to the cell surface rather than the vacuole (Payne *et al.*, 1988). The possible involvement of clathrin in transport of membrane proteins to the vacuole will be an important issue to be clarified.

Another interesting aspect of the delivery of membrane proteins to the vacuole concerns the vacuolar membrane H^+-ATPase (V-ATPase). The V-ATPase is a multi-subunit enzyme responsible for acidification of the vacuolar compartment (Anraku *et al.*, 1992). Considerable effort has been directed at purification and identification of the individual subunits of the ATPase (Kane and Stevens, 1992). The V-ATPase is composed of at least eight different subunits ranging in size from 17 kD to 100 kD. The genes encoding seven of these have been cloned and sequenced. The subunits of the V-ATPase can be grouped into peripheral membrane proteins (V_1 complex) and integral membrane proteins (V_0 complex). As such, the V-ATPase represents an interesting problem from the perspective of macromolecular assembly. Presumably, there must be

coordination of synthesis of integral and peripheral membrane components, and accessory factors have been described that appear to be necessary for the efficient assembly or full activity of the V-ATPase (Bauerle *et al.*, 1993; Ho *et al.*, 1993a; Ho *et al.*, 1993b; Kane *et al.*, 1992). Determination of the spatial and temporal aspects of V-ATPase assembly and the factors regulating it, in addition to the targeting of the enzyme to the vacuolar membrane, will be important developments. Clearly, resolution of the issue of default vs. signal-mediated delivery of membrane proteins to the vacuole will have profound ramifications upon the targeting of the V-ATPase to the vacuole membrane.

Acidification of the vacuole appears to play a role in the sorting of soluble vacuolar proteins. In this regard, yeast may be similar to mammalian cells where acidic conditions are required for dissociation of ligand from receptor in the endocytic and biosynthetic pathways leading to the lysosome (Kornfeld and Mellman, 1989; Mellman, 1992). Treatment of yeast with agents that raise vacuolar pH or with V-ATPase inhibitors result in the missorting of CPY and PrA (Banta *et al.*, 1988; Rothman *et al.*, 1989). A recent study has reported that buffering the media pH to 7.5 also induces a vacuolar protein sorting defect (Klionsky *et al.*, 1992a). A more specific approach has been to examine the effects on vacuolar protein sorting in strains deleted for the genes encoding subunits of the vacuolar ATPase. While independent studies have found differing degrees of vacuolar protein missorting, it is clear that disruption of the yeast vacuolar H^+-ATPase leads to defects in the delivery of vacuolar proteins (Klionsky *et al.*, 1992b; Yamashiro *et al.*, 1990). Subcellular fractionation and sucrose density gradients suggest that in V-ATPase subunit mutants

vacuolar protein precursors accumulate in prevacuolar compartments that may correspond to the late Golgi and a putative endosome-like compartment (Yaver *et al.*, 1993). This study also suggests that the accumulation of precursor forms of vacuolar proteins in these mutant strains reflects their missorting rather than a defect in precursor processing in the vacuole.

VPS gene products also appear to be involved in vacuolar acidification, possibly by affecting V-ATPase assembly or function. Early studies identified a subset of *vps* mutants that are defective in the acidification of the vacuole in addition to vacuolar protein localization defects (Banta *et al.*, 1988; Raymond *et al.*, 1990; Rothman *et al.*, 1989). It appears likely that the vacuole acidification defect observed in strains such as *vps3* is not the sole cause of the vacuolar protein missorting phenotype. While strains deleted for the *VPS3* gene are highly defective for the sorting of CPY (Raymond *et al.*, 1990; Rothman and Stevens, 1986), strains deleted for a peripheral membrane subunit of the V-ATPase exhibit only a modest defect in CPY mislocalization (Yamashiro *et al.*, 1990). Consistent with this idea, Raymond et al. have identified a subset of the *vps* mutants (Class D, see Vacuole Morphology) which are defective in vacuole acidification and fail to assemble peripheral membrane subunits of the V-ATPase (Raymond *et al.*, 1992). One interpretation of these results is that the vacuolar protein sorting defects associated with such *vps* mutants leads to an inability to properly assemble the vacuolar H^+-ATPase. The vacuole acidification defect would then be an indirect result of the missorting of a component critical to the assembly of a functional V-ATPase.

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Chapter 2:

A Novel Protein Kinase Homolog Essential for Protein Sorting to the Yeast Lysosome-like Vacuole

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A Novel Protein Kinase Homolog Essential for Protein Sorting to the Yeast Lysosome-like Vacuole

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Summary

The *VPS15* gene encodes a novel protein kinase homolog that is essential for the efficient delivery of soluble hydrolases to the yeast vacuole. Point mutations altering highly conserved residues within the Vps15p kinase domain result in the secretion of multiple vacuolar proteases. In addition, the in vivo phosphorylation of Vps15p is defective in these kinase domain mutants, suggesting that Vps15p may regulate specific protein phosphorylation reactions required for protein sorting to the yeast vacuole. Subcellular fractionation studies further demonstrate that the 1455 amino acid Vps15p is peripherally associated with the cytoplasmic face of a late Golgi or vesicle compartment. This association may be mediated by myristate as Vps15p contains a consensus signal for N-terminal myristoylation. We propose that protein phosphorylation may act as a molecular "switch" within intracellular protein sorting pathways by actively diverting proteins from a default transit pathway (e.g., secretion) to an alternative pathway (e.g., to the vacuole).

Introduction

The highly compartmentalized nature of the eukaryotic cell requires that specific mechanisms exist to sort and deliver proteins efficiently from their site of synthesis in the cytoplasm to their final intracellular or extracellular destinations. The general pathway followed by proteins destined for the lysosomal/vacuolar compartment of eukaryotic cells has been extensively studied and serves as one of the best-understood paradigms of an intracellular protein sorting process (Kornfeld and Mellman, 1989; Klionsky et al., 1990). Like those proteins destined for secretion, lysosomal proteins are translocated across the membrane of the endoplasmic reticulum (ER) and then travel from the ER to the Golgi complex. However, the transit pathway of lysosomal proteins diverges within the Golgi apparatus, where these proteins are sorted away from secretory protein traffic and are targeted toward the lysosome.

The delivery of proteins to the lysosome appears to be a complex process involving a relatively large number of distinct steps whose execution must be precisely controlled both spatially and temporally. These steps include the specific recognition of lysosomal proteins, their packaging into transport vesicles, delivery and fusion of these vesicles with the correct target organelle, release of the vesicular contents, and the subsequent recycling of transport components for further rounds of protein sorting. Biochemical and genetic studies have identified *cis*-acting targeting signals required for the initial recognition event in lysosomal and vacuolar protein delivery. In many types of mammalian cells, the N-linked carbohydrate chains of soluble lysosomal proteins are modified with mannose-6-phosphate residues, which are recognized by specific integral membrane receptors that mediate lysosomal delivery (Kaplan et al., 1977; reviewed in Kornfeld and Mellman, 1989). In contrast, the targeting signals of yeast vacuolar proteins do not involve a specific carbohydrate modification and instead appear to reside directly within the polypeptide backbone of these proteins (Johnson et al., 1987; Valls et al., 1987; Klionsky et al., 1988; Klionsky and Emr, 1990). However, beyond this initial recognition event, very little is currently known about the basic cellular mechanisms underlying the lysosomal or vacuolar protein delivery pathways.

In the yeast Saccharomyces cerevisiae, the application of several genetic selections has resulted in the isolation of a large number of mutants that exhibit defects in vacuolar protein localization and/or processing (Jones, 1977; Bankaitis et al., 1986; Robinson et al., 1988; Rothman and Stevens, 1986; Rothman et al., 1989; reviewed in Klionsky et al., 1990). Instead of delivering vacuolar hydrolases to the vacuole, these *vps* (for "vacuolar protein sorting defective") mutants missort vacuolar enzyme precursors to the yeast cell surface (Bankaitis et al., 1986; Rothman and Stevens, 1986; Rothman et al., 1989; Robinson et al., 1988). Protein secretion and protein glycosylation appear to be normal in most of the *vps* mutants, indicating that the defects in these mutants are specific for the targeting of vacuolar proteins (Rothman and Stevens, 1986; Robinson et al., 1988). Genetic comparisons among these mutants have demonstrated that they collectively define more than 47 unique complementation groups (see Klionsky et al., 1990). These results suggest that the delivery of proteins to the yeast vacuole is a complex process requiring the coordinated participation of a relatively large number of gene products. In addition to identifying cellular components directly involved in the specific segregation, packaging, and delivery of vacuolar proteins, these yeast sorting mutants should also define genes whose products are involved in the regulation, or control, of these processes.

The *VPS15* gene product is an essential component of the yeast vacuolar protein sorting apparatus. Mutations in the *VPS15* gene result in severe defects in the localization of several soluble vacuolar hydrolases, including carboxypeptidase Y (CPY), proteinase A, and proteinase B (Robinson et al., 1988). In contrast, two vacuolar membrane proteins, α-mannosidase and alkaline phosphatase, appear to be properly localized to the vacuole in *vps15* mutants (Robinson et al., 1988; Klionsky and Emr, 1990; C. Raymond and T. Stevens, personal communication). In addition, *vps15* mutant cells appear to possess a somewhat enlarged but morphologically normal vacuolar com-

Table 1. Strains Used in This Study

Strain	Genotype	Reference
S. cerevisiae		
SEY6210	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*	Robinson et al. (1988)
SEY6211	*MATa leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 ade2-101 suc2-Δ9*	Robinson et al. (1988)
SEY15-2	SEY6210 *vps15-2*	Robinson et al. (1988)
SEY15-9	SEY6211 *vps15-9*	Robinson et al. (1988)
PHY112	SEY6210 *vps15Δ1::HIS3*	This study
PHY113	SEY6211 *vps15Δ1::HIS3*	This study
SEY6210.5	*MATα/MATa leu2-3,112/leu2-3,112 ura3-52/ura3-52 his3-Δ200/ his3-Δ200 trp1-Δ901/trp1-Δ901 suc2-Δ9/suc2-Δ9 ADE2/ade2-101 lys2-801/LYS2*	This study
E. coli		
MC1061	*araD139 (araABOIC-leu)7679 Δ(lac)X74 galU galK hsdR rpsL*	Casadaban and Cohen (1980)
JM101	*F' traD36 lacI^q ZΔM15 proAB/supE thiΔlac(lac-pro)*	Miller (1972)
BW313	*F' lysA/dut ung thi-1 relA spoT1*	Kunkel (1985)

partment (Banta et al., 1988). These observations indicate that *vps15* mutants are competent for vacuole assembly and suggest that the wild-type *VPS15* gene product might be specifically involved in the intracellular sorting and delivery of soluble vacuolar proteins.

Here we report on the cloning and sequencing of the wild-type *VPS15* gene and on the identification and characterization of its protein product. The *VPS15* gene encodes a protein that exhibits significant sequence similarity to the catalytic domains of the serine/threonine family of protein kinases. We demonstrate that the mutational inactivation of the Vps15 protein (Vps15p) kinase domain results in a severe vacuolar protein sorting defect. In addition, in vivo phosphate labeling experiments demonstrate that Vps15p is a phosphoprotein and that its phosphorylation is dependent on the presence of a wild-type Vps15p kinase domain. In all, our results suggest that Vps15p regulates specific protein phosphorylation reactions that are required for the efficient delivery of proteins to the yeast vacuole.

Results

Cloning and Characterization of the *VPS15* Locus

All of the 14 originally identified *vps15* alleles result in a severe vacuolar protein sorting defect, which is generally characterized by the secretion of >90% of the newly synthesized CPY as a Golgi-modified precursor molecule (Robinson et al., 1988; P. H., unpublished data). In addition, eight of the *vps15* alleles result in a severe temperature-sensitive (ts) growth defect; however, the extent of the vacuolar protein sorting defect is the same at both the permissive and nonpermissive growth temperatures (Robinson et al., 1988). The wild-type *VPS15* gene was cloned by complementation of the ts growth phenotype associated with the *vps15-2* allele. SEY15-2 cells (*vps15-2, ura3-52*; see Table 1) were transformed with a yeast genomic DNA library constructed in the multicopy plasmid YEp24 (Carlson and Botstein, 1982). Of approximately 10,000 Ura$^+$ transformants analyzed, 2 were observed to be temperature resistant. Plasmid DNA was isolated from these two transformants, amplified in Escherichia coli, and reintroduced into both SEY15-2 and SEY15-9 cells. Both plasmids, pPHY15-1 and pPHY15-2, were found to correct the ts growth defects associated with these *vps15* yeast strains. Restriction enzyme mapping demonstrated that the two complementing plasmids contained overlapping genomic DNA inserts. Because the ~9 kb genomic insert of pPHY15-1 (Figure 1A) was contained entirely within that of

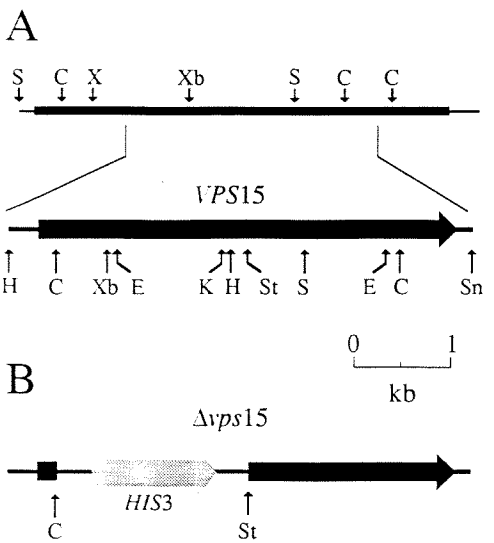

Figure 1. Characterization and Disruption of the *VPS15* Locus

(A) Restriction enzyme map of the ~9 kb genomic DNA insert in plasmid pPHY15-1. The bold line represents the yeast genomic DNA insert and the thin line the YEp24 vector sequences. The lower map is an enlargement of the 4.8 kb HindIII–SnaBI *VPS15* minimum complementing fragment with important restriction enzyme positions shown. Restriction enzymes are as follows: ClaI (C), EcoRI (E), HindIII (H), KpnI (K), SalI (S), SnaBI (Sn), StuI (St), XhoI (X), and XbaI (Xb). The large arrow indicates the *VPS15* coding region and the direction of transcription.

(B) A *VPS15* gene deletion/disruption is shown where the 2.2 kb ClaI–StuI fragment from within the *VPS15* coding region was replaced with the yeast *HIS3* gene. The large stippled arrow repesents the *HIS3* coding region.

pPHY15-2, only pPHY15-1 was analyzed further. Integrative mapping techniques demonstrated that the cloned DNA originated from a region of the yeast genome corresponding to the *vps15* mutant locus (see Experimental Procedures).

The *VPS15*-complementing activity was localized to a 4.8 kb HindIII–SnaBI fragment (Figure 1A) that complemented the *vps15* growth defect when present on a single copy (pPHY15C) as well as a multicopy (pPHY15E) plasmid. The ability of the pPHY15C plasmid to complement the other mutant phenotypes associated with the *vps15-2* allele was also tested. We directly analyzed the localization of CPY in yeast cells by labeling intact spheroplasts with Tran^{35}S-label, fractionating the cultures into supernatant (extracellular media) and pellet (cell-associated) fractions, and then immunoprecipitating with antisera to CPY. In wild-type yeast cells, >95% of the newly synthesized CPY was present as a 61 kd mature species in an intracellular fraction, indicative of correct delivery to the vacuole. In contrast, *vps15-2* cells secreted >95% of the CPY into the extracellular media fraction as a Golgi-modified 69 kd (p2) precursor form (similar to Δ*vps15* in Figure 6B). When the pPHY15C plasmid was introduced into the *vps15-2* mutant, this severe CPY sorting defect was completely corrected (data not shown). *vps15* mutants have also been found to be extremely sensitive to osmotic stress, as demonstrated by their inability to grow on media containing 1.5 M NaCl (Banta et al., 1988). The pPHY15C plasmid was able to complement fully the osmotic sensitivity of the *vps15-2* mutant (data not shown). Therefore, the cloned DNA present within the pPHY15C plasmid is capable of complementing all of the phenotypes associated with *vps15* cells.

Disruption of the *VPS15* Locus Results in a ts Growth Defect

To assess the phenotypic consequences of a *VPS15* null allele, we constructed a deletion/disruption of the *VPS15* gene. A linear fragment of the *VPS15* gene in which the 2.2 kb ClaI–StuI internal fragment had been replaced with the yeast *HIS3* gene (Figure 1B) was used to transform SEY6210.5 diploid cells (*his3-Δ200/his3-Δ200*) to histidine prototrophy by replacing one wild-type copy of the *VPS15* gene with the disrupted allele through homologous recombination (Rothstein, 1983). The structure of the disrupted allele was verified with a polymerase chain reaction (PCR) DNA amplification analysis (data not shown; see Herman and Emr, 1990). Two independent His$^+$ transformants were sporulated and their progeny were subjected to tetrad analysis. In the 27 tetrads analyzed, all haploid progeny were viable, indicating that the *VPS15* gene is not essential for vegetative growth at 26°C. Because our original selection had identified eight *vps15* alleles that exhibited a recessive ts growth defect, we examined the growth of these haploid progeny at 37°C. In all tetrads, the two His$^+$ progeny were unable to grow at 37°C, indicating that the *VPS15* gene product is required for growth at this elevated temperature. The ts progeny were observed to arrest growth after one or two cell divisions at 37°C. A second disruption of the *VPS15* gene confirmed these results (see Experimental Procedures). Therefore, the *VPS15* gene is required for vegetative growth only at elevated growth temperatures.

The Δ*vps15* strains exhibited the same spectrum of phenotypes as the original *vps15* mutants. As expected, Δ*vps15* cells mislocalized >95% of their CPY to the cell surface as the p2 precursor form (Figure 6B). Introduction of the wild-type *VPS15* gene (plasmid pPHY15C) completely corrected this sorting defect (Figure 6B). The Δ*vps15* yeast strains were also unable to grow on media supplemented with 1.5 M NaCl, and this osmotic sensitivity was complemented by the wild-type *VPS15* gene (data not shown). Δ*vps15* cells also possess a morphologically normal vacuole compartment.

The *VPS15* Gene Encodes a Protein That Has Significant Sequence Similarity to Protein Kinases

The 5.6 kb XhoI–ClaI fragment of pPHY15-1 (see Figure 1A) was sequenced using standard dideoxy chain termination methods as described in Experimental Procedures. The nucleotide sequence (Figure 2) identifies a single long open reading frame of 4365 bp contained entirely within the 4.8 kb HindIII–SnaBI *VPS15*-complementing fragment. This open reading frame has the potential to encode a protein of 1455 amino acids with a predicted molecular weight of 166,000. A hydrophobicity analysis of the predicted protein sequence (Kyte and Doolittle, 1982) indicates that Vps15p is relatively hydrophilic and appears to possess no N-terminal signal sequence or transmembrane domains (data not shown). Therefore, this protein would not be expected to enter the secretory pathway.

A comparison of the predicted amino acid sequence of Vps15p to other known protein sequences revealed that the N-terminal 300 amino acids of this protein share a significant degree of sequence similarity with the catalytic domains of the serine/threonine family of protein kinases (Figure 3). The most striking similarities were seen with the γ (catalytic) subunit of phosphorylase b kinase from rabbit skeletal muscle (Reimann et al., 1984), the protein product of the Schizosaccharomyces pombe *wee1*$^+$ gene (Russell and Nurse, 1987), and the ribosomal protein S6 kinase from Xenopus laevis (Jones et al., 1988). A lesser degree of sequence conservation was seen with the tyrosine protein kinases, such as pp60^{src} (Takeya and Hanafusa, 1983). Over the entire protein kinase catalytic domain, Vps15p shares 25% sequence identity with phosphorylase b kinase and 23% with the Wee1 protein. These values are consistent with the level of sequence conservation generally observed between unrelated protein kinases. In fact, phosphorylase b kinase and Wee1p share 25% sequence identity with each other over this region.

Protein kinases are generally classified into two relatively broad groups based on their substrate specificity: those that phosphorylate serine or threonine residues and those that modify tyrosine (see Hanks et al., 1988). The catalytic domains of both the serine/threonine and tyrosine protein kinases are modular structures in which regions of very high sequence conservation are interspersed with regions of little similarity (Hunter, 1987;

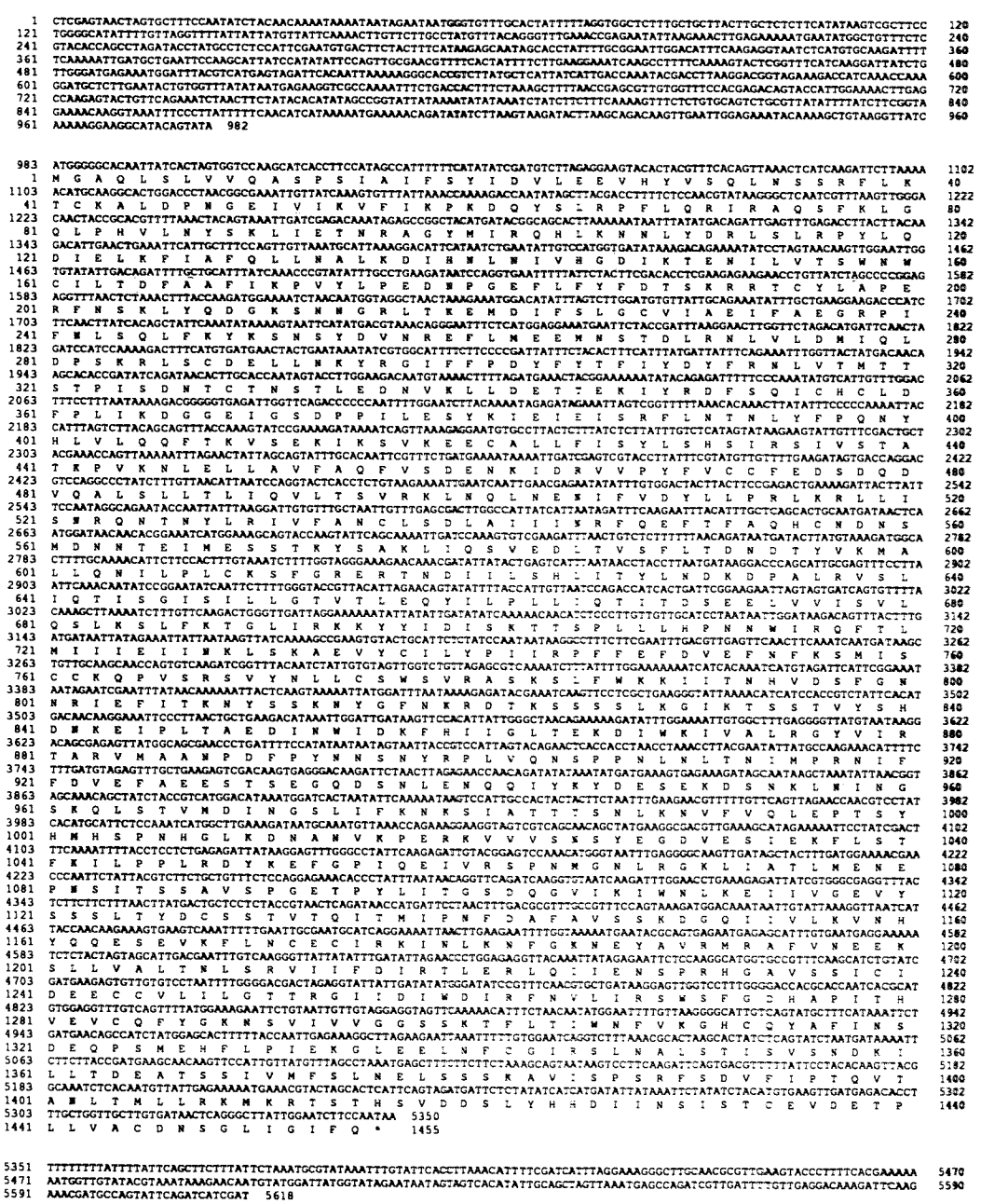

Figure 2. Nucleotide Sequence of the *VPS15* Locus

The DNA sequence of the *VPS15* coding strand and the predicted amino acid sequence of its product are shown. Amino acids are given in single-letter code.

Hanks et al., 1988). The highly conserved subdomains are presumably important for catalytic function either directly, as constituents of an active site, or indirectly, as structural elements required for the formation of the active site. The observed sequence similarity between Vps15p and protein kinases is clustered about those regions that are most highly conserved in the different protein kinase catalytic domains (Figure 3). Importantly, the Vps15p sequence

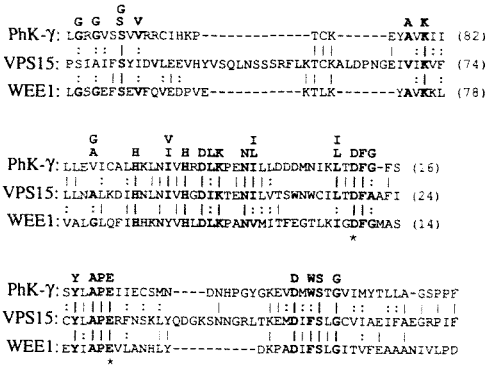

Figure 3. Comparison of the Vps15p Sequence with the Kinase Domains of Phosphorylase b Kinase and the Product of the *wee1*$^+$ Gene

The predicted sequence of Vps15p is compared with the catalytic domains of the two serine/threonine-specific protein kinases, phosphorylase b kinase from rabbit skeletal muscle (PhK-γ; Reimann et al., 1984) and the product of the S. pombe *wee1*$^+$ gene (Wee1p; Russell and Nurse, 1987). In the comparisons, identical amino acids are designated with a bar, and conservative changes are indicated with two dots. Gaps in the amino acid sequence are represented by dashed lines. The numbers given in parentheses indicate the number of amino acids separating the sequences shown. Residues that are very highly conserved in protein kinase catalytic domains are indicated in boldface above the sequence comparisons (see Hanks et al., 1988). The asterisks indicate the two amino acids altered in this study.

contains the consensus triplet A-P-E (see Figure 3), a conserved sequence element that is often referred to as an indicator of protein kinase catalytic domains (Hunter and Cooper, 1986).

An especially high degree of sequence conservation is seen in the central core of the protein kinase domain (shown in the bottom two comparisons of Figure 3), where Vps15p and phosphorylase b kinase share 42% sequence identity over a stretch of 96 amino acids. Included within this region is a short stretch of amino acids that has been used as an indicator of protein kinase substrate specificity (Hanks, 1987). Generally, serine/threonine-specific kinases possess a sequence resembling the consensus element DLKPEN, in which the lysine residue is absolutely conserved, while the tyrosine-specific kinases have either DLRAAN or DLAARN. The Vps15p sequence of DIKTEN closely resembles the serine/threonine consensus element, suggesting that Vps15p belongs to the serine/threonine family of protein kinases. A lower degree of sequence conservation with other protein kinases was seen in the most N-terminal regions of the Vps15p kinase-like domain (Figure 3; see Discussion). The observation that Vps15p possesses sequence similarity to the catalytic domain of protein kinases suggests that Vps15p may be a protein kinase and that protein phosphorylation may play an important role in controlling protein delivery to the yeast vacuole.

A visual examination of the predicted amino acid sequence of Vps15p identified a potential site for the attachment of myristic acid at the N-terminus of this protein. Myristic acid is a 14 carbon fatty acid that is cotranslationally added to an N-terminal glycine residue of specific eukaryotic proteins via an amide linkage (reviewed in Towler et al., 1988a). An extensive analysis of the substrate specificity of the yeast myristoyl CoA:protein N-myristoyltransferase (NMT) has suggested a consensus sequence for N-terminal myristoylation of $Gly_1-X_2-Z_3-Z_4-Ser_5-Z_6$ (Towler et al., 1987, 1988b). The yeast NMT enzyme exhibits an absolute specificity for a glycine residue at position 1 (following removal of the initiating methionine); the primary amino group of this glycine residue appears to be critical for the binding of substrate by yeast NMT (Towler et al., 1988a). In addition, a serine residue at position 5 favors a high affinity interaction of the protein/peptide substrate with the yeast NMT enzyme (Towler et al., 1988b). Finally, position 2 should be a small, uncharged amino acid (indicated by an X), and neutral residues (indicated by a Z) are preferred at the somewhat more permissive positions 3, 4, and 6. The Vps15p sequence of (Met)-$Gly_1-Ala_2-Gln_3-Leu_4-Ser_5-Leu_6$ fits this consensus, suggesting that Vps15p may be myristoylated in vivo (see below). The large C-terminal domain of Vps15p (>1000 amino acids) exhibits no significant sequence conservation with any other known protein sequence.

Vps15p Associates with a Yeast Membrane Fraction

Polyclonal antisera were raised against a trpE-Vps15 fusion protein and used in immunoprecipitation experiments from radiolabeled yeast cell extracts. These antisera detected a single ~170 kd protein in wild-type extracts that was not recognized by the preimmune control sera and was absent from Δ*vps15* yeast cell extracts (Figure 4). In addition, in yeast cells harboring a multicopy *VPS15* plasmid, the level of this 170 kd protein increased 30- to 50-fold (Figure 4). Therefore, these polyclonal antisera specifically recognize the product of the *VPS15* gene. An examination of the synthesis levels of Vps15p relative to CPY (~0.1% of total cell protein) by densitometric methods indicates that Vps15p constitutes less than 0.002% of the total yeast cell protein in logarithmically growing cultures (data not shown). Pulse-chase experiments indicate that Vps15p is a stable protein with a half-life of at least 60 min (Figure 4). It is interesting to note that a relatively high level of Vps15p overproduction does not result in a vacuolar protein sorting defect (see Figure 6B).

The absence of a signal sequence and any potential transmembrane domains in the predicted Vps15p sequence suggests that this protein does not enter the secretory pathway. Immunoprecipitation experiments with yeast cells treated with tunicamycin, a potent inhibitor of N-linked glycosylation, are consistent with this prediction. The Vps15p detected in tunicamycin-treated cells migrates with the same apparent molecular weight as the wild-type protein on SDS–polyacrylamide gels, suggesting that none of the 15 potential N-linked glycosylation sites within the Vps15p sequence are utilized (Figure 4). Two additional observations indicate that Vps15p is in contact with the cytosol and not sequestered within the lumen of an intracellular organelle. First, protease protection experiments with osmotically lysed spheroplasts indicate that Vps15p is exposed to the yeast cytoplasm. In these

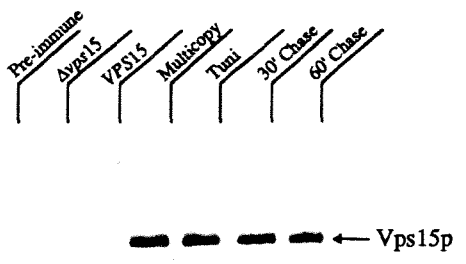

Figure 4. Identification and Characterization of the Vps15 Protein

Either eight (Δ*vps15* and *VPS15*) or two (all other lanes) OD_{600} units of yeast cells were labeled with Tran^{35}S-label in YNB glucose minimal medium for 20 min at 30°C. The radiolabeled cells were broken with glass beads and immunoprecipitations were performed from the clarified cell extracts as described in Experimental Procedures. In the first lane (Pre-immune), SEY6210 cells harboring the *VPS15* multicopy plasmid pPHY15E were immunoprecipitated with the preimmune control sera. In all other lanes, the Vps15p immune sera were used. The next three lanes show the effect of *VPS15* gene dosage on the synthesis levels of the ~170 kd Vps15p. No protein is detected in PHY112 cell extracts (Δ*vps15*). This protein is overexpressed 30-to 50-fold in SEY6210 cells possessing the pPHY15E plasmid (Multicopy) relative to the level seen in SEY6210 cells (*VPS15*). To assess the N-linked carbohydrate modification, SEY6210 cells with the plasmid pPHY15E were pretreated with 20 μg/ml tunicamycin for 15 min prior to labeling (Tuni). In the last two lanes, methionine and cysteine were added to a final concentration of 2 mM and the cultures were chased for 30 min or 60 min prior to cell lysis (30' Chase and 60' Chase). The position of Vps15p (~170 kd) is indicated.

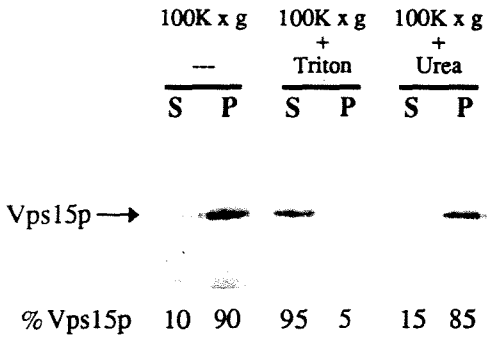

Figure 5. Subcellular Fractionation of the Vps15 Protein

SEY6210 cells harboring the *VPS15* multicopy plasmid pPHY15E were spheroplasted, labeled with Tran^{35}S-label, and osmotically lysed. The clarified cell lysates were incubated for 10 min at 0°C with either 2% Triton X-100, 2 M urea, or no additions and then centrifuged at 100,000 × g for 30 min at 4°C. Quantitative immunoprecipitations were performed from the supernatant and pellet fractions with antisera specific for Vps15p. A comparison of the relative levels of Vps15p detected following centrifugation relative to the level found in the total cell extract indicated that Vps15p recovery was greater than 80%.

experiments, spheroplasts were labeled with Tran^{35}S-label and gently lysed by the addition of DEAE–dextran under conditions that disrupt the yeast plasma membrane but maintain the structural integrity of internal organelles (see Klionsky and Emr, 1990). Following this lysis, Vps15p was found to be completely degraded by exogenously added proteinase K, whereas lumenal constituents of the ER, Golgi, and vacuolar compartments were resistant to this proteolysis (data not shown). In addition, biochemical labeling of yeast cells with tritiated myristic acid has demonstrated that Vps15p is myristoylated in vivo (P. Herman, unpublished data). Since the yeast NMT enzyme activity is cytoplasmic (see Towler et al., 1988a), the N-terminus of Vps15p must be exposed to the yeast cytosol. These data clearly demonstrate that Vps15p is in contact with the yeast cytosol.

We used differential centrifugation techniques to determine more precisely the intracellular location of Vps15p in vivo. In our initial experiments, yeast spheroplasts were radiolabeled and osmotically lysed, and the clarified lysates were immediately centrifuged at 100,000 × g for 30 min. Vps15p was then immunoprecipitated from the resulting supernatant and pellet fractions. Greater than 90% of Vps15p was detected in the pellet fraction, indicating that this protein is associated with a particulate fraction of yeast cell extracts (Figure 5). The nature of this Vps15p association was investigated by treating the clarified yeast lysates with either 2 M urea, 2% Triton X-100, or 1 M NaCl prior to centrifugation. Although treatment with 2 M urea and 1 M NaCl had very little effect, extraction with 2% Triton X-100 resulted in the solubilization of >95% of the particulate Vps15p (Figure 5 and data not shown). These data therefore suggest that Vps15p is associating with a membrane fraction of yeast cell extracts. This is especially interesting since the N-terminus of Vps15p possesses a consensus site for the addition of myristic acid and appears to be myristoylated in vivo. The N-terminal myristoylation of several proteins, including the product of the *c-src* proto-oncogene and the α subunits of G proteins, has been demonstrated to be essential for their membrane association (Buss et al., 1986; Jones et al., 1990).

In an attempt to characterize further the membrane association of Vps15p, radiolabeled yeast spheroplasts were osmotically lysed and subjected to a set of differential centrifugations, and the relative level of Vps15p within each fraction was assessed by immunoprecipitation. The majority, ~90%, of Vps15p was detected in the P100 fraction (Table 2). In contrast, p1 CPY (a marker for ER and early Golgi; Franzusoff and Schekman, 1989), p2 CPY (marker for Golgi; Stevens et al., 1986), and mature alkaline phosphatase (vacuolar membrane protein; Klionsky and Emr, 1990) were all found predominantly in the P13 fraction (Table 2). Interestingly, Kex2p, which is probably a resident of a late Golgi compartment (Julius et al., 1984), is also found largely within the P100 fraction (Table 2). Therefore, Vps15p exhibits a fractionation profile very similar to that of Kex2p. In addition to this late Golgi compartment, the P100 fraction probably includes the vesicular intermediates that transit between secretory pathway organelles (Walworth et al., 1989), presumably including those vesi-

Table 2. Differential Centrifugation with the Vps15 Protein

	P13	P100	S100
Vps15p	5%	90%	5%
p1 CPY	90%	5%	5%
p2 CPY	80%	15%	5%
ALP	90%	10%	<2%
Kex2p	25%	75%	<2%
PGK	5%	5%	90%

The relative levels of Vps15p and relevant protein markers in the 13,000 × g pellet (P13), 100,000 × g pellet (P100), and 100,000 × g supernatant (S100) fractions of yeast cell extracts were assessed by quantitative immunoprecipitation with the appropriate antisera as described in Experimental Procedures. PGK is a marker for the cytoplasmic fraction, p1 CPY for the ER and early Golgi compartments, p2 CPY for more distal Golgi compartments, alkaline phosphatase for the vacuolar membrane, and Kex2p for a late Golgi compartment.

cles trafficking between the Golgi and vacuolar compartments. Therefore, these experiments suggest that Vps15p is associating with a late Golgi compartment and/or membrane vesicles in yeast cells.

The Vps15p Kinase Domain Is Required for Vacuolar Protein Localization

To assess the functional relevance of the observed sequence similarity between Vps15p and protein kinases, we altered specific residues within the Vps15p kinase domain and examined the phenotypic consequences of these changes. Two different kinase domain mutations, each in a separate protein kinase subdomain, were constructed and analyzed. We based our *VPS15* mutations on previous mutational analyses performed on other known protein kinases. In the tyrosine kinase pp60^{src}, a series of single amino acid alterations was incorporated into the highly conserved A-P-E sequence triplet (Bryant and Parsons, 1984). All of the changes, including a glutamic acid to lysine substitution, resulted in dramatically lowered protein kinase activity and in the loss of pp60^{src} transforming ability. Based on this study, we constructed a mutant *vps15* allele that encoded a protein with an arginine residue replacing the glutamic acid normally present at amino acid 200 within the A-P-E sequence motif of Vps15p (E_{200}→R; Figure 6).

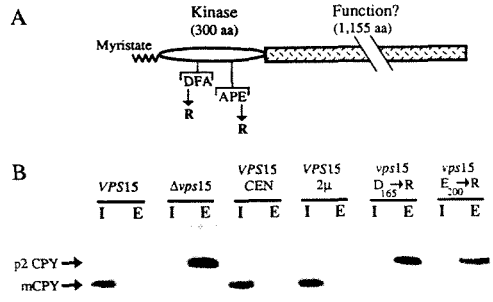

Figure 6. Intracellular Sorting of CPY

(A) A schematic representation of Vps15p showing the potential N-terminal myristic acid modification, the 300 amino acid protein kinase domain, and the large C-terminal domain of unknown function. The two kinase domain alterations used throughout this study are shown: D_{165}→R and E_{200}→R.

(B) Yeast spheroplasts were labeled with Tran^{35}S-label for 20 min at 30°C and then chased for 30 min following the addition of methionine and cysteine to 2 mM. The labeled cultures were centrifuged for 2 min at 13,000 × g and separated into pellet (I, intracellular) and supernatant (E, extracellular) fractions. The level of CPY in each fraction was assessed by quantitative immunoprecipitation with antisera to CPY. The strains examined were SEY6210 (*VPS15*), PHY112 (*Δvps15*), PHY112 with the pPHY15C plasmid (*VPS15* CEN), PHY112 with the pPHY15E plasmid (*VPS15* 2μ), PHY112 with the pPHY15C-K1 plasmid (*vps15* D_{165}→R), and PHY112 with the pPHY15C-K2 plasmid (*vps15* E_{200}→R). The positions of mature and p2 CPY are indicated. Neither of the kinase mutant alleles is able to complement the *Δvps15* CPY sorting defect.

For the second kinase domain mutant, a *vps15* allele that encoded a protein with an arginine substituted for aspartic acid at position 165 was constructed (D_{165}→R; Figure 6). An alteration of the corresponding aspartic acid in the product of the *CDC28* gene of S. cerevisiae results in a loss of in vitro protein kinase activity and *CDC28* gene function (Mendenhall et al., 1988). Plasmids encoding either the wild-type or one of the kinase domain mutant forms of Vps15p were introduced into *Δvps15* cells (PHY112; see Table 1). The ability of the mutant *vps15* alleles to complement the vacuolar protein sorting defects associated with the *Δvps15* mutant was examined. In *Δvps15* cells, greater than 95% of the newly synthesized CPY is secreted from the cell as a Golgi-modified p2 precursor (Figure 6B; see above). Introduction of the wild-type *VPS15* gene into the null mutant completely corrects this CPY sorting defect (Figure 6B). In contrast, neither of the *VPS15* kinase domain mutants is able to even partially complement the *Δvps15* protein sorting defect (Figure 6B), indicating that these mutations result in the biological inactivation of the *VPS15* gene product. These *vps15* mutant alleles are also unable to complement the ts growth defects and osmotic sensitivity associated with the *Δvps15* yeast strains (data not shown).

Pulse-chase immunoprecipitation experiments demonstrated that the defects associated with these altered forms of Vps15p are not due to decreased synthesis rates or decreased stability of the mutant proteins (see below). A wild-type Vps15p kinase domain therefore is required for the delivery of proteins to the yeast vacuole, suggesting that protein phosphorylation may be involved in the regulation of this protein sorting process.

Phosphorylation of Vps15p Requires a Wild-Type Vps15p Kinase Domain

Many protein kinases appear to catalyze specific autophosphorylation reactions (Krebs, 1986), although the biological significance of this phosphorylation in most cases is not known. We attempted to analyze whether Vps15p participated in a similar autophosphorylation reaction by first determining if this protein is phosphorylated in vivo and then whether or not this phosphorylation reaction is affected by mutations in the Vps15p kinase domain. To test if Vps15p is a phosphoprotein, we radiolabeled yeast cells with $^{32}PO_4$, lysed the cells with glass

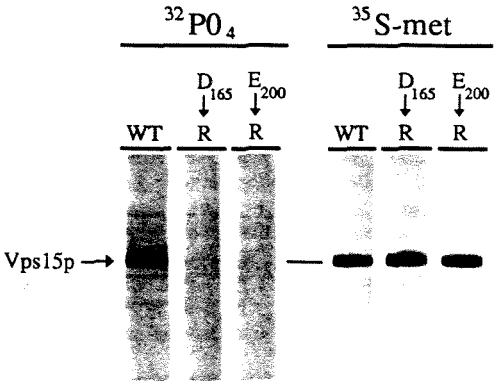

Figure 7. In Vivo Phosphorylation of the Vps15 Protein

Yeast cells were labeled with $^{32}PO_4$ for 20 min at 30°C and immunoprecipitated with antisera specific for Vps15p as described in Experimental Procedures. PHY112 (Δ*vps15*) cells harboring the following plasmids were examined: pPHY15E (WT), pPHY15E-K1 (D_{165}→R), and pPHY15E-K2 (E_{200}→R). The same strains were also labeled with Tran^{35}S-label for 20 min at 30°C, chased with methionine and cysteine (2 mM) for either 0, 30, 60 or 90 min, and then immunoprecipitated with the Vps15p antisera to assess the relative synthesis rates and stability of the three forms of Vps15p. Similar amounts of the three proteins were detected at all chase times; the 30 min chase point is shown. The position of Vps15p (~170 kd) is indicated.

beads, and then performed immunoprecipitations with antisera specific for Vps15p. The antisera detected a single labeled protein of ~170 kd in the extracts of yeast cells harboring a multicopy *VPS15* plasmid, indicating that Vps15p is phosphorylated in vivo (Figure 7). The dependence of this phosphorylation on the presence of a wild-type Vps15p kinase domain was examined by analyzing the phosphorylation of the proteins encoded by the two kinase domain mutant alleles in identical labeling experiments. Both of these mutant proteins were very poorly phosphorylated; the signal was decreased greater than 20-fold relative to that of the wild-type protein (Figure 7). However, both mutant proteins exhibit wild-type synthesis rates and stability (Figure 7), indicating that the low levels of phosphate incorporation are not due to reduced levels of these proteins in the cell. Therefore, the Vps15p kinase domain appears to be required for the in vivo phosphorylation of this protein.

Discussion

VPS15 gene function is required for the efficient delivery of soluble proteins to the yeast vacuole. With the aim of understanding the molecular role of the *VPS15* gene product in this protein sorting process, we have cloned and sequenced the wild-type *VPS15* gene and initiated a characterization of its protein product. A comparison of the predicted amino acid sequence of Vps15p with other known protein sequences revealed that Vps15p exhibits significant sequence similarity to the catalytic domains of the serine/threonine family of protein kinases (Figure 3). The observed sequence conservation is clustered about regions of the kinase domain that are very highly conserved among all known protein kinases (Hunter, 1987; Hanks et al., 1988). These sequence elements have been implicated in the ATP-binding and phosphotransferase activities associated with protein kinases (Brenner, 1987; Hanks et al., 1988). This sequence similarity therefore raises the interesting possibility that Vps15p is a protein kinase and that protein phosphorylation reactions regulate specific steps of the yeast vacuolar protein delivery pathway.

We constructed mutations within two separate regions of the Vps15p kinase domain (D_{165}FA→RFA and APE$_{200}$→APR) and assessed their effects on *VPS15* gene function and Vps15p phosphorylation. Phosphate labeling experiments demonstrated that Vps15p is phosphorylated in vivo (Figure 7). Furthermore, this phosphorylation was dependent on the presence of a wild-type Vps15p kinase domain, since both kinase domain mutant forms of Vps15p are very poorly phosphorylated in vivo (Figure 7). Alterations at the corresponding amino acid residues of other known protein kinases have been demonstrated to result in a dramatic decrease in associated kinase activity (Bryant and Parsons, 1984; Mendenhall et al., 1988). These data are therefore consistent with this kinase-like domain of Vps15p catalyzing a specific autophosphorylation reaction.

An alternative explanation is that the introduced amino acid changes alter Vps15p structure such that it can no longer serve as a substrate for another yeast protein kinase. However, several observations suggest that this latter possibility is unlikely. The Vps15 kinase domain mutant proteins are as stable as wild-type Vps15p in vivo, and, like the wild-type protein, these mutant proteins are also associated with the P100 fraction of yeast cell extracts (P. H., unpublished data). In addition, we have recently constructed a third mutation in a separate kinase subdomain of Vps15p (D_{147}→T of the DIKTEN sequence motif) and have found that this change also results in a significant decrease in the Vps15p phosphorylation signal (J. H. S. and P. H., unpublished data). Therefore, the alteration of three distinct Vps15p kinase domain motifs results in a dramatic loss of Vps15p phosphorylation in vivo. Altogether, these data suggest a direct role for the Vps15p kinase domain in the regulation of specific protein phosphorylation reactions. The two aspartic acid residues that were altered in Vps15p, D_{147} and D_{165}, correspond to amino acids that are conserved in a variety of phosphotransferase systems, including protein kinases and aminoglycoside phosphotransferases (Brenner, 1987). It has been suggested that these two aspartic acid residues may bind the ATP phosphate groups through an intermediate Mg^{2+} ion (Brenner, 1987), and it is therefore very interesting that changes in either of these residues result in a decrease in Vps15p activity.

We have demonstrated a functional role for the Vps15p kinase domain by analyzing vacuolar protein sorting in yeast cells possessing only the kinase-deficient forms of Vps15p. In these cells, as with Δ*vps15* cells, greater than 95% of the newly synthesized CPY is secreted in a Golgi-

modified precursor form (Figure 6B). Therefore, mutational alteration of the Vps15p kinase domain results in the biological inactivation of this protein. This, together with our observations concerning the dependence of Vps15p phosphorylation on the Vps15p kinase domain, suggests a role for protein phosphorylation in the vacuolar protein delivery pathway.

Although a high degree of sequence conservation is observed between protein kinases and Vps15p, relatively weak similarity is detected in the most N-terminal regions of the kinase domain (Figure 3). These sequence differences suggest that this protein may be a novel kinase homolog. This region of the kinase catalytic domain usually contains a Gly-X-Gly-X-X-Gly/Ser sequence element (where X refers to any amino acid), which has been termed a "nucleotide-fold" (see Walker et al., 1982). A similar sequence motif has been observed in both ATP- and GTP-binding proteins (Walker et al., 1982; McCormick et al., 1985; Dever et al., 1987), and X-ray crystallographic data of E. coli elongation factor Tu and the human c-H-*ras* protein have suggested that this subdomain interacts with the phosphate groups of the bound nucleotide (la Cour et al., 1985; de Vos et al., 1988). In Vps15p, the first and second glycine residues of this motif are replaced with a serine and an alanine residue, respectively (Figure 3). Since it has been suggested that an essential feature of the conserved glycines in this region is their small size (de Vos et al., 1988), it is possible that the conservative sequence substitutions observed in Vps15p may not alter the function of this kinase subdomain. A recently identified protein kinase homolog encoded by the Drosophila melanogaster *ninaC* locus has the sequence Ala-X-Gly-X-X-Ala, where alanines replace two of the glycines usually found in this sequence motif (Montell and Rubin, 1988).

Another interesting possibility is that Vps15p constitutes only a partial protein kinase domain. Vps15p may be part of a hetero-oligomeric complex in which Vps15p contributes the majority of the kinase catalytic domain and a second protein in the complex contributes the nucleotide-fold region and nearby sequences. Different kinase molecules already display some level of structural diversity with respect to the arrangement of their regulatory and catalytic domains. In some protein kinases, both domains are present on the same polypeptide chain (e.g., EGF receptor; see Ullrich and Schlessinger, 1990), while in others, these domains are present on distinct proteins (e.g., cAMP-dependent protein kinase; see Edelman et al., 1987). If the Vps15p kinase catalytic domain does possess such a novel structural arrangement, then it may be possible to identify a candidate gene that encodes this second component by examining the in vivo phosphorylation of Vps15p in other *vps* mutant backgrounds.

A more complete understanding of the role of Vps15p in vacuolar protein sorting will require the identification of other cellular components that functionally interact with this protein. The previous identification of a large number of *vps* mutants provides us with a starting point for this analysis. Potential regulators of Vps15p activity might be identified by assessing the extent of Vps15p phosphorylation in other *vps* mutant backgrounds. In addition, an analysis of the in vivo phosphorylation of other *VPS* gene products in *VPS15* and *vps15* yeast strains could identify potential substrates of the Vps15p kinase. Interestingly, two lines of evidence suggest that the products of the *VPS15* and *VPS34* genes may functionally interact in vivo (J. H. S. and P. H., unpublished data; see Herman and Emr, 1990). The overproduction of Vps34p in *vps15* kinase domain mutants partially suppresses both the growth defects and the vacuolar protein missorting defects associated with these mutants. In addition, chemical cross-linking experiments in total yeast cell extracts have indicated that Vps15p and Vps34p physically interact. Preliminary experiments also have demonstrated that Vps34p is a phosphoprotein in vivo, and we are currently analyzing this phosphorylation in kinase-deficient *vps15* yeast strains.

Subcellular fractionation and protease protection experiments (Figure 5 and Table 2) indicate that Vps15p is associated with the cytoplasmic face of an intracellular membrane, probably that of a late Golgi compartment or of a transport intermediate between the Golgi complex and the vacuole (e.g., vesicles). We are therefore presented with the problem of understanding how a protein kinase present on the cytoplasmic side of a secretory pathway organelle(s) is able to influence the delivery of specific lumenal constituents within this compartment. Some insight into this problem might be provided by observations from other systems that implicate protein phosphorylation as a key regulator of protein sorting. Specific protein phosphorylation reactions appear to act within protein sorting pathways at branch positions where proteins must choose between two or more different transport fates. The proper sorting of two transmembrane receptors within an early endosomal compartment appears to be dependent on specific protein phosphorylation reactions. Wild-type EGF receptors are targeted for lysosomal degradation, whereas kinase-inactive EGF receptors are observed to recycle back to the cell surface (Felder et al., 1990; Honegger et al., 1990). In addition, the phosphorylation of a specific serine residue within the cytoplasmic tail of the polymeric immunoglobulin receptor is required for its efficient transcytosis across polarized epithelial cells (Casanova et al., 1990). In the absence of this phosphorylation, these receptors appear to recycle back to the cell surface. In this study, we observed that a wild-type Vps15p kinase domain is required for the localization of proteins to the yeast vacuole. In each of these examples, the diversion of proteins from what is likely to be a default transport pathway into an auxiliary route appears to require specific protein phosphorylation reactions. Therefore, protein phosphorylation may act as a molecular "switch" within intracellular protein sorting pathways by actively diverting proteins from a default route into an alternative delivery pathway.

Other studies of eukaryotic protein secretion have suggested that GTP hydrolysis is an essential step for the vesicular transport of proteins between all secretory compartments (Balch, 1989) and that each interorganellar transfer event appears to involve a unique GTP-binding protein (see, for example, Salminen and Novick, 1987;

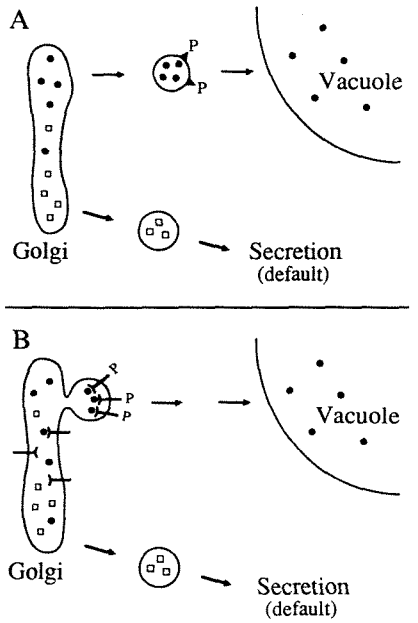

Figure 8. Possible Roles for Vps15p-Mediated Protein Phosphorylation in Vacuolar Protein Sorting

In (A), the phosphorylation of a specific vesicle surface protein designates that vesicle for delivery to, or fusion with, the vacuole. Alternatively, in (B), Vps15p-mediated phosphorylation might be required for the packaging of vacuolar proteins into the appropriate transport vesicles or for the formation of these transport vesicles. The phosphorylation of the cytoplasmic tails of transmembrane receptors specific for soluble vacuolar proteins may direct their proper packaging into transport vesicles destined for the yeast vacuole. The sorting of proteins to the yeast vacuolar compartment has been shown to be an active process that requires the recognition of specific sorting determinants present within the polypeptide backbone of vacuolar proteins. In the absence of this recognition, vacuolar proteins are secreted from yeast cells (reviewed in Klionsky et al., 1990; shown as the default pathway). See the text for further discussion.

Segev et al., 1988). It has been proposed that these GTP-binding proteins may be responsible for the unidirectionality observed in secretory protein traffic (Bourne, 1988) and may possibly designate a transport vesicle's final destination (Goud et al., 1988). Protein phosphorylation could then be thought of as a second, or an alternative, level of control superimposed upon this basic regulatory circuitry at specific branchpoints within a protein delivery pathway. In vacuolar protein sorting, Vps15p might function as such a switch in at least two different ways (Figure 8). The Vps15p-mediated phosphorylation of a specific vesicle surface protein (possibly Vps15p itself) could serve to direct the delivery, or fusion, of this transport carrier to the vacuolar membrane.

Alternatively, Vps15p might function in earlier steps of this sorting pathway, possibly in the selective packaging of vacuolar proteins into their appropriate vesicular carriers or in the formation of these vesicles. The observation that *vps15* cells are competent for vacuole assembly is most consistent with the latter packaging model, in which vesicular traffic to the vacuole could proceed almost normally except that the vesicles would lack much of their usual lumenal content. Vps15p might facilitate the selective packaging of appropriate vacuolar proteins by phosphorylating the cytoplasmic tails of transmembrane receptors specific for these soluble vacuolar hydrolases. Through the application of genetics together with a recently developed in vitro reconstitution assay for vacuolar protein sorting (Vida et al., 1990), we hope to be able to develop an understanding of the precise role Vps15p plays in the vacuolar protein sorting pathway of yeast. This understanding should provide insights into the more general role of protein phosphorylation in intracellular protein sorting processes.

Experimental Procedures

Strains and Media

The S. cerevisiae and E. coli strains used in this study are listed in Table 1. Standard genetic techniques were used to construct the yeast strains (Sherman et al., 1979). Standard yeast (Sherman et al., 1979) and E. coli (Miller, 1972) media were used and supplemented as needed.

Recombinant DNA Methods

All recombinant DNA manipulations were performed as described previously (Maniatis et al., 1982; Ausubel et al., 1987). The 4.8 kb *VPS15*-containing fragment from the upstream HindIII site to the SnaBI site in pPHY15-1 (see Figure 1A) was subcloned into either pSEYC58 or pSEY8 (Emr et al., 1986) to produce pPHY15C or pPHY15E, respectively. The plasmid pPHY15Δ1 was constructed by first subcloning the 3.8 kb XhoI–SalI fragment of pPHY15-1 (Figure 1A) into pUC8 and then replacing the 2.2 kb ClaI–StuI fragment within *VPS15* with the yeast *HIS3* gene (Figure 1B). The second *VPS15* disruption plasmid, pPHY15Δ2, was made by subcloning the 1.1 kb EcoRI–KpnI fragment of pPHY15-1 into the yeast *TRP1*-integrating vector pPHYI10 (Herman and Emr, 1990). The 3.8 kb XhoI–SalI fragment of pPHY15-1 was subcloned into pPHYI10 to produce the integrative mapping plasmid, pPHY122. The plasmid pPHY135 was constructed by subcloning the 1.4 kb EcoRI fragment of pPHY15-1 into pBluescript II KS$^+$ (Stratagene). The 1.2 kb ClaI fragment of pPHY135 was then subcloned into the ClaI site of pATH2 to construct a gene fusion of the E. coli *trpE* gene and *VPS15*.

Site-directed mutagenesis of the *VPS15* gene was performed using *dut*$^-$ *ung*$^-$ E. coli as previously described (Kunkel, 1985; Ausubel et al., 1987). The 1.8 kb XhoI–XbaI fragment of pPHY15-1 was subcloned into SalI- and XbaI-digested M13mp18 RF DNA to produce M13V15. Single-stranded uracil-containing M13V15 DNA was isolated from the E. coli strain BW313 (*dut*$^-$ *ung*$^-$), and the oligonucleotides 5′-GTATATTGACGCGTTTTGCTGCA-3′ and 5′-TCTAGCCCCGCGGAGGTTTAAC-3′ were used to mutagenize the *VPS15* sequences to produce M13V15-K1 and M13V15-K2, respectively. The mutagenized *VPS15* sequences were moved back into the full-length *VPS15* gene by exchanging the 2.4 kb HindIII fragment of pPHY15C with the mutated HindIII fragment from M13V15-K1 or M13V15-K2 to produce pPHY15C-K1 and pPHY15C-K2, respectively. Equivalent exchanges were performed with the pPHY15E plasmid to produce pPHY15E-K1 and pPHY15E-K2, respectively. K1 corresponds to a change of D_{165} to R and K2 to a change of E_{200} to R (see Figure 6A).

Yeast Genetic Methods

Standard yeast genetic methods were used throughout this study (Sherman et al., 1979). Yeast transformation was achieved by the method of alkali cation treatment (Ito et al., 1983) and transformants were selected on SD media.

The *VPS15* gene was cloned by complementation of the severe ts growth defect associated with the *vps15-2* allele. SEY15-2 cells (*vps15-2, ura3-52*) were transformed with a yeast genomic DNA library constructed in the YEp24 plasmid (DNA kindly provided by M. Carlson;

Carlson and Botstein, 1982). Ura$^+$ transformants were selected at 26°C and were subsequently replicated to 37°C YPD plates. Plasmids conferring a temperature-resistant phenotype upon the cells were isolated and analyzed.

Two different schemes were used to generate gene disruption/deletions of the *VPS15* locus. In the first, the plasmid pPHY15Δ1 was digested with HindIII and SalI and the 2.3 kb fragment was gel purified. This linearized DNA was then used to transform either the diploid strain SEY6210.5 or the haploid strains SEY6210 and SEY6211 (see Table 1) to histidine prototrophy. With the second method, an integrative disruption of the *VPS15* gene was constructed by digesting pPHY15Δ2 DNA with EcoRV (the EcoRV site is internal to the *VPS15* EcoRI–KpnI fragment) and transforming the diploid yeast strain SEY6210.5 to tryptophan prototrophy. Integration would result in the replacement of the wild-type *VPS15* locus with two noncomplementing, but overlapping, fragments of the *VPS15* gene. In 23 tetrads examined the Trp$^+$ phenotype segregated 2:2 and absolutely cosegregated with a ts growth defect. In both constructions, the genomic DNA structure about the *VPS15* locus was verified with a PCR amplification method previously described (Herman and Emr, 1990).

For integrative mapping, the plasmid pPHY122 was digested with EcoRV to direct its integration to the chromosomal homolog of the cloned DNA (Rothstein, 1983). SEY15-2 cells were transformed with this linearized DNA, and four independent Trp$^+$ transformants were analyzed and found to be temperature resistant. (The XhoI–SalI fragment encodes a truncated form of Vps15p that complements the *vps15-2* ts growth defect.) Two of these transformants were crossed to SEY6211, and the resultant diploids were sporulated and their meiotic progeny were analyzed by tetrad analysis. A 4 Ts$^+$:0 Ts$^-$ segregation pattern was observed for all 21 tetrads analyzed, indicating that the identified cloned DNA represented the authentic *VPS15* locus.

DNA Sequencing and Sequence Analysis

Restriction fragments encompassing the region from the XhoI site to the rightmost ClaI site in Figure 1A were subcloned into the appropriate pBluescript vectors and sequenced using standard dideoxy chain termination methods (Sanger et al., 1977) as previously described (Herman and Emr, 1990).

The predicted protein sequence of the *VPS15* gene product was compared with the contents of the National Biomedical Research Foundation (NBRF) protein data base and the GenBank DNA data base with the FASTA and TFASTA programs (Pearson and Lipman, 1988), respectively. The comparisons were performed with the University of Wisconsin Genetics Computer Group sequence analysis package for VAX/VMS computers (Devereux et al., 1984).

Preparation of Vps15p Antisera

A gene fusion was constructed between the E. coli *trpE* gene and *VPS15* using the pATH vector system (Dieckmann and Tzagoloff, 1985). The 1.2 kb ClaI–EcoRI fragment of *VPS15* was subcloned into the pATH2 polylinker (see above for details) to produce the plasmid pPHY139. This plasmid encodes a trpE–Vps15 fusion protein containing 246 amino acids of Vps15p, from amino acid number 21 to 266. The trpE–Vps15p fusion was induced, prepared, and used to immunize New Zealand White male rabbits as described previously (Herman and Emr, 1990).

Cell Labeling and Immunoprecipitation

Immunoprecipitations from whole yeast cells labeled with Tran^{35}S-label (ICN Radiochemicals) were performed as described previously (Herman and Emr, 1990) except that urea-cracking buffer (10 mM sodium phosphate [pH 7.2], 6 M urea, 1% SDS, 1% β-mercaptoethanol) was substituted for the boiling buffer and the final sample buffer. If Vps15p is boiled in normal SDS-containing buffers, in the absence of urea, it forms an aggregate that is unable to enter the SDS–polyacrylamide resolving gel. This molecular aggregate is efficiently dissociated by the addition of urea. To assess N-linked oligosaccharide modification, yeast cells were incubated in the presence of 20 μg/ml tunicamycin for 15 min prior to labeling. The CPY fractionation immunoprecipitations were performed as previously described (Robinson et al., 1988). Radiolabeled proteins were electrophoresed on 8% SDS–polyacrylamide gels. Following electrophoresis the gels were fixed in 50% methanol, 10% acetic acid, and 10% trichloroacetic acid (TCA) and treated with Autofluor (National Diagnostics).

The subcellular fractionation experiments were performed as described previously (Herman and Emr, 1990) with the following modifications. The yeast strain SEY6210, harboring the plasmid pPHY15E, was grown to mid-logarithmic phase, spheroplasted, and labeled with Tran^{35}S-label for 15 min at 30°C. The cells were then osmotically lysed and the resulting clarified lysate was centrifuged at 13,000 × g for 15 min at 4°C. The 13,000 × g supernatant was carefully removed and centrifuged at 100,000 × g for 30 min at 4°C. The 100,000 × g supernatant fraction (S100) was made 5% with respect to TCA and precipitated for 20 min on ice. The 13,000 × g pellet (P13) and 100,000 × g pellet (P100) fractions were resuspended in 5% TCA and also held on ice for 20 min. Immunoprecipitations were performed as described above with the appropriate antiserum. The antiserum to PGK was a gift from Dr. Jeremy Thorner and to Kex2p from Dr. William Wickner. The antisera to CPY (Robinson et al., 1988) and alkaline phosphatase (Klionsky and Emr, 1990) were described previously. The extraction studies with Vps15p were performed as previously described (Herman and Emr, 1990).

In Vivo Phosphorylation Assays

Yeast cells were grown to mid-logarithmic phase in Wickerham's minimal medium supplemented with 0.2% yeast extract (Wickerham, 1946; Robinson et al., 1988). Five OD_{600} units of cells were collected by centrifugation, resuspended in 1 ml of LPSM medium (Reneke et al., 1988) supplemented with 5 mM $MgSO_4$, and incubated for 30 min at 30°C. Bovine serum albumin (to a final concentration of 1 mg/ml) and 500 μCi of $^{32}PO_4$ (Amersham) were then added, and the cells were incubated for 20 min at 30°C. The labeling was terminated by the addition of TCA to a final concentration of 5%. The TCA pellet was washed twice with acetone, dried, and resuspended in 100 μl of the urea-cracking buffer. One milliliter of Tween-20 immunoprecipitation buffer (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 0.1 mM EDTA, 0.5% Tween-20) and 10 μl of a 100 mg/ml bovine serum albumin solution were added, and immunoprecipitations were performed with antisera to Vps15p as described above except that the protein A–Sepharose beads were washed as follows: twice with Tween-20 IP buffer; once with Tween–urea buffer (100 mM Tris–HCl [pH 7.5], 200 mM NaCl, 2 M urea, 0.5% Tween-20); once with IP salts buffer (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 0.1 mM EDTA); once with 1% β-mercaptoethanol; and once with 0.5 M LiCl, 0.1 M Tris–HCl (pH 7.5).

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Chapter 3:

A Genetic and Structural Analysis of the Yeast Vps15 Protein Kinase: Evidence for a Direct Role of Vps15p in Vacuolar Protein Delivery

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A genetic and structural analysis of the yeast Vps15 protein kinase: evidence for a direct role of Vps15p in vacuolar protein delivery

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The yeast *VPS15* gene encodes a novel protein kinase homolog that is required for the sorting of soluble hydrolases to the yeast vacuole. In this study, we extend our previous mutational analysis of the *VPS15* gene and show that alterations of specific Vps15p residues, that are highly conserved among all protein kinase molecules, result in the biological inactivation of Vps15p. Furthermore, we demonstrate here that short C-terminal deletions of Vps15p result in a temperature-conditional defect in vacuolar protein sorting. Immediately following the temperature shift, soluble vacuolar hydrolases, such as carboxypeptidase Y and proteinase A, accumulate as Golgi-modified precursors within a saturable intracellular compartment distinct from the vacuole. This vacuolar protein sorting block is efficiently reversed when mutant cells are shifted back to the permissive temperature; the accumulated precursors are rapidly processed to their mature forms indicating that they have been delivered to the vacuole. This rapid and efficient reversal suggests that the accumulated vacuolar protein precursors were present within a normal transport intermediate in the vacuolar protein sorting pathway. In addition, this protein delivery block shows specificity for soluble vacuolar enzymes as the membrane protein, alkaline phosphatase, is efficiently delivered to the vacuole at the non-permissive temperature. Interestingly, the C-terminal Vps15p truncations are not phosphorylated *in vivo* suggesting that the phosphorylation of Vps15p may be critical for its biological activity at elevated temperatures. The rapid onset and high degree of specificity of the vacuolar protein delivery block in these mutants suggests that the primary role of Vps15p is to regulate the sorting of soluble hydrolases to the yeast vacuolar compartment.

Key words: lysosome/protein kinase/protein sorting/vacuole

Introduction

The cytoplasmic environment of eukaryotic cells is subdivided into a number of functionally distinct membrane-enclosed organelles. In order to maintain the functional integrity of these organelles, specific mechanisms must exist to allow the cell efficiently to sort and deliver proteins from their common site of synthesis in the cytoplasm to their appropriate final destinations. The secretory pathway of eukaryotic cells is responsible for the proper modification and delivery of proteins to the cell surface and to a variety of intracellular compartments (Pfeffer and Rothman, 1987). The delivery of proteins to the lysosomal, or vacuolar, compartment of eukaryotic cells is mediated by the secretory pathway and is one of the best characterized examples of an intracellular protein sorting process (Kornfeld and Mellman, 1989; Klionsky *et al.*, 1990). Following translocation across the membrane of the endoplasmic reticulum, lysosomal proteins transit to the Golgi complex together with proteins destined for secretion from the cell. Within a late Golgi compartment, lysosomal proteins are sorted away from the secretory protein traffic and are targeted to the lysosome. This routing of proteins from the default secretion path is an active process requiring specific sorting information present within lysosomal proteins.

In mammalian cells, the best characterized lysosomal sorting system involves the specific modification of soluble lysosomal enzymes with a mannose-6-phosphate moiety (Kaplan *et al.*, 1977; reviewed in Kornfeld and Mellman, 1989). This carbohydrate modification is recognized in the Golgi apparatus by specific membrane receptors that mediate delivery of the modified proteins to the lysosome. In contrast, the *cis*-acting sorting information present in several yeast vacuolar proteins is not associated with any specific carbohydrate modification and instead appears to reside within the polypeptide backbone of these proteins (Johnson *et al.*, 1987; Valls *et al.*, 1987; Klionsky *et al.*, 1988; Klionsky and Emr, 1990). Despite the gains made in our understanding of the initial recognition of lysosomal and vacuolar proteins, very little is presently known about the cellular components that function subsequently to bring about the proper packaging and delivery of these proteins to the lysosomal and vacuolar compartments.

In the yeast, *Saccharomyces cerevisiae*, an extensive genetic analysis of vacuolar protein localization has been undertaken in an attempt to develop a better understanding of the *trans*-acting machinery responsible for mediating protein delivery to the vacuole (reviewed in Klionsky *et al.*, 1990). Two independent genetic selections have identified a large number of yeast mutants defective in the localization of multiple soluble vacuolar hydrolases, including carboxypeptidase Y (CPY), proteinase A (PrA) and proteinase B (PrB) (Bankaitis *et al.*, 1986; Rothman and Stevens, 1986; Robinson *et al.*, 1988; Rothman *et al.*, 1989). Rather than delivering these proteins to the vacuole, *vps* mutants (for vacuolar protein sorting defective) missort these enzymes to the cell surface as Golgi-modified precursors. In addition to these localization defects, many of the *vps* mutants exhibit

additional phenotypes including defects in vacuole biogenesis and segregation, severe temperature-sensitive (*ts*) growth defects and defects in the sorting of vacuolar membrane proteins (Banta *et al.*, 1988; Robinson *et al.*, 1988; Herman and Emr, 1990; Raymond *et al.*, 1990). Complementation analyses between the *vps* mutants and other related sets of mutants, have demonstrated that there are at least 47 complementation groups required for the efficient targeting of vacuolar proteins in yeast (Klionsky *et al.*, 1990). This high level of genetic complexity indicates that vacuolar protein delivery is a complex process requiring the direct, or indirect, participation of a relatively large number of gene functions. In order to gain an understanding of the basic cellular processes underlying vacuolar and lysosomal protein delivery, we have initiated efforts to identify specific *VPS* gene products and to characterize, genetically and biochemically, the step(s) of the sorting pathway at which these products function (Banta *et al.*, 1990; Herman and Emr, 1990; Herman *et al.*, 1991).

Several experimental observations indicate that the yeast *VPS15* gene product may play a central role in regulating the sorting of soluble hydrolases to the vacuole (Herman *et al.*, 1991). First, mutations in the *VPS15* gene result in severe defects in the localization of several vacuolar hydrolases, including CPY, PrA and PrB (Robinson *et al.*, 1988; Herman *et al.*, 1991). These defects appear to be relatively specific for soluble constituents of the yeast vacuole as the localization of two vacuolar membrane proteins, alkaline phosphatase (ALP) and α-mannosidase, appears to be less defective in *vps15* mutants (Robinson *et al.*, 1988; Klionsky and Emr, 1989; Herman *et al.*, 1991). Second, the cloning and sequencing of the *VPS15* gene has indicated that the predicted Vps15 protein (Vps15p) sequence exhibits significant similarity to the catalytic domains of the serine/threonine family of protein kinases. Mutations altering highly conserved residues within the Vps15 protein kinase domain result in the biological inactivation of this protein and eliminate *in vivo* phosphorylation of Vps15p (Herman *et al.*, 1991). Finally, subcellular fractionation studies indicated that Vps15p may be associated with the cytoplasmic face of a late Golgi or vesicle compartment (Herman *et al.*, 1991). Since vacuolar protein sorting appears to occur within a late Golgi compartment (Graham and Emr, 1991; see Klionsky *et al.*, 1990), this intracellular location is consistent with Vps15p having a direct influence upon vacuolar protein sorting. On the basis of these observations, we proposed that Vps15p-mediated phosphorylation events may play a key role in regulating protein delivery to the yeast vacuole and that protein phosphorylation reactions, in general, might act as a molecular 'switch' within the eukaryotic secretory pathway to divert proteins from a default transit pathway (e.g. secretion) to an alternative pathway (e.g. to the vacuole).

In this study, we extend our mutational analysis of the Vps15p kinase domain and also examine the phenotypic consequences of alterations within the myristic acid attachment site and the large carboxy-terminal (C-terminal) domain of Vps15p. We find that relatively short C-terminal deletions of Vps15p result in a rapid, severe and highly specific, temperature-conditional defect in vacuolar protein delivery. Biochemical analysis of the *ts* vacuolar protein sorting defects, and their efficient reversal, indicates that Vps15p is directly involved in the delivery of soluble hydrolases to the yeast vacuolar compartment.

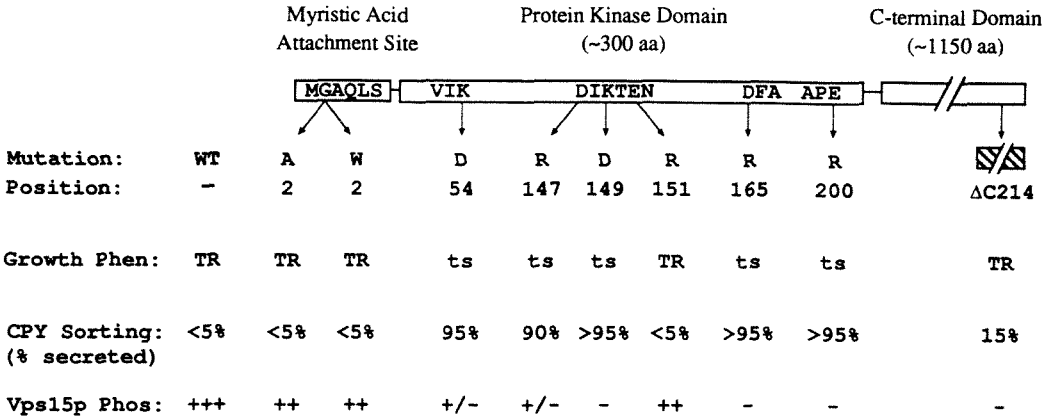
Results

Mutational analysis of the Vps15p kinase domain

Our initial analysis of the predicted Vps15p amino acid sequence suggested that this protein could be divided into three separate sequence domains (Herman *et al.*, 1991; Figure 1). The immediate N-terminal sequence of Vps15p contains a potential attachment site for myristic acid, a rare 14-carbon fatty acid (see below). The next ~300 amino acids of Vps15p exhibit significant sequence similarity to the catalytic domains of protein kinases. We assessed the functional significance of the Vps15p kinase domain sequences by altering two of the most conserved kinase motifs of Vps15p and analyzing the phenotypic consequences of these changes. Both of the alterations, D165R (i.e. D165 → R) and E200R, resulted in the biological inactivation of Vps15p (Herman *et al.*, 1991; see Figure 1). This mutational analysis suggested that Vps15p functions as a protein kinase *in vivo* and that specific protein phosphorylation reactions are required for the efficient delivery of proteins to the yeast vacuole. The third domain consists of the remaining C-terminal sequences of Vps15p (>1100 amino acids). This region of Vps15p exhibited no significant similarities to any other known protein sequence. The tripartite nature of the 1455 amino acid Vps15p is shown schematically in Figure 1.

The first kinase motif examined includes the Vps15p sequence DIKTEN and corresponds to the kinase domain region that exhibits the highest degree of sequence conservation between different protein kinases (Hanks *et al.*, 1988). In addition, the sequence conservation observed in this region of protein kinases also serves as an indicator of kinase substrate specificity (Hanks, 1987). The lysine residue within this sequence motif (K149 in the Vps15p sequence) is conserved in all known serine/threonine protein kinase sequences. In tyrosine-specific protein kinases, this lysine residue is replaced with either an alanine or an arginine residue (however, see Ben-David *et al.*, 1991; Featherstone and Russell, 1991). Using oligonucleotide-directed mutagenesis, we constructed a *VPS15* allele that encoded a protein with aspartic acid replacing this lysine at position 149 (K149D). This alteration resulted in the biological inactivation of Vps15p in each of three different assays; the K149D mutant exhibited a severe *ts* growth defect, mislocalized >95% of the newly synthesized CPY to the cell surface in a Golgi-modified precursor form (p2 CPY) and was defective for Vps15p phosphorylation *in vivo* (Figures 1 and 2).

Two additional alterations, D147R and E151R, were constructed within this kinase domain motif. The aspartic acid at position 147 of Vps15p is very highly conserved and is found in a corresponding position in all known protein kinases, both serine/threonine and tyrosine-specific (Hanks *et al.*, 1988). In addition, the aspartic acids D147 and D165 correspond to residues that appear to be conserved in a variety of phosphotransferase systems, including protein kinases and aminoglycoside phosphotransferases (Brenner, 1987). It was suggested that this common pair of aspartic acids could be involved in the binding of the ATP phosphate groups. The substitution of an arginine residue for the aspartic acid at position 147 resulted in a severe reduction in Vps15p activities. The D147R mutant exhibits a severe *ts* growth defect and mislocalizes ~90% of the newly synthesized CPY to the cell surface as p2 CPY (Figures 1 and 2). In addition, the D147R protein is very poorly



Mutation:	**WT**	**A**	**W**	**D**	**R**	**D**	**R**	**R**	**R**	
Position:	**-**	**2**	**2**	**54**	**147**	**149**	**151**	**165**	**200**	**ΔC214**
Growth Phen:	**TR**	**TR**	**TR**	**ts**	**ts**	**ts**	**TR**	**ts**	**ts**	**TR**
CPY Sorting: (% secreted)	**<5%**	**<5%**	**<5%**	**95%**	**90%**	**>95%**	**<5%**	**>95%**	**>95%**	**15%**
Vps15p Phos:	**+++**	**++**	**++**	**+/-**	**+/-**	**-**	**++**	**-**	**-**	**-**

Fig. 1. Summary of the mutational analyses of *VPS15*. A schematic representation of the tripartite nature of the Vps15p sequence is shown. The alterations made at each residue are indicated below the bar diagram of Vps15p along with the amino acid position of this residue in the Vps15p sequence. The growth phenotype (GrowthPhen) indicates the ability of the different *vps15* mutants to grow at 38°C on YPD plates. The next row indicates the extent of the CPY sorting defect in the different mutants. To analyze CPY localization, the engineered *vps15* alleles were introduced into a Δ*vps15* yeast strain (PHY112) on a centromere-containing plasmid. Yeast spheroplasts were labeled with Tran^{35}S-label, fractionated into supernatant (extracellular) and pellet (intracellular) fractions and then immunoprecipitated with antisera specific for the vacuolar hydrolase CPY. The relative levels of the different forms of CPY in each fraction were determined and the values in the table indicate the percentage of the total CPY that is found as p2 CPY in the extracellular fraction. The final row (Vps15p Phos) is a relative measure of the extent of ^{32}P incorporation into Vps15p in the different mutants. The *vps15* alleles were introduced into the strain PHY112 on a 2μ multicopy plasmid. These different yeast strains were then labeled with [^{32}P]orthophosphate and immunoprecipitations were performed from yeast cell extracts with antisera specific for Vps15p. '++' indicates a signal ~30–50% of wild-type; '+', ~5–10% of wild-type; and '–' indicates that no detectable phosphorylation was observed. The relative synthesis rates and stability of each of the mutant Vps15 proteins were assessed in a series of pulse–chase immunoprecipitation experiments. Δ*vps15* yeast strains, expressing the appropriate Vps15p mutant protein, were pulse labeled with Tran^{35}S-label for 5–10 min and then chased for 0, 45 or 90 min following the addition of unlabeled methionine and cysteine. The relative levels of Vps15p present at each time point were determined by quantitative autoradiography following immunoprecipitation with antisera specific for Vps15p and separation on SDS–polyacrylamide gels. In addition, the steady-state levels of most of the altered Vps15p proteins were analyzed by immunoblotting experiments with antisera specific for Vps15p. Together, these analyses indicated that the steady-state level of each of the mutant proteins was very similar to that of wild-type Vps15p (data not shown; see Herman *et al.*, 1991).

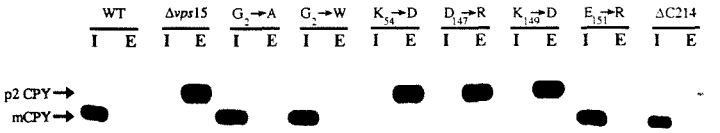


Fig. 2. Intracellular sorting of CPY in yeast *vps15* mutants. Yeast spheroplasts were labeled with Tran^{35}S-label for 20 min at 26°C and then chased for 30 min following the addition of methionine and cysteine to 25 mM. The labeled cultures were centrifuged for 2 min at 13 000 *g* and separated into pellet (I, intracellular) and supernatant (E, extracellular) fractions. The level of CPY in each fraction was assessed by quantitative immunoprecipitation with antisera to CPY. In each sample, the strain examined was PHY112 (Δ*vps15::HIS3*) carrying a particular *vps15* allele on a centromere-containing plasmid. WT refers to the wild-type allele (pPHY150) and Δ*vps15* to PHY112 harboring the vector plasmid (pPHYC18). For all other samples, the particular *vps15* allele present would encode a Vps15p protein with the indicated alteration (see Figure 1). The positions of mature (61 kDa) and p2 CPY (69 kDa) are indicated.

phosphorylated *in vivo*. The D147R protein incorporates only ~5–10% of the $^{32}PO_4$ incorporated by the wild-type Vps15p in a similar labeling experiment.

The final alteration of the DIKTEN sequence motif, E151R, was constructed on the basis of observations made with several serine/threonine-specific kinases that suggested that this residue was critical for kinase interaction with substrate (Taylor *et al.*, 1990). In general, protein kinases having a preference for basic amino acids preceding the substrate phosphorylation site have an acidic residue conserved at this position. Conversely, a basic amino acid tends to be present in kinases with a preference for acidic groups near the target phosphorylation site. We therefore assessed the phenotypic effects of substituting a basic residue, arginine, for the glutamic acid (E151) normally present at this position in Vps15p. As seen in Figure 1, this alteration had only a modest effect on Vps15p activity as judged by our three assays. While 38°C growth and CPY sorting appeared normal with the E151R mutant (Figures 1 and 2), the level of $^{32}PO_4$ incorporation into this altered Vps15p was only ~35–50% of that seen with the wild-type Vps15p *in vivo* (Figure 1).

Finally, we altered Vps15p at lysine residue K54 in the kinase domain. This residue corresponds to an invariant lysine observed in both serine/threonine and tyrosine protein kinases (Hanks *et al.*, 1988; Taylor *et al.*, 1990). This lysine

residue has been suggested to be within, or near, the ATP binding domain of protein kinases as it can be specifically labeled with ATP analogs such as *p*-fluorosulfonyl 5′-benzoyl adenosine (Zoller *et al.*, 1981; Kamps and Sefton, 1984). We constructed a mutant *VPS15* allele that encodes a protein with an aspartic acid replacing this lysine, K54D, and have found that this alteration dramatically reduces Vps15p activity *in vivo*. The K54D mutant exhibits a severe *ts* growth defect and mislocalizes ~95% of the newly synthesized CPY to the cell surface (Figures 1 and 2). In addition, this mutant is also very defective for the *in vivo* phosphorylation of Vps15p (Figure 1).

Vps15p is modified with myristic acid at its N terminus

The 14-carbon fatty acid, myristate, is added co-translationally to the N-terminal glycine residue of many cellular proteins following the removal of the initiating methionine (reviewed in Schultz *et al.*, 1988; Towler *et al.*, 1988b). Our previous studies indicated that the N-terminal sequence of Vps15p, (Met1)-Gly2-Ala3-Gln4-Leu5-Ser6-Leu7, was in good agreement with a proposed consensus sequence for myristic acid addition and therefore suggested that Vps15p could be myristoylated at its N terminus (Herman *et al.*, 1991). To analyze directly whether Vps15p was myristoylated *in vivo*, we radiolabeled yeast cells with tritiated myristic acid for 60 min and then performed immunoprecipitations with antisera specific to Vps15p. Vps15p was observed to be specifically labeled in these experiments suggesting that it is modified by the addition of myristic acid *in vivo* (Figure 3). Further support for this assertion was obtained from our analysis of two different *vps15* mutants that possessed alterations in the myristic acid attachment site. In these mutants, the critical glycine residue at position 2 was changed to either an alanine, G2A, or a tryptophan, G2W (Figure 1). Consistent with results from previous studies on the substrate specificity of the yeast myristoyl CoA:protein N-terminal myristoyltransferase (Towler *et al.*, 1987, 1988a), neither of the mutant Vps15p proteins were labeled by the [^{3}H]myristic acid in these experiments (Figure 3).

Although these two alterations abolished the myristoylation of Vps15p, the non-myristoylated Vps15 proteins appeared to possess near wild-type levels of biological activity *in vivo*. The G2A and G2W mutants both exhibited near wild-type growth rates at 38°C and neither mutant was defective for CPY delivery to the vacuole at 26°C (Figures 1 and 2). Previous cell-fractionation studies had indicated that Vps15p was associated with a membrane fraction of yeast cell extracts (Herman *et al.*, 1991). Since the N-terminal myristoylation of several eukaryotic proteins results in their association with a specific intracellular membrane, we examined whether the non-myristoylated Vps15p proteins remained associated with a membrane fraction. Differential centrifugation experiments demonstrated that, like wild-type Vps15p, the G2A mutant protein was associated with a 100 000 *g* pellet fraction and could be extracted from this fraction with 1% Triton X-100 (data not shown). Therefore, the membrane association of Vps15p does not appear to be mediated solely by the N-terminal myristic acid moiety. As with the kinase domain mutant, E151R (see above), the non-myristoylated Vps15 proteins were observed to be partially defective in the *in vivo* Vps15p phosphorylation reaction, as the phosphorylation signal was only ~35–50% of that observed with the wild-type protein (Figure 1).

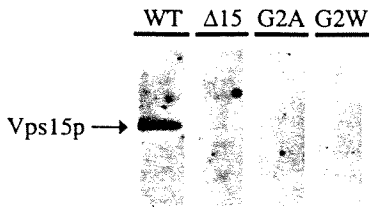


Fig. 3. *In vivo* myristoylation of the Vps15p protein. Yeast cells were labeled with [^{3}H]myristic acid for 60 min at 30°C and immunoprecipitated with antisera specific for Vps15p as described in Materials and methods. The strains examined were PHY112 (*Δvps15*) harboring the multicopy plasmids with the indicated *VPS15* alleles. WT refers to the wild-type *VPS15* allele (pPHY15E) and Δ15 to PHY112 harboring the vector alone (pPHYC18). The position of Vps15p (~170 kDa) is shown.

C-terminal Vps15p truncations exacerbate the effects of alterations in the Vps15p myristoylation site and kinase domain

In order to investigate the role of the large C-terminal domain in Vps15p function, we assessed the biological consequences of removing specific C-terminal sequences from Vps15p. The largest truncation mutant, removing the C-terminal 214 amino acids of Vps15p (ΔC214), exhibited a near wild-type growth rate at 38°C but was slightly defective for CPY sorting at 26°C (Figures 1 and 2). Interestingly, the ΔC214 protein was not phosphorylated *in vivo* suggesting that the C-terminal 214 amino acids of Vps15p either contain the site of phosphorylation or are specifically required for phosphorylation elsewhere within Vps15p (Figure 1; see below).

We subsequently introduced the G2A and E151R alterations into the ΔC214 truncated version of Vps15p (Figure 4A). Both double mutants, G2A/ΔC214 and E151R/ΔC214, exhibited a severe *ts* growth defect and missorted >95% of their CPY to the cell surface (Figure 4). Therefore, the ΔC214 truncation greatly exaggerates the effects of both the G2A and E151R alterations. The relative importance of the ΔC214 truncation in these double mutant interactions is illustrated by an analysis of a G2A/E151R double mutant. This double mutant exhibits a temperature-resistant growth phenotype and >95% of its CPY is properly sorted to the vacuole. Each of the single and double mutant Vps15p proteins described above were demonstrated to be associated with the P100 membrane fraction of yeast cell extracts indicating that their phenotypic effects were not due to the disruption of the Vps15p membrane association (data not shown).

We constructed a series of deletions at the 3′ end of the *VPS15* gene in order to map more precisely the C-terminal domain responsible for the observed synergy (see Materials and methods). These *vps15* deletion alleles encode truncated Vps15 proteins lacking either 167, 128 or 30 C-terminal amino acids. The deletions were constructed both in a wild-type *VPS15* background and in the G2A mutant. Interestingly, each of the double mutants, including G2A/ΔC30, exhibited an extreme *ts* growth defect (data not shown). As expected, each of the single ΔC deletion alleles was able to complement fully the *Δvps15 ts* growth phenotype. These results suggest that a C-terminal deletion of as little as 30 amino acids is sufficient for the synergistic interaction observed with the G2A alteration. In addition, these double

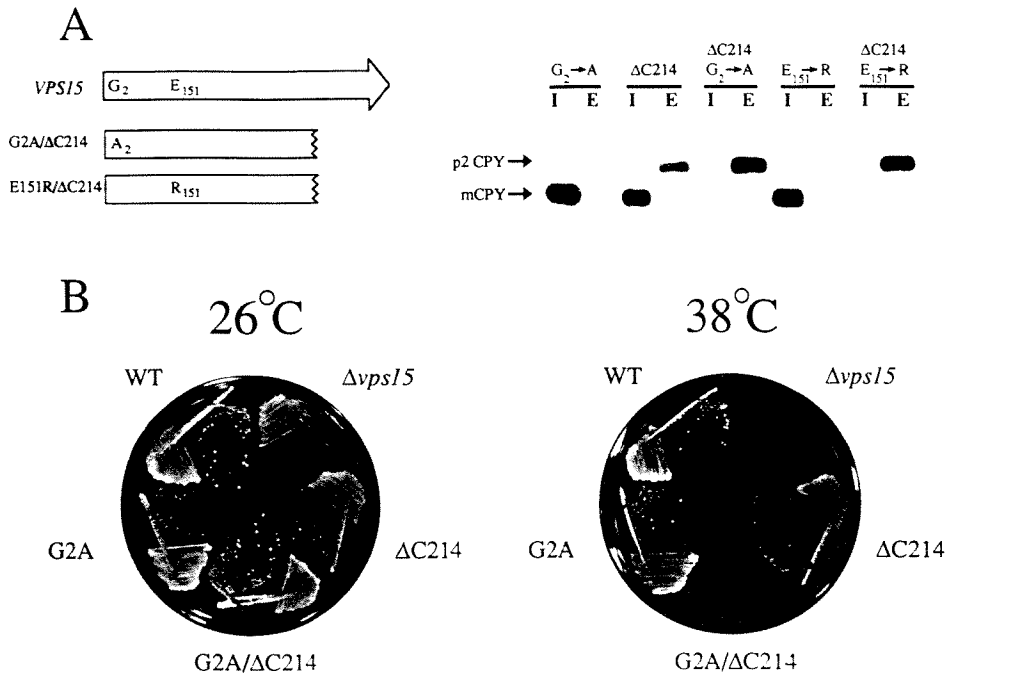


Fig. 4. Analysis of *vps15* double mutants. (**A**) Intracellular sorting of CPY in *vps15* double mutants. A schematic drawing illustrating the two *vps15* double mutants analyzed. A CPY sorting analysis is shown on the right-hand side. Yeast spheroplasts were labeled and processed as described in the legend to Figure 2. The strains were PHY112 harboring different *vps15* alleles on a low copy plasmid (pPHYC18). The mutants encoded Vps15p proteins with the single or double alterations as indicated. (**B**) Analysis of the *ts* growth defect of *vps15* double mutants. PHY112 cells harboring the indicated *vps15* alleles on a low copy yeast plasmid were streaked onto YPD plates and incubated at either 26 or 38°C. WT refers to the wild-type allele (pPHY15C) and Δ*vps15* refers to the vector control (pPHYC18).

mutant effects suggest a role for the Vps15p myristic acid modification in vacuolar protein sorting.

C-terminal deletion mutants of VPS15 C-terminal deletion mutants exhibit a rapid and specific temperature-conditional block in vacuolar protein sorting

The extreme defects observed with the G2A/ΔC214 and E151R/ΔC214 double mutants suggested that the C-terminal domain of Vps15p plays a significant role in Vps15p function. Although the C-terminal deletion mutants exhibited only a minor CPY sorting defect at 26°C, we found that this vacuolar protein sorting defect was greatly exaggerated at elevated growth temperatures. CPY sorting and/or processing in these mutants was analyzed in whole yeast cells at either 26 or 38°C as described in Materials and methods. In wild-type cells, all of the CPY radiolabeled at either 26 or 38°C was present at the 61 kDa mature species, indicative of efficient vacuolar delivery (Figure 5). In contrast, in Δ*vps15* cells all of the CPY was present as the 69 kDa Golgi-modified p2 precursor. In the case of the C-terminal deletion mutants, only a slight CPY processing defect was observed at 26°C, varying from ~5% p2 CPY with the ΔC30 mutant to ~15% with ΔC214 (Figure 5). However, at 38°C, all of the C-terminal deletion mutants were extremely defective for CPY maturation, accumulating >95% of the newly synthesized CPY in a p2 precursor form (Figure 5). These results suggested that each of these mutants exhibited an extreme *ts* defect in vacuolar protein delivery (see below). We have classified this type of allele as *tsf*,

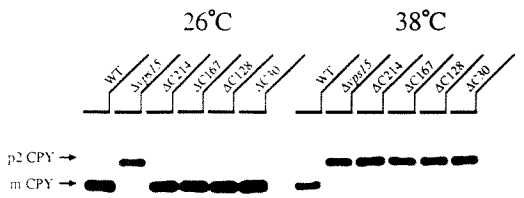


Fig. 5. Temperature-sensitive CPY processing in *vps15* C-terminal deletion mutants. Yeast cells were pre-incubated at either 26 or 38°C for 5 min prior to label addition. The cells were labeled with Tran^{35}S-label for 5 min, methionine and cysteine were added to a final concentration of 25 mM and the cells were chased for an additional 30 min. The labeling and chase were performed at the same temperature as the pre-incubation. The cells were then processed for immunoprecipitation with antisera specific for CPY as described in Materials and methods. All strains analyzed were PHY112 containing a low copy, centromere-containing yeast plasmid with the specific *vps15* allele indicated. WT refers to the wild-type allele (pPHY150) and Δ*vps15* to the vector control (pPHYC18). The positions of mature and p2 CPY are indicated.

or temperature-sensitive for function, to distinguish them from other *vps15* alleles (including null alleles) that result in a *ts* growth defect and a severe CPY missorting phenotype at both the permissive and non-permissive growth temperatures.

We analyzed the fate of the p2 CPY in these 38°C-blocked

cells in a series of pulse–chase temperature-shift experiments. In these experiments, yeast spheroplasts were briefly labeled with Tran^{35}S-label at 26°C and a chase was then initiated by the addition of unlabeled methionine and cysteine. One aliquot of the labeled culture was rapidly shifted to 38°C and chased for 30 min at that temperature. The remainder of the culture was kept at 26°C and also chased for 30 min. The cultures were then fractionated into an intracellular and an extracellular fraction and immunoprecipitations were performed with antisera specific for CPY. Following the short labeling period, the majority of the CPY was present as the ER-modified p1 form in both the wild-type and ΔC30 mutant (Figure 6). In wild-type cells, following the 30 min chase at either 26 or 38°C, all of the radiolabeled CPY was present in the intracellular fraction as a 61 kDa mature form, indicative of correct delivery to the vacuolar compartment. In the ΔC30 mutant, after 30 min of chase at 26°C, ~95% of the CPY was processed to its mature form and was found in the intracellular fraction (Figure 6). However, after 30 min of chase at 38°C, >95% of the radiolabeled CPY was present as the 69 kDa p2 precursor. Surprisingly, >90% of this p2 CPY was not secreted from the cell but was instead found associated with the spheroplast pellet (Figure 6). The observed block in p2 CPY secretion at 38°C was not specific to spheroplasts as we have performed similar temperature-shift experiments with whole yeast cells and have obtained identical results (see below).

In all previously examined *vps15* mutants (as well as all other *vps* mutants examined to date), p2 CPY was not retained within the cell pellet, but was instead efficiently secreted from the cells (Figure 2; Herman *et al.*, 1991). One possible explanation for the observed lack of p2 CPY secretion is that the ΔC30 mutant may exhibit a general block in protein secretion at 38°C. However, the observation that the ΔC30 mutant is able to grow at 38°C with a near wild-type growth rate is not consistent with this explanation. Nevertheless, we directly tested this possibility by examining the secretion of a 33 kDa cell wall protein, CWP33, in ΔC30 and wild-type yeast cells at 38°C. The CWP33 protein is a major constituent of the yeast cell wall and is released from the wall upon treatment with zymolase (Sanz *et al.*, 1987). The passage of this protein through the secretory pathway has been extensively studied (Sanz *et al.*, 1987; Toyn *et al.*, 1988) and it serves as a good marker of secretory protein flow to the cell surface. Using antibody directed specifically against this cell wall protein, we were able to show that ΔC30 mutant spheroplasts secrete the CWP33 protein into the media as efficiently as wild-type yeast at both 26 and 38°C (Figure 6). Therefore, the intracellular retention of p2 CPY in ΔC30 cells at 38°C is not due to a general block in protein secretion in these mutants.

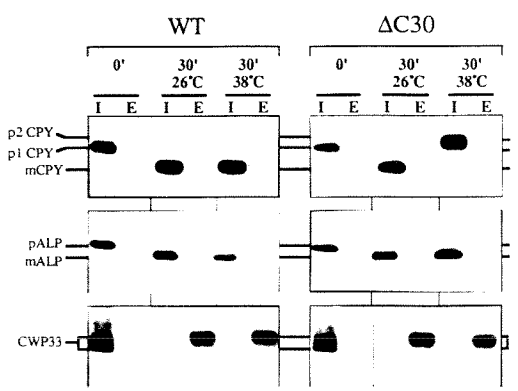


Fig. 6. Temperature-shift analysis of the *ts* defects associated with the ΔC30 mutant. Yeast spheroplasts were labeled with Tran^{35}S-label for 3 min at 26°C and methionine and cysteine were then added to 25 mM to initiate the chase period. One aliquot was chased at 26°C for 30 min and the other at 38°C for 30 min. The labeled cultures were then centrifuged for 2 min at 13 000 *g* and separated into a pellet (I, intracellular) and a supernatant (E, extracellular) fraction. The level of either CPY, ALP or CWP33 in each fraction was assessed by quantitative immunoprecipitation with antisera specific for the appropriate protein. WT refers to PHY112 cells harboring the pPHY150 plasmid (wild-type) and ΔC30 to PHY112 cells with a low copy plasmid carrying the indicated C-terminal deletion allele.

Altogether, our results suggest that the 38°C-induced block in the *vps15* ΔC30 mutant is specific for vacuolar protein traffic as protein secretion appears to continue unabated in this mutant at the elevated temperature. In order to examine the specificity of the vacuolar delivery block more carefully, we analyzed the fate of two additional vacuolar proteins, PrA and ALP, in ΔC30 mutant spheroplasts at 38°C. In identical temperature-shift experiments, we observed that the soluble hydrolase PrA behaved much like CPY; the majority of the radiolabeled PrA was associated with the cell pellet in a Golgi-modified precursor form (data not shown). In contrast, precursor ALP was processed to its mature form with near wild-type kinetics in the ΔC30 mutant blocked at 38°C (Figure 6). Therefore, the temperature-conditional block in vacuolar protein processing appears to be specific for soluble vacuolar hydrolases, as at least one vacuolar membrane protein is processed normally at 38°C. The efficient processing of ALP in ΔC30 cells at 38°C suggests that ALP is delivered to the vacuolar compartment since this proteolytic processing event is mediated by the vacuolar enzyme, PrA (Klionsky *et al.*, 1990). This assertion is further supported by indirect immunofluorescence experiments and biochemical analyses of ALP processing that indicate that ALP is efficiently localized to the vacuolar compartment in *vps15* mutants that display severe CPY localization defects (Klionsky and Emr, 1989; C.Raymond and T.Stevens, personal communication). In addition, as both CPY and ALP are processed in the vacuole in a PrA-dependent manner, the above results indicate that the proCPY accumulated at 38°C is not present in the same intracellular compartment as ALP and is therefore not likely to have reached the vacuolar compartment in temperature-shifted ΔC30 cells.

The onset of p2 CPY secretion is delayed in vps15 ΔC30 cells at 38°C

In a Δ*vps15* mutant essentially all of the CPY is present as the p2 precursor form and the great majority of this p2 CPY was secreted from the cell at both 26 and 30°C (Figure 2 and Herman *et al.*, 1991). We therefore decided to analyze p2 CPY secretion in a *vps15* null mutant at 38°C to test whether *vps15* mutants, in general, were competent for the secretion of vacuolar protein precursors at this elevated temperature. PHY112 (Δ*vps15*::*HIS3*) and wild-type yeast spheroplasts were labeled briefly at 26°C and then were chased at 38°C for 30 min as described above. Under these

conditions, all of the CPY in wild-type cells was processed to mature CPY and was associated with the spheroplast pellet (Figure 7). In the *Δvps15* yeast strain, >95% of the radiolabeled CPY was in its p2 precursor form and most of this was secreted from the cells into the media (Figure 7). Therefore, *vps15* mutants appear to be competent for p2 CPY secretion at 38°C.

In contrast to the above results with a *Δvps15* yeast strain, in the *vps15* ΔC30 mutant, >90% of the radiolabeled CPY was retained within the spheroplast pellet as p2 CPY after a 30 min chase at 38°C (see Figure 6). The fundamental difference between the *Δvps15* and ΔC30 mutants in these temperature-shift experiments is that prior to the imposition of the 38°C block *Δvps15* mutants are completely defective for vacuolar protein sorting while ΔC30 cells exhibit only a very slight CPY sorting defect (Figures 5 and 7). We reasoned that if ΔC30 cells were blocked at 38°C for an extended period of time it might be possible to saturate the intracellular compartment accumulating p2 CPY. At this point, ΔC30 cells would therefore begin to mimic *Δvps15* cells and missort newly synthesized CPY to the cell surface. Since the ΔC30 mutant is able to grow at the elevated temperature with a near wild-type growth rate, the 38°C block could be imposed for any desired length of time. To analyze the effects of pre-incubation at 38°C on p2 CPY secretion, we labeled ΔC30 and wild-type whole cells for 5 min at 38°C after 0, 30 or 60 min of pre-incubation at this temperature. The cultures were then chased for 30 min at 38°C, converted to spheroplasts and separated into media and spheroplast fractions. Immunoprecipitations were then performed with antisera specific to CPY.

In wild-type cells, essentially all of the radiolabeled CPY was present in the spheroplast fraction as mCPY for all three times of pre-incubation (data not shown). With the ΔC30 mutant, we observed that varying the length of the 38°C pre-incubation had a significant effect upon the fate of CPY trafficking through the secretory pathway. When no pre-incubation was performed, >95% of the radiolabeled CPY accumulated in an intracellular fraction as p2 CPY (Figure 7). However, when ΔC30 cells were pre-incubated for either 30 or 60 min prior to labeling, we observed that the majority of the newly synthesized CPY was secreted from the cells as the Golgi-modified p2 precursor (Figure 7). After 60 min of pre-incubation at 38°C, essentially all of the p2 CPY was detected in the extracellular fraction. Therefore, the ΔC30 mutant accumulates p2 CPY within a saturable compartment following a temperature shift to 38°C.

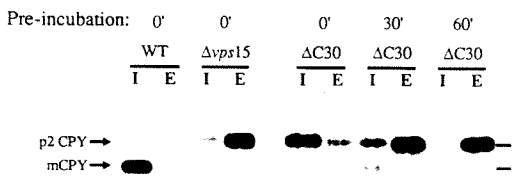


Fig. 7. p2 CPY secretion in the ΔC30 mutant. For the first two pairs of lanes, PHY112 spheroplasts harboring either pPHY150 (WT, wild-type allele) or pPHYC18 (*Δvps15*, vector) were labeled with Tran^{35}S-label for 3 min at 26°C. The cultures were then shifted to 38°C and chased for 30 min at this temperature following the addition of methionine and cysteine to 25 mM. The spheroplasts were processed as described below. For the final three pairs of lanes, PHY112 cells harboring a single copy yeast plasmid with the ΔC30 allele were pre-incubated for 0, 30 or 60 min at 38°C. The cells were then labeled with Tran^{35}S-label for 5 min at 38°C and chased for an additional 30 min at 38°C. The chase was initiated by the addition of methionine and cysteine to a final concentration of 25 mM. The whole cells were converted to spheroplasts, separated into a pellet (I, intracellular) and a supernatant (E, extracellular) fraction by centrifugation at 13 000 *g* for 2 min and immunoprecipitated with antisera specific to CPY. The positions of mature and p2 CPY are indicated.

The vps15 ΔC30 temperature-dependent block in CPY sorting is reversible

When the ΔC30 mutant is rapidly shifted to 38°C, p2 CPY and other soluble vacuolar precursors accumulate within an intracellular compartment. We examined the reversibility of this 38°C block in order to gain some insight into the nature of this compartment. As above, we briefly labeled ΔC30 spheroplasts at 26°C and then chased with unlabeled methionine and cysteine for 30 min at 38°C. After this chase period, ~95% of the radiolabeled CPY is cell-associated and in its p2 precursor form (Figure 8). This culture was subsequently split in half; one aliquot remained at 38°C for an additional 20 min and the other was placed at 26°C for 20 min. The additional 20 min chase at 38°C did not significantly alter the CPY distribution in the ΔC30 cells (Figure 8). The p2 CPY that was blocked after 30 min at 38°C remained blocked after an additional 20 min at 38°C. In contrast, >90% of the p2 CPY that accumulated at 38°C was processed to its mature form upon shifting the ΔC30

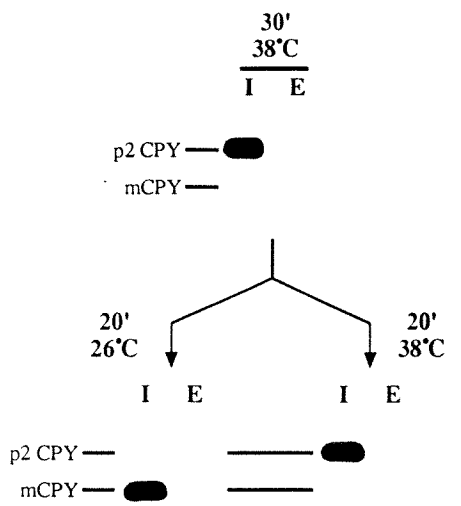


Fig. 8. Reversibility of *ts* vacuolar protein delivery defect of ΔC30 mutants. PHY112 spheroplasts harboring a single copy yeast plasmid with the ΔC30 allele were labeled for 3 min at 26°C and chased for 30 min at 38°C following the addition of methionine and cysteine to 25 mM. The labeled culture was then split into three and processed as follows. One aliquot was centrifuged for 2 min at 13 000 *g* and separated into a pellet (I, intracellular) and a supernatant (E, extracellular) fraction. The level of CPY in each fraction was assessed by quantitative immunoprecipitation with antisera to CPY. The second aliquot was chased for an additional 20 min at 38°C and the third for 20 min at 26°C. These latter two aliquots were then processed as the first. The positions of mature and p2 CPY are indicated.

cells back to 26°C suggesting that this CPY has been efficiently delivered to the yeast vacuolar compartment (Figure 8). Our results therefore indicate that the great majority of the p2 CPY that accumulates within 38°C-blocked ΔC30 cells remains competent for subsequent vacuolar delivery during the time that the block is imposed. Moreover, the efficient reversal of this temperature block suggests that the compartment housing the p2 CPY may in fact represent a normal functional intermediate in the vacuolar protein delivery pathway.

A short C-terminal domain of Vps15p is required for its phosphorylation in vivo

Vps15p has been demonstrated to be a phosphoprotein *in vivo* and mutational analyses of the Vps15p kinase domain have suggested that this phosphate incorporation may be due to a specific autophosphorylation reaction (Figure 1; Herman *et al.*, 1991). The ΔC214 truncated Vps15p protein was very defective for this *in vivo* phosphorylation reaction (Figure 1). Since the ΔC214 mutant also exhibits a *ts* CPY sorting defect, it is possible that the phosphorylation of Vps15p is important for it to achieve, or maintain, an active conformation at elevated growth temperatures. We therefore analyzed the *in vivo* phosphorylation of each of the C-terminal Vps15p truncation proteins. All of the C-terminal deletion mutants including ΔC30, were very defective for the *in vivo* phosphorylation of Vps15p (Figure 9). In order to determine if the observed lack of Vps15p phosphorylation was specifically due to the deletion of sequences within the C-terminal 30 amino acids of Vps15p (total of 1455 amino acids), we constructed an internal deletion of amino acids 1412–1427, ΔI16, and analyzed the phosphorylation of this Vps15p mutant protein. The ΔI16 protein was observed to be phosphorylated to a wild-type level (Figure 9). This result indicates that the lack of Vps15p phosphorylation in the C-terminal deletion mutants is not due to a general misfolding of Vps15p proteins that possess deletions in their C termini. Rather, our results suggest that a short C-terminal domain of Vps15p is specifically required for its phosphorylation *in vivo*. Furthermore, since deletions of this domain result in a *ts* vacuolar protein sorting defect, the phosphorylation of Vps15p may be essential for its biological activity at elevated temperatures.

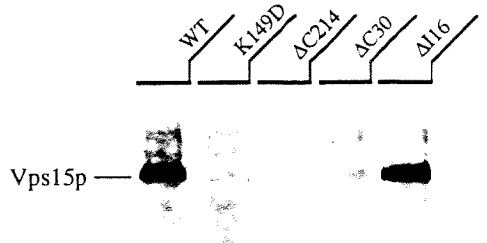


Fig. 9. *In vivo* phosphorylation of the *VPS15* C-terminal deletion mutants. Yeast cells were labeled with $^{32}PO_4$ for 30 min at 30°C and immunoprecipitated with antisera specific for Vps15p as described in Materials and methods. PHY112 cells harboring the indicated *vps15* alleles on multicopy plasmids were analyzed. WT refers to plasmid pPHY15E (wild-type allele). The position of Vps15p (~170 kDa) is shown.

Discussion

In a previous study, we identified a membrane-associated protein kinase, encoded by the yeast *VPS15* gene, that is essential for the efficient vacuolar delivery of multiple soluble hydrolases. The severe phenotypic consequences of mutations within the *VPS15* protein kinase domain, together with the specificity of *vps15* defects for soluble constituents of the vacuole, suggested that Vps15p might regulate specific protein phosphorylation reactions required for the delivery of soluble proteins to the yeast vacuole. In addition to the N-terminal 300 amino acid protein kinase domain, Vps15p also possesses a myristic acid addition site at its N terminus and a large C-terminal domain of >1100 amino acids that exhibited no significant similarity to any other known protein sequence. In this study, we have extended our previous mutational analysis of Vps15p and have now examined multiple alterations in each of these three Vps15p domains.

Vps15p kinase domain mutants

The Vps15p sequence exhibits a significant degree of similarity to the catalytic domains of the serine/threonine family of protein kinases. With this present study, we have now constructed mutations in four distinct Vps15p kinase subdomains and have altered specific amino acid residues that are highly conserved among all protein kinase molecules. This high level of conservation, together with observations from chemical modification experiments, suggests that these amino acids are directly involved in catalytic function (see Hanks *et al.*, 1988; Taylor *et al.*, 1990). Alterations within each of the four Vps15p kinase motifs result in severe defects in both the delivery of soluble hydrolases to the vacuole and the *in vivo* phosphorylation of Vps15p (Figure 1). Therefore these mutational studies suggest that the Vps15p kinase regulates Vps15p phosphorylation and that it may do so directly, by catalyzing a specific autophosphorylation reaction. We are currently attempting to demonstrate Vps15p kinase activity *in vitro* in order to determine if the *in vivo* phosphorylation of Vps15p does indeed correspond to an autophosphorylation reaction. Since the phenotype of *VPS15* kinase domain mutations (e.g. D165R in Figure 1) are generally more extreme than those associated with a loss of Vps15p phosphorylation (e.g. ΔC30 mutant in Figure 1), the Vps15p kinase domain likely regulates additional protein phosphorylation reactions *in vivo*. The severe vacuolar protein sorting defects associated with Vps15p kinase domain alterations would therefore be due to defects in multiple Vps15p-mediated protein phosphorylation reactions involving as yet unknown cellular substrates of the Vps15p kinase. Furthermore, the relatively efficient sorting of CPY to the vacuole in *vps15* mutants that are defective for the *in vivo* phosphorylation of Vps15p suggests that the phosphorylation of Vps15p is not essential for the delivery of soluble hydrolases to the yeast vacuole.

Vps15p is modified at its N terminus by the addition of myristic acid

Biochemical labeling experiments with [^{3}H]myristic acid indicated that Vps15p is modified *in vivo* by the addition of the 14-carbon fatty acid, myristate, at its N terminus. However, this lipophilic moiety does not appear to mediate the observed membrane association of Vps15p as non-myristoylated forms of this protein remain associated with a similar membrane fraction of yeast cell extracts. Although

the majority of myristoylated proteins are associated with specific intracellular membranes, there are several examples of proteins that are myristoylated but soluble, including the catalytic domain of the cAMP-dependent protein kinase (Towler *et al.*, 1988b). The biological significance of the myristic acid in these soluble proteins is generally not known. In the case of the cAMP-dependent protein kinase, the myristate does not appear to be important for catalytic activity as the myristoylated and non-myristoylated forms of the catalytic subunit have identical kinase activities *in vitro* (Slice and Taylor, 1989). We have found that the non-myristoylated form of Vps15p is phosphorylated to a lesser extent than the wild-type protein *in vivo*. If the observed *in vivo* phosphorylation of Vps15p is due to a specific autophosphorylation reaction, it will be interesting to determine if the myristate moiety added to the N terminus of Vps15p directly influences the catalytic activity of this enzyme.

C-terminal truncations of Vps15p result in a temperature-conditional defect in vacuolar protein sorting

The identification of conditional alleles of a given genetic locus generally allows for the development of a more complete understanding of the precise role and stage at which the gene product acts in the biochemical or developmental pathway under study. For example, in the analysis of the yeast *sec* mutants, the accumulation of specific intermediates at the non-permissive temperature allowed Schekman and his co-workers to assign particular gene functions to different positions throughout the secretory pathway (Schekman and Novick, 1982). Our initial genetic selection uncovered multiple *vps15* alleles that were *ts* for growth; however, all of these alleles resulted in an equally severe CPY sorting defect at both the permissive and non-permissive growth temperatures (Robinson *et al.*, 1988). During our present analysis of a series of C-terminal Vps15p deletions, we found that relatively short C-terminal truncations of Vps15p, removing as little as 30 amino acids, resulted in a severe *ts* defect in the delivery of CPY to the vacuole. This *ts* defect is especially dramatic in the ΔC30 mutant where CPY delivery is essentially wild-type at the permissive temperature but almost completely blocked when cells are shifted to the restrictive temperature of 38°C. Upon imposition of the temperature block, ΔC30 cells accumulate ~95% of the newly synthesized CPY within an intracellular compartment as a p2 precursor molecule. This intracellular retention of p2 CPY was a somewhat unexpected result as all of the previously characterized *vps15* mutants efficiently secrete p2 CPY. Furthermore, the *ts* vacuolar protein delivery defects associated with the ΔC30 mutant appear to be specific for soluble vacuolar hydrolases. ALP, a vacuolar membrane protein, is efficiently processed to its mature form at the non-permissive temperature in ΔC30 mutants. Since ALP and CPY are both processed in the vacuole in a PrA-dependent manner (reviewed in Klionsky *et al.*, 1990), the efficient maturation of ALP suggests that ΔC30 cells are processing-proficient and that CPY is apparently sequestered within an intracellular compartment distinct from the vacuole. Therefore the *ts* defects observed in the C-terminal deletion mutants are apparently due to a failure to deliver CPY to the vacuole properly.

The *ts* vacuolar protein delivery block in the Δ30 mutant exhibits an extremely rapid rate of onset as an essentially complete block in CPY processing can be established in < 1 min of incubation at the non-permissive temperature. The rapid onset of the mutant phenotype suggests that the *VPS15* gene product is quickly inactivated at 38°C and that this inactivation almost immediately manifests itself as a defect in CPY delivery to the vacuole. This, in turn, provides strong genetic evidence that Vps15p is directly involved in the sorting and/or delivery of proteins to the vacuole. Therefore the observed vacuolar protein sorting defects in *vps15* mutants are not likely to result as a secondary consequence of the loss of Vps15p function. Although the exact manner in which the C-terminal domain influences Vps15p activity is unclear, the observation that all of the C-terminal truncated forms of Vps15p, including ΔC30, are not phosphorylated *in vivo* suggests that the phosphorylation of Vps15p is important for its biological activity at elevated temperatures. The C-terminal 30 amino acids of Vps15p may either include the site of Vps15p phosphorylation or else constitute a specific domain required for phosphorylation elsewhere within this protein. We are presently attempting to map the specific site(s) of protein phosphorylation in Vps15p in order to determine how this phosphorylation, and the C-terminal domain in general, contribute to Vps15p function.

The immediate phenotypic consequence of a loss of Vps15p activity therefore appears to be the intracellular accumulation of Golgi-modified precursor forms of soluble vacuolar hydrolases, such as CPY and PrA. These precursors appear to be present within a specific intracellular compartment that is distinct from the vacuole. This compartment does not appear to represent an aberrant, dead-end structure since the resident p2 CPY can be efficiently processed to its mature form following a shift back to the permissive temperature. Moreover, the rapid and efficient reversal of the 38°C delivery block in ΔC30 cells suggests that the accumulated p2 CPY was present within a normal intermediate of the vacuolar protein transport pathway. Since very little is presently known about the transport intermediates functioning between the Golgi complex and the vacuole, the identification and characterization of this p2 CPY compartment could provide some fundamental insights into the vacuolar protein transport process. It is interesting to note that an electron microscopic analysis of *vps15* mutants indicated that 80 nm vesicles and abnormal membranous structures that resembled Golgi-derived Berkeley bodies accumulated within the cytoplasm of these cells (Banta *et al.*, 1988).

Models for Vps15p function in vacuolar protein sorting

CPY synthesized in ΔC30 cells immediately following a shift to 38°C accumulates within a saturable intracellular compartment as its p2 precursor form. This p2 CPY remains cell-associated during at least 50 min of subsequent incubation at 38°C. These data suggest that this p2 CPY is sequestered within a compartment that is unable to communicate with the cell surface. One possibility is that this p2 CPY has been sorted away from secretory protein traffic and packaged into a specific transport intermediate, possibly a membrane vesicle, committed for delivery to the vacuole. If Vps15p activity is required for either the delivery or fusion of these vesicles to the vacuole then the thermal

inactivation of Vps15p could result in the accumulation of p2 CPY within this intracellular compartment. In addition, this block in vacuolar protein delivery would also prevent the recycling of transport factors back to the Golgi where they could catalyze additional rounds of transport. In this model, p2 CPY would be packaged into vesicles until a cellular component essential for either this packaging, or the formation of the transport intermediate, became limiting. Once the system was saturated, p2 CPY would enter the default secretion pathway and be delivered to the cell surface. In an alternative model, Vps15p activity could be specifically required for the packaging of certain receptor−ligand (e.g. receptor-p2 CPY) complexes into transport vesicles destined for the vacuole. A loss of Vps15p activity would lead to an accumulation of p2 CPY, bound by its specific transmembrane receptor, in a late Golgi compartment. In this second model, the accumulated p2 CPY in the *vps15* ΔC30 mutant at 38°C would therefore be present within a late Golgi compartment. Secretion would then result from the saturation of these p2 CPY-specific receptors.

Although *vps15* mutants exhibit severe defects in the localization of several soluble vacuolar hydrolases, vacuolar membrane proteins, such as ALP, appear to be delivered to the vacuole in *vps15* cells. This observation has several interesting implications for Vps15p function within the vacuolar protein delivery pathway (Figure 10). ALP and CPY could transit together from a late Golgi compartment to the vacuole within the same vesicular transport intermediate. Since ALP is efficiently delivered to the vacuole in the *vps15* ΔC30 mutant, the cellular machinery required for the formation and delivery of these transport vesicles must be present and functional. Therefore, Vps15p would presumably function prior to these transport events and could be required for the packaging of p2 CPY into the vacuole-bound transport intermediates (Figure 10). On the other hand, CPY and ALP might be delivered to the vacuole by two independent pathways involving separate and distinct carriers. In this model, Vps15p could act at any step along the CPY delivery pathway but would not be required for transit along the ALP route (Figure 10). The intracellular compartment that accumulates p2 CPY in the ΔC30 *vps15* mutant is therefore a candidate for the transport intermediate trafficking between the Golgi and vacuolar compartments in the CPY-specific delivery pathway. Purification and compositional studies of this compartment could provide important insights into the precise mechanisms regulating vacuolar/lysosomal protein sorting in yeast and other eukaryotes.

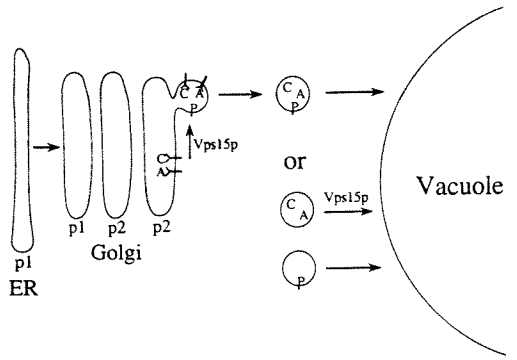


Fig. 10. Possible roles for Vps15p in vacuolar protein sorting. Two possible roles for Vps15p in the vacuolar protein delivery pathway are shown. In the top model, soluble vacuolar hydrolases, such as CPY and PrA, are shown trafficking to the vacuole within the same transport vesicle as vacuolar membrane proteins such as ALP. Vps15p is shown as functioning in the specific packaging of soluble vacuolar proteins into transport carriers. In the bottom model, soluble and membrane proteins are shown to be transiting to the vacuole via distinct transport intermediates (e.g. vesicles). In this model, Vps15p is specifically required for the recognition or fusion of the soluble hydrolase-containing vesicles with the vacuolar compartment. The different vacuolar proteins are indicated as follows: C, CPY; A, PrA; and P, ALP. The p1 and p2 designations refer to the form of CPY or PrA present in the indicated secretory pathway compartment.

Materials and methods

Strains and media

Escherichia coli strains BW313 (*F' lysA/dut ung thi-1 relA spoT1*; Kunkel, 1985) and CJ236 (*dut1 ung1 thi-1 relA1*/pCJ105 [cam^{r}F1]; Kunkel *et al.*, 1987) were used for the oligonucleotide-directed mutagenesis experiments and JM101 (*F' traD36 lacI^{q}ZΔM15 proAB/supE thiDlac Δlac-pro*; Miller, 1972) was used for all other purposes. The *S.cerevisiae* strains PHY112 (*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 Δvps15::HIS3*; Herman *et al.*, 1991) and SEY6210 (*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*; Robinson *et al.*, 1988) were used for all experiments described in this study. Standard yeast and *E.coli* media were used and supplemented as needed (Miller, 1972; Sherman *et al.*, 1979).

Yeast methods

Standard yeast genetic methods were used throughout this study (Sherman *et al.*, 1979). Yeast cells were transformed by the method of alkali cation treatment (Ito *et al.*, 1983) and transformants were selected on the appropriate SD media.

Plasmid construction and oligonucleotide-directed mutagenesis

Recombinant DNA manipulations were performed as described previously (Ausubel *et al.*, 1987; Maniatis *et al.*, 1982). Two yeast shuttle vectors, pPHYC18 (*CEN, URA3*; Herman and Emr, 1990) and pJSY324 (*2μ, TRP1*) were used for most of the plasmid constructions described within this study. The yeast vector pJSY324 was constructed by subcloning the 1.6 kb *Hpa*I−*Hin*dIII fragment of YEp13, containing the yeast 2μ plasmid origin of replication, into the *Aat*II site of pRS304 (Sikorski and Hieter, 1989). The *Hin*dIII 3' recessed ends were filled in with Klenow polymerase and the appropriate deoxynucleotides prior to ligation. The yeast *VPS15* plasmids pPHY15-1, pPHY15C and pPHY15E were described previously (Herman *et al.*, 1991).

Oligonucleotide-directed mutagenesis of the *VPS15* gene was performed using *dut$^-$ ung$^-$ E.coli* as described previously (Kunkel, 1985; Ausubel *et al.*, 1987). Two different mutagenesis procedures were used to generate the mutants described in Figure 1. The first scheme was used to produce the G2 → A and E151 → R alterations and was described previously (Herman *et al.*, 1991). For the second, the *VPS15* gene was subcloned as a 4.8 kb *Sca*I−*Sna*BI fragment from pPHY15-1 into the *Sma*I site of both pJSY324 and pBluescriptII KS$^+$ (Stratagene) to produce pJSY324.15 and pBP.15, respectively. These *VPS15*-containing plasmids were introduced into the *dut$^-$ ung$^-$ E.coli* strain CJ236 and single-stranded plasmid DNA was isolated and mutagenized as described in the Bio-Rad MutaGene kit manual. The mutagenized *VPS15* DNA was subsequently subcloned as a *Bam*HI−*Xho*I fragment into pPHYC18 and pJSY324 as required. The wild-type *Sca*I−*Sna*BI fragment was also cloned into the *Sma*I site of pPHYC18 to generate pPHY150.

The *VPS15* C-terminal deletion plasmids were constructed by subcloning the appropriate *VPS15* restriction fragments from pPHY15-1 into the *Sma*I site of pPHYC18. The following restriction fragments were gel-isolated and, if required, were treated with either T4 DNA polymerase to remove 3' overhangs, or Klenow polymerase to fill in 3' recessed ends: *Sca*I−*Bsp*HI (ΔC30), *Sca*I−*Hgi*AI (ΔC128) and *Sca*I−*Xmn*I (ΔC167). For the double mutants combining either the G2 → A or E151 → R alteration with a specific C-terminal deletion, the above fragments were excised from either pPHYC15-G2A (G2 → A) or pPHYC15-E151R (E151 → R), respectively.

The largest C-terminal deletion, removing 214 amino acids of the Vps15p, was constructed as follows. First, the 3.6 kb *ClaI* fragment of pPHY15-1 was subcloned into the *ClaI* site of pPHYC18 to produce the plasmid pPHY127. The 1.8 kb *XbaI* fragment from M13V15 RF DNA (see Herman *et al.*, 1991), wild-type or mutagenized, was gel-isolated and used to replace the *XbaI* fragment of pPHY127, thereby reconstructing a *VPS15* gene truncated at the 3' internal *ClaI* site.

Cell labeling and immunoprecipitation

Immunoprecipitations from whole yeast cells labeled with Tran^{35}S-label (ICN Radiochemicals) were performed as described previously (Herman and Emr, 1990; Herman *et al.*, 1991). For the analysis of the temperature-conditional CPY processing defect in the *VPS15* C-terminal deletion mutants, yeast cells were pre-incubated at either 26 or 38°C for 5 min prior to labeling. The cells were then labeled with Tran^{35}S-label for 5 min and chased for 30 min at the same temperature. The chase was initiated by the addition of unlabeled methionine and cysteine to 25 mM. CPY fractionation immunoprecipitations from radiolabeled yeast spheroplasts were performed as described previously (Robinson *et al.*, 1988).

For the *ts* shift experiments, typically 20 OD_{600} cell equivalents of yeast spheroplasts were labeled for 3 or 4 min at 26°C with Tran^{35}S-label in 2.5 ml of spheroplast labeling media (Wickerham's minimal proline media supplemented with 1.3 M sorbitol; Wickerham, 1946). The culture was then divided into two aliquots of 1.5 and 1.0 ml. To the first aliquot an equal volume of 47–48°C chase solution (spheroplast labeling media plus 50 mM methionine, 50 mM cysteine and 0.4% yeast extract) was added to rapidly bring up the culture temperature. The chase was then continued for 30 min at 38°C. To the second aliquot, an equal volume of 26°C chase solution was added and 1.0 ml was removed and fractionated for subsequent immunoprecipitation as follows. The spheroplast cultures were centrifuged at 13 000 *g* for 10 s and the supernatant was carefully removed and TCA was added to a final concentration of 5%. The spheroplast pellet was resuspended in 1.0 ml of 5% TCA. The TCA precipitations were held on ice for at least 20 min. The remaining half of the second aliquot was chased for 30 min at 26°C. After 30 min of chase, 1.0 ml of both the 38 and 26°C cultures were fractionated as described above. One-half, or 1.0 ml, of the 38°C culture was shifted to 26°C and the other half was kept at 38°C for an additional 20 min. After this additional chase, the samples were processed as described above. For the whole cell temperature-shift experiments, a similar experimental protocol was followed except that the labeled aliquots removed for analysis were added to an equal volume of ice-cold stop solution (50 mM Tris–HCl, pH 7.5, 2 M sorbitol, 40 mM NaN_3, 40 mM NaF, 20 mM dithiothreitol) and held on ice for 5 min. Zymolyase-100T (Seikagako Kogyo Co.) was added to 20 $\mu g/OD_{600}$ cell equivalents and spheroplasting was carried out for 30 min at 30°C. The cultures were then fractionated as described above for the spheroplast labelings. In some experiments, the chase solution was supplemented by the addition of 0.04 vol of 1 M Tris–HCl, pH 7.0, and/or α_2-macroglobulin to a final concentration of 1 mg/ml. The antiserum specific for CWP33 was a generous gift from P.Sanz.

The *in vivo* phosphate labeling of yeast cells and the subsequent immunoprecipitation of Vps15p were performed as described previously (Herman *et al.*, 1991) except that the protein A–Sepharose beads were washed as follows: once with Tween-20 IP buffer (0.5% Tween-20, 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1 mM EDTA) and once with IP buffer 2 (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1 mM EDTA).

For the analysis of Vps15p myristoylation, yeast cells were grown to mid-logarithmic phase in Wickerham's minimal media supplemented with 0.2% yeast extract (Wickerham, 1947; Robinson *et al.*, 1988). Five OD_{600} units of cells were collected by centrifugation and resuspended in 1 ml of the same media. Cerulenin (Sigma) was added to a final concentration of 20 μg/ml and the cells were incubated for 20 min at 30°C. Following this pre-incubation, 1.0 mCi of [9,10(*n*)-^{3}H]myristic acid (New England Nuclear) was added and the cells were labeled for 60 min at 30°C. The relatively short labeling period was chosen to minimize the conversion of the labeled myristic acid to other fatty acids, such as palmitate. The labeling was terminated by the addition of TCA to a final concentration of 5%. The protein A–Sepharose beads were washed as described above for the *in vivo* phosphorylations.

Functional analysis of vps15 mutants

For this study, we assayed the biological activities of the engineered *vps15* alleles by introducing these alleles into a *Δvps15* yeast strain (PHY112) on either a low copy centromere-containing plasmid (pPHYC18), or multicopy 2μ circle derivative (pJSY324). The low copy plasmids were tested for their ability to complement the severe *ts* growth defect (at 38°C) and the CPY localization defects associated with *Δvps15* yeast strains. The *in vivo* phosphorylation of the mutant Vps15p proteins was assayed by labeling *Δvps15* yeast strains, carrying the appropriate *vps15* alleles on a 2μ multicopy plasmid, with [^{32}P]orthophosphate and then immunoprecipitating with antisera specific for Vps15p.

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Chapter 4:

Phosphatidylinositol 3-Kinase Encoded by Yeast *VPS34* Gene Essential for Protein Sorting

Phosphatidylinositol 3-Kinase Encoded by Yeast *VPS*34 Gene Essential for Protein Sorting

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The *VPS*34 gene product (Vps34p) is required for protein sorting to the lysosome-like vacuole of the yeast *Saccharomyces cerevisiae*. Vps34p shares significant sequence similarity with the catalytic subunit of bovine phosphatidylinositol (PI) 3-kinase [the 110-kilodalton (p110) subunit of PI 3-kinase], which is known to interact with activated cell surface receptor tyrosine kinases. Yeast strains deleted for the *VPS*34 gene or carrying *vps*34 point mutations lacked detectable PI 3-kinase activity and exhibited severe defects in vacuolar protein sorting. Overexpression of Vps34p resulted in an increase in PI 3-kinase activity, and this activity was specifically precipitated with antisera to Vps34p. *VPS*34 encodes a yeast PI 3-kinase, and this enzyme appears to regulate intracellular protein trafficking decisions.

A variety of peptide growth factors and hormones, such as platelet-derived growth factor (PDGF), nerve growth factor (NGF), and insulin mediate their cellular effects by interaction with cell surface receptor tyrosine kinases. The interaction of these ligands with their cognate receptors induces a series of intracellular signaling events, including stimulation of protein tyrosine kinase activity and the formation and activation of multiprotein complexes at the plasma membrane (*1*–*3*). These receptor complexes contain enzymes that take part in phosphoinositide metabolism, including phospholipase C (PLC) and PI 3-kinase (*1*). PLC-γ is known to cleave PI 4,5-bisphosphate [PI(4,5)P_2] to generate the second messengers diacylglycerol, which activates protein kinase C, and inositol 1,4,5-trisphosphate (IP_3), which can mobilize intracellular Ca (*4*). The association of PI 3-kinase with receptor tyrosine kinases has permitted the identification of a family of phosphoinositides phosphorylated at the 3 position of the inositol ring that appear to act in a signaling pathway distinct from that resulting from turnover of PI(4,5)P_2 (*5*). None of the three reaction products of PI 3-kinase—PI 3-monophosphate (PI3P), PI 3,4-bisphosphate [PI(3,4)P_2], or PI 3,4,5-trisphosphate [PI(3,4,5)P_3]—is a substrate of the known PLC isozymes, suggesting that the lipid compounds themselves function as second messengers in cell signaling (*6*, *7*).

Mammalian PI 3-kinase exists as a heterodimer of an 85-kD (p85) and a 110-kD (p110) subunit (*8*–*10*). The cDNA encoding p110 from bovine brain has been cloned, and the p110 protein has been characterized as the catalytically active subunit of the PI 3-kinase complex (*11*). The p85 subunit is believed to mediate the binding of the p110 subunit to active receptor protein tyrosine kinases through its Src homology 2 (SH2) domains (*12*). This association of p85-p110 with the receptor might lead to the activation of the lipid kinase (*13*, *14*). The binding of tyrosine-phosphorylated peptides to the SH2 domains of p85-p110 in vitro stimulates the PI 3-kinase activity fivefold (*2*).

The amino acid sequence deduced from the cDNA sequence of the catalytic subunit of PI 3-kinase from bovine brain is similar to the sequence of the yeast *VPS*34 gene product (*11*), which is essential for the efficient sorting of vacuolar hydrolases in yeast (Fig. 1) (*15*). In eukaryotic cells, vacuolar (lysosomal) enzymes and proteins destined for secretion are transported together through the early stages of the secretory pathway (endoplasmic reticulum and Golgi apparatus) (*16*, *17*). Protein transport is mediated by vesicles that bud from one compartment and then dock and fuse with the next compartment in the pathway (*18*). Vacuolar (lysosomal) hydrolases are actively sorted away from proteins destined for the cell surface in a late Golgi compartment (*19*, *20*). Mutations in the *VPS*34 gene lead to the missorting and secretion of Golgi apparatus–modified precursor forms of several vacuolar hydrolases, including carboxypeptidase Y (CPY) and proteinase A (PrA) (*15*, *17*). Subcellular fractionation experiments have shown that the product of the *VPS*34 gene, Vps34p, is associated through protein-protein interactions with an organelle distinct from the vacuole (*15*, *21*).

PI 3-kinase activity is readily detected in yeast cell extracts (*22*). To determine whether *VPS*34 encodes PI 3-kinase, we assayed for PI 3-kinase activity in extracts from wild-type yeast cells, cells with the

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VPS34 gene deleted (Δ*vps34*), and cells that overproduce Vps34p (Fig. 2A). The crude membrane fraction sedimented at 100,000*g* (P100) from wild-type yeast cells contained 90% of the cellular PI 3-kinase activity, whereas the supernatant (S100) contained only 10%. High amounts of PI 4-kinase activity, which phosphorylates the 4 position of the inositol ring (*23*), were present in both cellular fractions. The fact that 90% of the PI 3-kinase activity was present in the crude membrane pellet (P100), whereas only 50% of the Vps34 protein was recovered in this pellet, indicates that membrane-associated Vps34p has a higher specific activity (Fig. 2B). PI 3-kinase activity was not detected in either the P100 and S100 fractions isolated from the Δ*vps34* strain. PI 4-kinase activity, however, was found in both the P100 and S100 fractions of the *vps34* null mutant strain (Fig. 2A).

Cell lysates from yeast cells containing the *VPS34* gene on a multicopy yeast plasmid contained fourfold more PI 3-kinase activity than was found in an equivalent amount of extract from wild-type cells. Unlike wild-type cells, however, the lipid kinase activity in extracts from the strain overproducing Vps34p was distributed nearly equally between the particulate (P100, 55%) and the soluble (S100, 45%) subcellular fractions (Fig. 2A). Approximately 20-fold more Vps34p was produced in the strain carrying the *VPS34* gene on a multicopy number plasmid than in wild-type cells. The fact that most (95%) of Vps34p was found in the S100 fraction indicates that there are a limited number of sites for association of Vps34p with the crude membrane fraction or that a modification of Vps34p is required for the association and that the modifying system is saturated when Vps34p is overproduced (Fig. 2B). The association of Vps34p with the membrane or with one or more proteins in the membrane appears to result in the activation of the Vps34 PI 3-kinase activity (Fig. 2A).

To verify that Δ*vps34* cells lack PI 3-kinase activity, we labeled the wild-type and Δ*vps34* strains in vivo with [^{3}H]*myo*-inositol. The labeled lipids were isolated, deacylated, and separated by high-performance liquid chromatography (HPLC). Wild-type yeast contained two compounds that eluted at the same positions as the glycerophosphatidylinositol 3-phosphate (gPI3P) and glycerophosphatidylinositol 4-phosphate (gPI4P) standards, respectively, whereas only gPI4P was found in the lipids recovered from the Δ*vps34* strain (Fig. 3). No multiply phosphorylated phosphoinositides [PI(3,4)P_2 or PI(3,4,5)P_3] were found under these conditions.

We obtained further evidence that the VPS34 gene encodes PI 3-kinase by demonstrating that PI 3-kinase activity can be specifically immunoprecipitated from cell extracts with antiserum that recognizes Vps34p. Protein A–Sepharose beads (Pharmacia) to which the antiserum to Vps34p was adsorbed were incubated with the S100 fraction isolated from the strain overproducing Vps34p. The resulting immune precipitate contained the PI 3-kinase activity (Fig. 4); no PI 3-kinase activity was detected in the supernatant fraction after the immune precipitation. By contrast, no PI

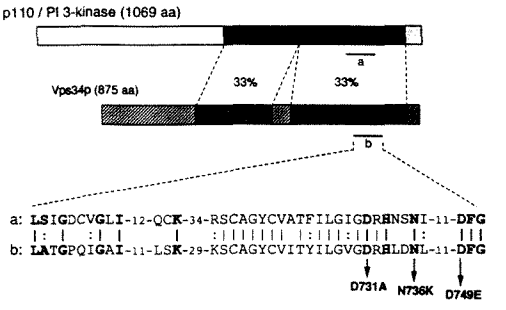

Fig. 1. Sequence similarity between Vps34p and the p110 subunit of the bovine brain PI 3-kinase. Black boxes correspond to regions of these proteins that share significant sequence similarity (33% amino acid identity). Shown below is a comparison of p110 (indicated by a) and Vps34p (indicated by b) sequences that contain the conserved sequence motifs present in the catalytic domains of protein kinases (bold letters) (*25, 26*). Single amino acid changes that were constructed by site-directed mutagenesis (*32*) of the *VPS34* gene are also shown. In (a) and (b), vertical bars indicate identical amino acid residues, colons indicate conservative amino acid substitutions, and the numbers indicate amino acid spacing between conserved sequence elements. Abbreviations for the amino acids (aa) are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

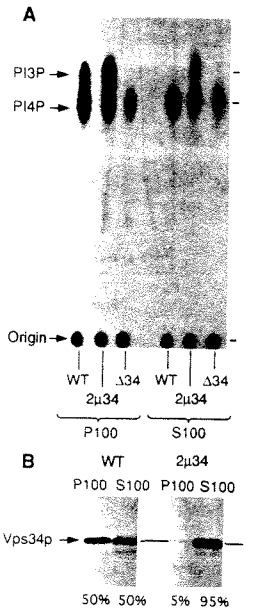

Fig. 2. PI 3-kinase activity of Vps34p and its subcellular localization. (**A**) Thin-layer chromatography (TLC) of PI kinase assay reaction products performed with the P100 and S100 fractions isolated from wild-type strain SEY6210 (WT), Δ*vps34* strain PHY102 (Δ34), and strain SEY6210 bearing the *VPS34* gene on a multicopy plasmid (2μ34) (*15*). Yeast cells were grown in yeast nitrogen base (YNB) medium (*33*), enzymatically converted into spheroplasts, lysed, and centrifuged at 500*g* for 5 min. The cleared cell extract was centrifuged at 100,000*g* for 30 min at 4°C. Protein (1 μg) from the pellet (P100) and supernatant (S100) fractions was assayed for PI kinase activity in the presence of [γ-^{32}P]ATP in a volume of 50 μl (*34*). Assays were incubated for 5 min at 25°C. Samples were separated on silica TLC plates (Merck) with the borate system (*35*). We determined the distribution of the total cellular PI 3-kinase activity between the subcellular fractions after normalizing for protein recoveries in the supernatant (70% of cellular protein) and the pellet (30% of cellular protein). For the wild-type strain, this resulted in a distribution of 90% of the PI 3-kinase activity in the P100 and 10% in the S100 fraction. For the strain overproducing Vps34p (2μ34), 55% of the PI 3-kinase activity was found in the P100 and 45% in the S100 fraction. We added sufficient exogenous substrate (10 μg of PI; Sigma) to attain linear reaction kinetics through a 10-min incubation period. No significant PI3P-phosphatase activity was detected under the assay conditions used. The reduced amount of PI4P produced in the Δ*vps34* strain extract is most likely due to the slow growth of the mutant strain. A Phosphor-Imager (Molecular Dynamics) was used for quantitation. (**B**) Distribution of Vps34p between the P100 and S100 fractions of SEY6210 (WT) and the strain overproducing Vps34p (2μ34). Spheroplasts were labeled in selective minimal media in the presence of Tran-^{35}S-label (10 μCi/$OD_{600\ nm}$, ICN) for 30 min and incubated at 30°C for 60 min after the addition of methionine and cysteine to a final concentration of 25 mM (*15*). The spheroplasts were lysed and separated into P100 and S100 fractions as described in (A). We used antiserum to Vps34p to immunoprecipitate Vps34p (*15*), and the immunoprecipitated proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE) (8% gel). The percentage distribution between P100 and S100 fractions was deteremined by densitometry of the autoradiogram (LKB Ultroscan XL) and is indicated under each lane. Relative to the wild-type strain, one-tenth as much sample from the overproducing strain (2μ34) was loaded onto the gel.

4-kinase activity was detected in the pellet fraction. It remained in the supernatant (Fig. 4). Preimmune serum did not precipitate any PI 3-kinase or PI 4-kinase activity.

The regions of sequence similarity between Vps34p and p110 are in the COOH-terminal parts of the proteins (Fig. 1). The degree of identity in this region is 33%, and the degree of similarity is >57% if conservative amino acid changes are included [comparison of amino acids 315 to 820 of Vps34p with amino acids 539 to 1014 of p110 with gap analysis (*24*)]. The regions of similarity between Vps34p and p110 include motifs that are also highly conserved in the catalytic domains of protein kinases (Fig. 1) (*25*). Vps34p and p110, however, contain only a subset of the structural features conserved among protein kinases. The crystal structure of the adenosine 3′,5′-monophosphate (cAMP)–dependent protein kinase (PKA) indicates that these conserved motifs participate in adenosine trisphosphate (ATP) binding and phosphate transfer (*26*). These motifs are the glycine-rich region (GXGXXG; G is glycine and X represents any amino acid), which interacts with nontransferable phosphates; the catalytic loop region (DXHXXN; D is aspartic acid, H is histidine, and N is asparagine); and the DFG (F is phenylalanine) motif, which is the smallest and most highly conserved structural feature among protein kinases.

We used site-directed mutagenesis to construct three *vps*34 point mutations to study the functional significance of the sequences shared between Vps34p and protein kinases. Asp^{731} was replaced by alanine (D731A), Asn^{736} was changed to lysine (N736K), and Asp^{749} was replaced by glutamic acid (D749E) (Fig. 1). These *vps*34 mutant alleles were introduced into a Δ*vps*34 strain on a multicopy plasmid vector. No PI 3-kinase activity was detected in extracts from any of the three mutants (Fig. 5A). In vivo [^{35}S]methionine labeling experiments demonstrated that all of the mutants expressed Vps34p (Fig. 5B), and the mutant proteins exhibited stability similar to that of the wild type. The various *vps*34 point mutant constructs did not complement any of the phenotypes associated with a Δ*vps*34 mutation (*15*). All three strains were temperature-sensitive for growth and had a severe defect in vacuolar protein sorting. Unlike wild-type cells in which the Golgi apparatus–modified precursor form of CPY (p2CPY) is transported to the vacuole and matured (mCPY), all of the p2CPY was secreted by the mutant cells. No CPY was detected inside these cells (Fig. 5C).

The data presented in this study implicate PI 3-kinase activity in the regulation of vacuolar protein sorting. The precise function of PI3P or of multiply phosphorylated 3-phosphoinositides is not yet known. At least two general models can be considered for the role of phosphoinositides in protein sorting. (i) They may alter the biophysical properties of the lipid bilayer in the sorting compartment: the increase of the PI3P (a charged phospholipid) in the outer leaflet of the membrane could change the molecular organization of the outer leaflet, thus increasing the curvature of the membrane and thereby stimulating vesicle emergence, as has been postulated in the lipid-bilayer couple hypothesis (*27*). (ii)

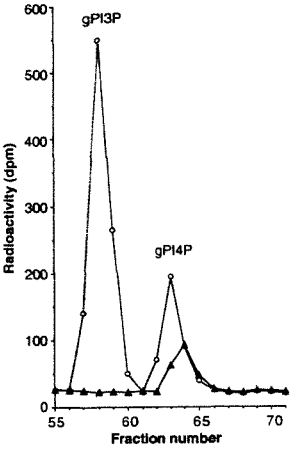

Fig. 3. In vivo PI kinase activity in wild-type and Δ*vps*34 strains. The HPLC elution profiles of tritiated glycerophosphoinositols isolated from SEY6210 (WT; open circles) and PHY102 (Δ*vps*34; closed triangles) are shown. Strains were grown in the presence of 30 μCi (6 μCi/ml) of [^{3}H]*myo*-inositol (18.8 Ci/mmol; Amersham) over six generations at 25°C in synthetic Wickerham's minimal growth medium without inositol (*36*). Vitamins and amino acids were added as required (Difco). Lipids were extracted with acidified chloroform–methanol and deacylated; the resulting glycerophosphoinositols were separated by HPLC (*37*). Fractions were analyzed in a liquid scintillation counter (Beckman LS1801). Tritiated standards of glycerophosphatidylinositol, gPI4P, and glycerophosphatidylinositol 4,5-bisphosphate were prepared from [^{3}H]PI, [^{3}H]PI4P and [^{3}H]PI(4,5)P_2, respectively, and we prepared a glycerophosphatidylinositol 3-monophosphate (gPI3P) standard by phosphorylating PI in vitro with purified bovine brain PI 3-kinase in the presence of [γ-^{32}P]ATP.

Fig. 4. Immunoprecipitation of PI 3-kinase activity as in Fig. 2A. PI kinase assays were done on the total S100 fraction of the Vps34p-overproducing strain (lane 1), the sedimented Vps34p immune complex (lane 2), and the supernatant from the immunoprecipitation (lane 3). Protein A–Sepharose beads were adsorbed to antibodies specific for Vps34p (*15*) and washed, and the supernatant fraction (S100) of SEY6210 containing *VPS*34 on a yeast multicopy plasmid (2μ34) was incubated with the beads for 4 hours at 4°C. PI kinase assays were done after we washed the immune complexes (four times with 0.5% Tween-20 in tris-buffered saline and six times with tris-buffered saline). After the immunoprecipitation reactions, 25 to 50% of the total PI kinase activities were recovered.

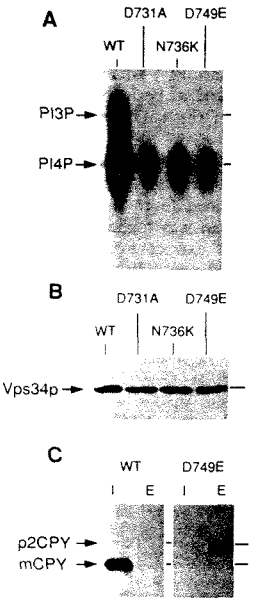

Fig. 5. The *vps*34 point mutations. (**A**) TLC of PI kinase assay reaction products done with the P100 fractions of wild-type strain SEY6210 (WT) and *vps*34 point mutants. All *vps*34 mutant alleles were present on multicopy plasmids in PHY102 (Δ*vps*34). (**B**) Expression of wild-type and mutant *VPS*34 proteins. Cells were labeled with Tran-^{35}S-label for 20 min at 30°C. Proteins precipitated in trichloracetic acid were resuspended and incubated with antiserum to Vps34p; proteins in the immunoprecipitate were separated by SDS-PAGE (10% gel) as described (*15*). (**C**) Sorting of CPY in wild-type yeast and in one of the point mutants (D749E). Nearly identical results were obtained with the D731A and N736K mutants. Spheroplasts were grown in selective minimal media in the presence of Tran-^{35}S-label for 5 min and chased for 30 min. The labeled cultures were centrifuged for 2 min at 13,000*g* and separated into sedimented (I, intracellular) and supernatant (E, extracellular) fractions. The amount of CPY in each fraction was assessed by quantitative immunoprecipitation with CPY-specific antisera, followed by SDS-PAGE as described (*31*).

Phosphoinositides could serve as specific membrane targets that bind proteins required for the formation of transport vesicles, such as the polypeptides of the adaptor complex that link clathrin to the cytoplasmic tails of certain transmembrane receptor proteins (for example, the mannose-6-phosphate receptor) (*28*). Vesicles from rat adipocytes that contain the glucose transporter also contain PI 4-kinase, which may regulate the transport (fusion) of these vesicles with the plasma membrane in response to insulin (*29*). In addition to its potential role in signaling cell proliferation, PI 3-kinase associated with receptor protein tyrosine kinases at the plasma membrane may also take part in the endocytosis and down-regulation (lysosomal degradation) of these receptors. In this way, the duration and the magnitude of the growth signal might be modulated. The association of Vps34p with the membrane appears to be mediated by the product of another *VPS* gene, *VPS15*. The *VPS15* gene encodes a membrane-associated protein kinase (Vps15) (*30*, *31*) that can be chemically cross-linked to Vps34p (*21*). This raises the possibility that the Vps15 and Vps34 proteins may function together as components of a signal transduction complex that regulates intracellular protein sorting decisions.

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Chapter 5:

A Membrane-Associated Complex Containing the Vps15 Protein Kinase and the Vps34 PI 3-Kinase is Essential for Protein Sorting to the Yeast Lysosome-Like Vacuole

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A membrane-associated complex containing the Vps15 protein kinase and the Vps34 PI 3-kinase is essential for protein sorting to the yeast lysosome-like vacuole

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The Vps15 protein kinase and the Vps34 phosphatidylinositol 3-kinase (PI 3-kinase) are required for the sorting of soluble hydrolases to the yeast vacuole. Overproduction of Vps34p suppresses the growth and vacuolar protein sorting defects associated with *vps15* kinase domain mutants, suggesting that Vps15p and Vps34p functionally interact. Subcellular fractionation and sucrose density gradients indicate that Vps15p is responsible for the association of Vps34p with an intracellular membrane fraction. Chemical cross-linking and native immunoprecipitation experiments demonstrate that Vps15p and Vps34p interact as components of a hetero-oligomeric protein complex. In addition, we show that an intact Vps15 protein kinase domain is required for activation of the Vps34 PI 3-kinase, suggesting that the Vps34 lipid kinase is regulated by a Vps15p-mediated protein phosphorylation event. We propose that Vps15p and Vps34p function together as components of a membrane-associated signal transduction complex that regulates intracellular protein trafficking decisions through protein and lipid phosphorylation events.

Key words: PI 3-kinase/protein kinase/protein sorting/vacuole/yeast

Introduction

The efficient sorting and delivery of proteins from a common cytoplasmic site of synthesis to their final intracellular locations is a fundamental characteristic of all eukaryotic cells. Proteins destined for the lysosome or vacuole transit the early stages of the secretory pathway before being segregated away from the bulk flow of secretory proteins headed for the cell surface (reviewed in Kornfeld and Mellman, 1989; Klionsky *et al.*, 1990). In a late Golgi compartment, lysosomal and vacuolar proteins are sorted in an active process that requires the presence of *cis*-acting sorting information. One well characterized signal for sorting to the mammalian lysosome is the mannose-6-phosphate modification present on soluble lysosomal proteins (reviewed in Kornfeld and Mellman, 1989; Kornfeld, 1992). Lysosomal proteins containing these phosphomannosyl residues bind to mannose-6-phosphate receptors in a late Golgi compartment. The receptor–ligand complex is delivered via clathrin-coated vesicles to an intermediate endosomal compartment where the lysosomal proteins dissociate from the receptors. Lysosomal proteins are then delivered from the endosome to the lysosome while the receptors recycle back to the Golgi complex where they are utilized for additional rounds of transport. Delivery of yeast proteins to the lysosome-like vacuole does not involve a carbohydrate-specific sorting signal. Instead, the sorting signal is present in the amino acid sequence of vacuolar proteins (Johnson *et al.*, 1987; Valls *et al.*, 1987, 1990; Klionsky *et al.*, 1988).

Genetic selections in *Saccharomyces cerevisiae* have resulted in the isolation of a large number of mutants defective in vacuolar protein sorting (Jones, 1977; Bankaitis *et al.*, 1986; Rothman and Stevens, 1986; Robinson *et al.*, 1988; Rothman *et al.*, 1989). These *vps* (vacuolar protein sorting defective) mutants missort and secrete the precursors of soluble vacuolar hydrolases such as carboxypeptidase Y (CPY), proteinase A (PrA) and proteinase B (PrB) (Bankaitis *et al.*, 1986; Rothman and Stevens, 1986; Robinson *et al.*, 1988; Rothman *et al.*, 1989). Genetic analysis of the *vps* mutants found that they constitute >40 complementation groups, implicating a large number of gene products in the process of vacuolar protein localization in yeast (reviewed in Klionsky *et al.*, 1990). Representative alleles from each complementation group were analyzed for their effects on vacuolar morphology, protein sorting and growth (see Klionsky *et al.*, 1990). The *vps15* and *vps34* complementation groups exhibit a very similar set of terminal phenotypes such that they appear to constitute a unique subset among the *vps* mutant collection. Strains deleted for either or both of the *VPS15* and *VPS34* genes have the following characteristics: (i) viable but temperature-sensitive (ts) for growth at 37°C (Robinson *et al.*, 1988; Herman and Emr, 1990; Herman *et al.*, 1991b); (ii) specific and severe defects in the sorting of soluble vacuolar hydrolases (Robinson *et al.*, 1988; Herman and Emr, 1990; Herman *et al.*, 1991a,b); (iii) sensitivity to osmotic stress (Banta *et al.*, 1988; Herman and Emr, 1990); (iv) defects in vacuole segregation at mitosis (Herman and Emr, 1990); and (v) possess a morphologically near-normal vacuole (Banta *et al.*, 1988; Herman and Emr, 1990). This set of common phenotypes suggests that the *VPS15* and *VPS34* gene products may act at the same step in the vacuolar protein sorting pathway.

The *VPS15* gene encodes a rare 1455 amino acid protein whose N-terminal 300 residues exhibit significant similarity to the serine/threonine family of protein kinases (Herman *et al.*, 1991b). Alteration of residues in the Vps15 protein (Vps15p) that are highly conserved among protein kinases results in a ts growth defect, the missorting and secretion of vacuolar proteins and abolishes the *in vivo* phosphorylation of Vps15p (Herman *et al.*, 1991a,b). Truncation of 30 amino acids from the C-terminus of Vps15p results in a temperature-conditional defect in protein sorting. Following shift to the non-permissive temperature, these mutant cells exhibit an immediate but reversible defect in the sorting of

soluble vacuolar hydrolases. This indicates that the Vps15 protein kinase plays a direct role in regulating protein sorting to the vacuole (Herman *et al.*, 1991a). Subcellular fractionation studies show that Vps15p is associated with the cytoplasmic face of an intracellular membrane, most likely a late Golgi or vesicle compartment (Herman *et al.*, 1991b). Together, these results have led to the proposal that Vps15p-mediated protein phosphorylation reactions may provide a molecular switch within intracellular protein sorting pathways by actively diverting proteins from a default transit pathway (e.g. secretion) to an alternative pathway (e.g. to the vacuole).

The *VPS34* gene product (Herman and Emr, 1990) shares extensive sequence similarity with the catalytic subunit of mammalian phosphatidylinositol 3-kinase (PI 3-kinase) (Hiles *et al.*, 1992). PI 3-kinase phosphorylates membrane PI and its more highly phosphorylated derivatives [PI(4)P and $PI(4,5)P_2$] at the D-3 position of the inositol ring (reviewed in Carpenter and Cantley, 1990). In mammalian cells, PI 3-kinase associates with several signal transducing receptor tyrosine kinases and is postulated to be involved in the generation of key second messenger molecules important in regulating cell growth and proliferation (reviewed in Cantley *et al.*, 1991). The 110 kDa catalytic subunit is 33% identical and 55% similar to the Vps34 protein (Vps34p) over the stretch of 450 amino acids that constitutes the C-terminal half of each protein (Hiles *et al.*, 1992). *S.cerevisiae* has been shown to possess PI 3-kinase activity (Auger *et al.*, 1989) and strains deleted for *VPS34* appear to lack this activity (Schu *et al.*, 1993). In addition, point mutations in *VPS34*, which alter residues conserved between the p110 and Vps34 lipid kinases, result in a dramatic decrease in PI 3-kinase activity and a severe vacuolar protein sorting defect (Schu *et al.*, 1993). Therefore, it appears that Vps34p-mediated phosphorylation of membrane PI is essential for vacuolar protein sorting in yeast.

We present here genetic and biochemical evidence for a direct interaction between the Vps15 protein kinase and the Vps34 lipid kinase. We report that overproduction of Vps34p suppresses the growth and vacuolar protein sorting defects associated with *vps15* kinase domain mutants. Subcellular fractionation data indicate that Vps15p mediates the association of Vps34p with an intracellular membrane fraction. Native immunoprecipitation and chemical cross-linking experiments demonstrate that Vps15p and Vps34p are physically associated. In addition, we find that Vps15 protein kinase activity is required for activation of the Vps34 PI 3-kinase, suggesting that the Vps34 lipid kinase is regulated by a Vps15p-mediated protein phosphorylation event. Taken together, we show that Vps15 protein kinase and Vps34 lipid kinase are components of a hetero-oligomeric protein complex and propose that this complex acts as a key regulator of protein sorting decisions in the late Golgi.

Results

Overproduction of the Vps34 lipid kinase suppresses the pleiotropic defects exhibited by vps15 kinase domain mutants

The common phenotypes exhibited by Δ*vps15* and Δ*vps34* strains suggested that the Vps15 protein kinase and the Vps34 PI 3-kinase may act together at the same step in the vacuolar protein sorting pathway. To test this, we looked for genetic interaction between *VPS15* and *VPS34*. We found that overproduction of Vps34p suppressed the pleiotropic defects associated with a *vps15* kinase domain mutant strain. The *vps15-E200R* kinase domain point mutant (APE_{200} → APR in Vps15p) displayed a ts growth defect identical to that of the Δ*vps15* strain (Figure 1A; Herman *et al.*, 1991b). The ~20-fold overproduction of Vps34p from a multicopy plasmid containing wild-type *VPS34* (Herman and Emr, 1990) (pJSY324.34) corrected the ts growth defect of the *vps15-E200R* mutant strain (Figure 1A). Importantly, an isogenic Δ*vps15* strain carrying the same *VPS34* multicopy plasmid was unable to grow at 37°C (Figure 1A). This indicates that overproduction of Vps34p cannot bypass the requirement for the Vps15 protein kinase.

Overproduction of Vps34p also suppressed the severe vacuolar protein sorting defects found in the *vps15-E200R* kinase domain mutant. Yeast cells were pulse-labeled with $Tran^{35}S$-label, chased, converted to spheroplasts and supernatant (media) and pellet (intracellular) fractions were treated with CPY-specific antisera. In a wild-type strain, >95% of the newly synthesized CPY was found in the intracellular fraction as the 61 kDa mature species (mCPY), indicative of arrival and proteolytic processing in the vacuole (Figure 1B). In contrast, a Δ*vps15* strain missorted and secreted >95% of CPY from the cells as the Golgi-modified p2 precursor form (p2CPY). The *vps15-E200R* kinase domain mutant displayed a sorting phenotype identical to that of the

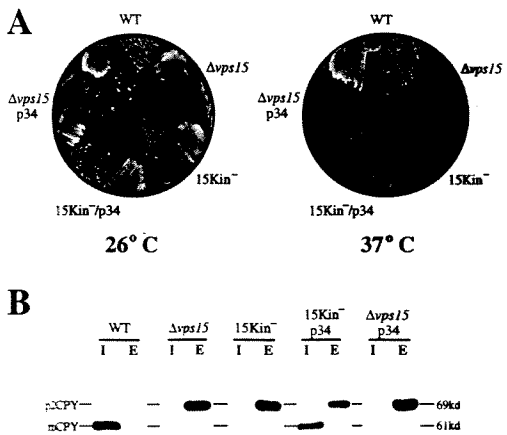

Fig. 1. Suppression of the growth and protein sorting defects of a *vps15* kinase domain mutant by overproduction of the Vps34 PI 3-kinase. (**A**) Analysis of growth characteristics of *vps15* mutants. The indicated strain was streaked onto YPD plates and incubated at either 26 or 37°C. The strains examined were: WT, SEY6210; Δ*vps15*, PHY112; 15Kin⁻, PHY112 harboring the *vps15-E200R* allele on a low copy number plasmid (pPHY15C-K2); 15Kin⁻/p34, PHY112 containing pPHY15C-K2 (*vps15-E200R*, *CEN*) and wild-type *VPS34* on a high copy number plasmid (pJSY324.34); Δ*vps15*/p34, pHY112 containing pJSY324.34. (**B**) Intracellular sorting and processing of CPY. Yeast cells were labeled with $Tran^{35}S$ label for 15 min and chased for 30 min at 30°C following the addition of methionine and cysteine to 2 mM. The labeled cells were converted to spheroplasts, centrifuged at 4000 *g* for 30 s and separated into pellet (I, intracellular) and supernatant (E, extracellular) fractions. Quantitative immunoprecipitation of CPY from each fraction was performed. The positions of p2CPY and mCPY are indicated. The strains examined are identical to those described in panel A.

Δ*vps15* strain (Figure 1B). In the *vps15-E200R* mutant strain harboring the multicopy *VPS34* plasmid, ~50% of newly synthesized CPY remained intracellular and was processed to the mature form, indicating that it was properly sorted and transported to the vacuole. Overproducing Vps34p in a Δ*vps15* strain did not rescue its sorting defect; >95% of CPY was secreted as the p2 precursor (Figure 1B). Thus, the overexpression of Vps34p suppressed the protein sorting and growth defects associated with a *vps15* kinase domain mutant, but not a Δ*vps15* strain. We have extended the suppression analysis to the vacuolar hydrolases PrA and PrB. The *vps15-E200R* mutant is also defective for the vacuolar sorting of both PrA and PrB, and overproduction of Vps34p suppressed the sorting defect to a level similar to that found for CPY (data not shown). In addition, we have also examined several other *vps15* kinase domain point mutants, including D147R, K149D and D165R (Herman *et al.*, 1991a), and found that they were also suppressed by overproduction of Vps34p (data not shown). Altogether, the common set of phenotypes exhibited by Δ*vps15* and Δ*vps34* strains and the fact that Vps34p overproduction will suppress the pleiotropic defects of *vps15* kinase domain mutants, but not a *vps15* null allelle, suggest that Vps15p and Vps34p functionally interact in the cell to facilitate vacuolar protein sorting.

Subcellular fractionation suggests that Vps15p is required for association of Vps34p with a sedimentable cell fraction

In an effort to define biochemically the functional interaction between Vps15p and Vps34p suggested by the genetic data, we investigated the subcellular distribution of these proteins. While neither protein contains a transmembrane domain to anchor it in the membrane, both proteins exhibit biochemical properties that suggest possible membrane association. Previous studies have indicated that a substantial fraction of Vps34p may be associated with a sedimentable multiprotein complex (Herman and Emr, 1990). Based on subcellular fractionation, protease accessibility and detergent extraction studies, Vps15p has been suggested to be peripherally associated with the cytoplasmic face of an intracellular membrane fraction, most likely the late Golgi or an intermediate compartment functioning between the Golgi and the vacuole (Herman *et al.*, 1991b).

To compare directly the subcellular distribution of Vps15p and Vps34p, yeast cells were converted to spheroplasts, labeled with Tran^{35}S-label for 30 min and chased for 1 h to approximate steady-state conditions. The labeled spheroplasts were subjected to gentle osmotic lysis and the lysate was centrifuged at 100 000 *g* for 30 min to generate pellet (P100) and supernatant (S100) fractions. The proteins in these fractions were precipitated with trichloroacetic acid (TCA) and subjected to quantitative immunoprecipitation using Vps15p- and Vps34p-specific antisera. In the wild-type yeast strain TVY1, Vps15p and Vps34p exhibited fractionation characteristics very similar to those previously reported: 70% of Vps15p was found in the P100 fraction and 30% in the S100 fraction, and Vps34p was distributed equally between P100 and S100 fractions (Figure 2). It is interesting to note that while wild-type yeast cells contain 2- to 3-fold more Vps34p than Vps15p, the relative amounts of Vps15p and Vps34p in the P100 fraction were very similar. These data are consistent with a model in which Vps15p and Vps34p associate with the same membrane compartment or protein complex. As Vps15p appears to be associated with an intracellular membrane fraction, it is possible that an association between Vps15p and Vps34p results in sedimentation of Vps34p. To test this, we examined the distribution of Vps34p in a Δ*vps15* strain. The absence of Vps15p resulted in a dramatic shift of Vps34p into the S100 fraction; 90% of Vps34p remained soluble after centrifugation at 100 000 *g* (Figure 2). These data indicate that Vps15p is required for the presence of Vps34p in the P100 fraction. One explanation for these results is that Vps15p directly recruits Vps34p to the membrane. If this were the case, overproduction of Vps34p might titrate the limited number of association sites defined by Vps15p. The association of Vps34p with a pelletable structure does indeed appear to be saturable. Overproducing Vps34p by expressing a *VPS34* multicopy plasmid in the TVY1 strain, which contains a single copy of the *VPS15* gene, resulted in >90% of Vps34p fractionating to the S100 (Figure 2). The amount of Vps34p in the P100 fraction was found to be approximately the same in strains expressing Vps34p from single or multicopy plasmids. These data indicate that high level expression of Vps34p has saturated the association sites responsible for its presence in the P100 fraction. Overproducing both Vps15p and Vps34p resulted in a 5-fold increase in the amount of pelletable Vps34p relative to overproducing Vps34p in a strain containing *VPS15* at single copy (data not shown). This supports the idea that Vps15p constitutes at least part of the membrane association site for Vps34p. The subcellular fractionation data are most consis-

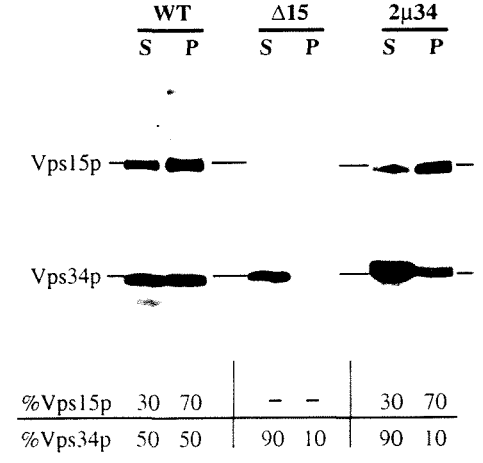

Fig. 2. Subcellular fractionation of Vps15p and Vps34p. 10 OD_{600} units of spheroplasts of the appropriate yeast strain were labeled with Tran^{35}S label for 30 min and chased for 1 h at 30°C. The labeled cells were osmotically lysed and the cleared lysate was centrifuged at 100 000 *g* for 30 min at 4°C. Quantitative immunoprecipitation of Vps15p (170 kDa) and Vps34p (95 kDa) was performed from the resulting supernatant (S100) and pellet (P100) fractions. The relative amount of Vps15p and Vps34p in each fraction was determined by densitometry of autoradiograms and is expressed as a percentage in each fraction. WT refers to TVY1, Δ15 refers to PHY112 and 2μ34 refers to TVY1 harboring *VPS34* on a multicopy plasmid (pJSY324.34). The 2μ34 lanes contained approximately one-half as much sample as the others.

tent with Vps15p directly regulating the presence of Vps34p in a pelletable fraction.

Vps15p and Vps34p associate with a membrane fraction on sucrose density gradients

The subcellular fractionation data suggest that Vps15p and Vps34p may be components of a pelletable multiprotein complex *in vivo*. Differential centrifugation experiments have shown that Vps15p exhibits fractionation characteristics which indicate that it is associated with a membrane fraction distinct from the endoplasmic reticulum, early Golgi or the vacuole (Herman *et al.*, 1991b). As Vps15p appears to be necessary for the presence of Vps34p in a P100 fraction, we investigated the possibility that both Vps15p and Vps34p are membrane-associated. To accomplish this, we used sucrose density gradients, which separate organelles and membrane-bound structures such as vesicles, on the basis of their buoyant density. The yeast strain TVY1 was converted to spheroplasts, labeled with Tran^{35}S-label, osmotically lysed and centrifuged at 100 000 *g* for 30 min exactly as described in the previous section. The P100 pellet was resuspended in 60% sucrose and overlayed with an equal volume of 35% sucrose as described previously by Paravicini *et al.* (1992). The gradient was spun at 170 000 *g* for 18 h, fractions were collected and immunoprecipitations performed. This single step gradient will not separate intracellular membrane organelles, however, it is quite useful in separating non-membrane from membrane-associated constituents. Vps15p and Vps34p were found to migrate out of the 60% sucrose layer and move into less dense fractions (Figure 3). It is striking that Vps15p and Vps34p showed nearly identical gradient fractionation characteristics and the relative abundance of each protein in a particular gradient fraction was very similar. A membrane protein of the late Golgi, Kex2p (Redding *et al.*, 1991), was analyzed in the same gradient as a membrane marker. The gradient fractionation characteristics of Kex2p were similar to Vps15p and Vps34p, which indicates that Vps15p and Vps34p behave in a sucrose gradient in a manner similar to this

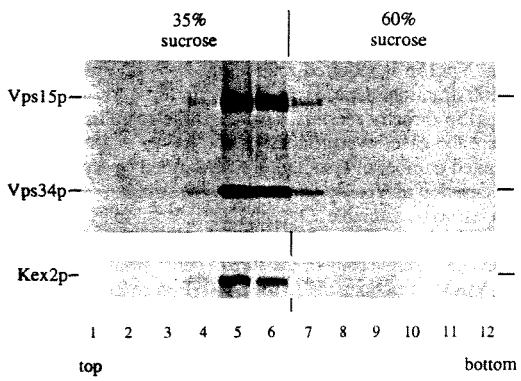

Fig. 3. Sucrose density gradient fractionation of Vps15p and Vps34p. 35 OD_{600} units of labeled TVY1 spheroplasts were osmotically lysed and the cleared lysate was centrifuged at 100 000 *g* for 30 min at 4°C. The resulting pellet was resuspended in 60% sucrose and overlayed with an equal volume of 35% sucrose. This step-gradient was centrifuged at 170 000 *g* for 18 h at 4°C. 12 fractions were collected from the top (fraction 1) to the bottom (fraction 12) and subjected to quantitative immunoprecipitation of Vps15p and Vps34p. The late Golgi membrane protein Kex2p was analyzed from the same gradient as a membrane marker.

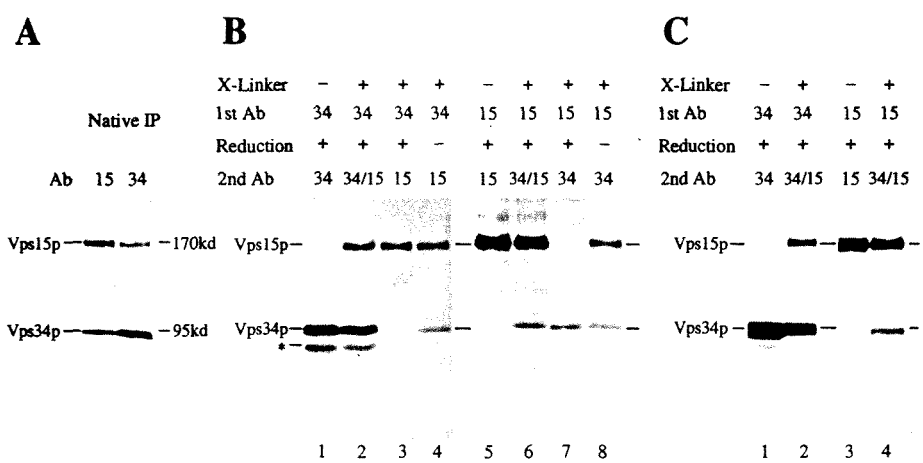

Fig. 4. Native immunoprecipitation and cross-linking of Vps15p and Vps34p. (**A**) Immunoprecipitation under non-denaturing conditions. 0.5 OD_{600} units of TVY1 spheroplasts harboring *VPS15* and *VPS34* multicopy plasmids were lysed in a Tween-20-containing buffer. The lysate was incubated with antisera specific for Vps15p or Vps34p (Ab). The immunoprecipitated proteins were electrophoresed by 8% SDS–PAGE and transferred to nitrocellulose as described in Materials and methods. The blot was incubated with both anti-Vps15p and anti-Vps34p antisera and immunoreactive species were detected using ^{35}S-labeled protein A and autoradiography. The positions of Vps15p and Vps34p are indicated. (**B**) Chemical cross-linking of Vps15p and Vps34p in a wild-type yeast strain. Labeled TVY1 spheroplasts were osmotically lysed and treated with the thiol-cleavable cross-linker DSP (X-linker). The cross-linked extract was subjected to quantitative immunoprecipitation under denaturing but non-reducing conditions using anti-Vps15p or anti-Vps34p antisera (1st Ab). The samples were treated with sample buffer with or without 2% 2-mercaptoethanol (Reduction) and then re-immunoprecipitated with the indicated antisera (2nd Ab). All samples were reduced immediately prior to electrophoresis. Each lane represents 10 OD_{600} units of labeled extract. The asterisk indicates a labeled species that non-specifically cross-reacts with anti-Vps34p antisera. (**C**) Chemical cross-linking of Vps15p and Vps34p in a strain overproducing both proteins. Labeled spheroplasts of the strain TVY1 containing *VPS15* and *VPS34* on multicopy plasmids (pPHY15E and pJSY324.34) were lysed and treated with DSP. The cross-linked extract was incubated with anti-Vps15p or anti-Vps34p antisera, reduced, then re-immunoprecipitated with the appropriate antisera as described in panel B. The samples were reduced and electrophoresed on an SDS–polyacrylamide gel. Each lane represents 2 OD_{600} units of labeled extract.

membrane protein (Figure 3). Previous studies have shown that other membrane proteins such as cytochrome *c* oxidase, NADPH cytochrome *c* reductase, and the plasma membrane ATPase also move out of the 60% sucrose load into less dense fractions in similar gradients (Walworth *et al.*, 1989). These gradients therefore demonstrate that the pelletable fraction of Vps15p and Vps34p is associated with an intracellular membrane fraction.

The Vps15 protein kinase and the Vps34 lipid kinase are members of a hetero-oligomeric protein complex

The similar phenotypes of Δ*vps15* and Δ*vps34* strains and the suppression of *vps15* kinase domain mutants by overproduction of Vps34p suggest that Vps15p and Vps34p are functionally interacting in the cell. The biochemical data presented in the previous sections indicate that Vps15p and Vps34p co-localize to an intracellular membrane in a manner consistent with physical interaction between the two proteins. To address directly the question of whether Vps15p and Vps34p are physically associated as members of a protein complex, we performed immunoprecipitations under non-denaturing conditions and chemical cross-linking experiments.

Yeast spheroplasts were gently lysed in a Tween-20-containing buffer and the lysates were subjected to native immunoprecipitation using antisera specific for either Vps15p or Vps34p. The immunoprecipitated material was electrophoresed by SDS−PAGE and transferred to nitrocellulose. The blots were then incubated with antisera to both Vps15p and Vps34p and immunoreactive material was visualized using ^{35}S-labeled protein A. It was found that Vps15p and Vps34p could be co-immunoprecipitated using antisera specific for either protein (Figure 4A). The co-precipitation of Vps15p and Vps34p under non-denaturing conditions suggests that they are physically associated *in vivo*.

The association between Vps15p and Vps34p also was investigated in chemical cross-linking experiments. The homobifunctional cross-linker dithio-*bis*(succinimidylpropionate) (DSP) contains a disulfide bond in the linker between the functional groups; therefore, reduction of cross-linked samples followed by electrophoresis using SDS−PAGE allows resolution of the individual components of a complex. Labeled yeast spheroplasts were gently lysed in a hypotonic buffer under conditions where the vacuole is lysed but the ER and Golgi remain intact (Eakle *et al.*, 1988) and the crude lysate was treated with DSP. The proteins in the lysate were TCA-precipitated and subjected to quantitative immunoprecipitation under denaturing but non-reducing conditions using antisera specific for either Vps15p or Vps34p. The immunoprecipitated cross-linked proteins were incubated in sample buffer containing 2-mercaptoethanol to reduce the disulfide bond in the cross-linker, then re-immunoprecipitated with the appropriate antisera and electrophoresed using SDS−PAGE.

Labeled extracts of the wild-type yeast strain TVY1 were treated with DSP cross-linker. Immunoprecipitation of cross-linked proteins with anti-Vps34p antisera, followed by reduction of the cross-linker and re-immunoprecipitation using both anti-Vps15p and anti-Vps34p antisera (Figure 4B, lane 2) or anti-Vps15p antisera alone (Figure 4B, lane 3), demonstrated that Vps34p and Vps15p can be co-immunoprecipitated using anti-Vps34p antisera. The reciprocal experiment using anti-Vps15p antisera followed by anti-Vps34p and anti-Vps15p antisera (Figure 4B, lane 6) or anti-Vps34p antisera alone (Figure 4B, lane 7) also demonstrated a physical association between the proteins. The co-precipitation of Vps15p and Vps34p under non-reducing conditions is absolutely dependent on the presence of cross-linker as neither antisera showed cross-reactivity in the absence of cross-linker (Figure 4B, lanes 1 and 5; Figure 4C, lanes 1 and 3). In addition, a titration of the DSP cross-linker (from 10−1000 μg/ml) demonstrated that the amount of cross-linked Vps15p−Vps34p complex increases with increasing cross-linker concentration up to a DSP concentration of 200 μg/ml (data not shown). The association of Vps15p and Vps34p was also examined in a strain containing *VPS15* and *VPS34* on multicopy plasmids. Immunoprecipitation of cross-linked extracts done in a manner identical to the wild-type strain described above also showed that Vps15p and Vps34p can be co-immunoprecipitated using antisera specific for either protein (Figure 4C). These data indicate that the ~20-fold overproduction of both proteins does not interfere with their ability to associate.

In the cross-linking experiments described above, the relative amounts of associated Vps15p and Vps34p appear to be similar (compare lanes 3 and 7 in Figure 4B). To obtain a crude estimate of the stoichiometry of Vps15p and Vps34p in the complex, the labeled TVY1 cross-linked samples were first immunoprecipitated with antisera specific for Vps34p. This should precipitate all cellular Vps34p and only the portion of Vps15p cross-linked to Vps34p. This sample was then re-immunoprecipitated with anti-Vps15p antisera without reducing the cross-linker. This step should only precipitate Vps34p that is associated with Vps15p. The sample was then reduced and electrophoresed using SDS−PAGE. The remaining immunoprecipitated Vps15p and Vps34p represents only the portion of the two proteins present within a cross-linkable complex (Figure 4B, lane 4). When the labeling efficiency of the proteins is taken into account by adjusting for the number of methionines and cysteines in each protein, it appears that Vps34p and Vps15p are present in roughly equimolar amounts in the protein complex. The same result is obtained if the cross-linked extract is first treated with anti-Vps15p antisera followed by anti-Vps34p antisera without reducing the cross-linker between antibody incubations (Figure 4B, lane 8). The estimate of an equimolar stoichiometry for Vps15p and Vps34p in the complex is also consistent with the subcellular and sucrose density gradient fractionation data described above.

Cross-linking and native immunoprecipitation experiments have the potential to reveal other components of the Vps15p−Vps34p complex. Longer exposures of gels containing the cross-linked proteins did show additional cross-linker-dependent immunoprecipitated labeled species (especially in the low molecular mass range), albeit at a lower intensity than Vps15p or Vps34p (data not shown). Native immunoprecipitation of Tran^{35}S-labeled yeast cell extracts also detected labeled proteins other than Vps15p and Vps34p (data not shown). While no other labeled proteins of intensity similar to Vps15p or Vps34p were detected in these experiments, this does not rule out the possibility that the complex contains members other than Vps15p and Vps34p. The association of other components with Vps15p and Vps34p may not be stable under the lysis conditions used, or the other member proteins may not contain cross-linkable

residues in close enough proximity to Vps15p or Vps34p to be cross-linked and immunoprecipitated with anti-Vps15p or anti-Vps34p antisera. Collectively, the native immunoprecipitation and cross-linking experiments demonstrate that Vps15p and Vps34p stably associate to form a membrane-associated protein complex.

Alterations in the kinase domain or myristoylation site of Vps15p do not affect the association of Vps15p with Vps34p

The addition of myristic acid to the N-terminus of several cellular proteins results in their association with a specific intracellular membrane (reviewed in Schultz *et al.*, 1988; Towler *et al.*, 1988). Myristoylation has also been suggested to be involved in protein–protein interaction on the basis of the hydrophobic nature of myristate (Schultz *et al.*, 1988). As Vps15p has been demonstrated to be myristoylated *in vivo* (Herman *et al.*, 1991a), we tested whether a non-myristoylated mutant form of Vps15p (G2A), in which the essential N-terminal glycine was changed to alanine by site-directed mutagenesis of the *VPS15* gene, is capable of association with Vps34p. In addition, because it was recently reported that the association of a substrate for protein kinase C with the plasma membrane is regulated by phosphorylation (Thelen *et al.*, 1991), we used the *vps15-E200R* kinase domain mutant to test the possibility that Vps15p autophosphorylation or Vps15p-mediated phosphorylation of Vps34p may be involved in Vps15p–Vps34p complex formation.

Yeast strains harboring the appropriate multicopy *VPS15* and *VPS34* plasmids were converted to spheroplasts, labeled with Tran^{35}S-label, osmotically lysed and treated with DSP cross-linker, as described above. The cross-linked proteins were immunoprecipitated with antisera specific for Vps34p, reduced and re-immunoprecipitated with antisera to both Vps15p and Vps34p. We found that neither the non-myristoylated G2A nor the E200R kinase domain mutant was defective in forming a complex with Vps34p (Figure 5). These mutants also showed fractionation characteristics for Vps15p and Vps34p that were nearly identical to the wild-type strain (data not shown). These data indicate that these mutant Vps15 proteins are localized normally and are capable of associating with Vps34p.

Vps15 protein kinase activity is required for activation of Vps34 phosphatidylinositol 3-kinase

We have demonstrated by genetic and biochemical techniques that the Vps15 protein kinase and the Vps34 PI 3-kinase both functionally and physically interact to facilitate the delivery of soluble proteins to the yeast vacuole. We have also previously shown that both Vps15 protein kinase and Vps34 lipid kinase activities are required for vacuolar protein sorting (Herman *et al.*, 1991a,b; Schu *et al.*, 1993). To test whether Vps15p is involved in the regulation of Vps34 lipid kinase activity, we examined Vps34p-associated PI 3-kinase activity in *vps15* mutant strains. Yeast extracts were centrifuged at 100 000 *g* to generate S100 and P100 fractions. These fractionated extracts were incubated with [γ-^{32}P]ATP and PI. The lipids were then extracted with methanol–chloroform and were separated by thin layer chromatography on silica gel plates (Walsh *et al.*, 1991). As previously shown, wild-type yeast cells contain PI 3-kinase activity that is predominately present in the P100 crude membrane pellet (Figure 6; Auger *et al.*, 1989; Schu *et al.*, 1993). A yeast strain deleted for *VPS34*, however, lacks detectable PI 3-kinase activity (Figure 6; Schu *et al.*, 1993). Strains deleted for *VPS15* are extremely defective in PI 3-kinase activity (Figure 6). It is interesting to note that while the Δ*vps34* strain contains essentially no detectable PI 3-kinase activity, the Δ*vps15* strain exhibits a very low but detectable level of PI 3-kinase activity. A possible interpretation of these results is that the PI 3-kinase activity detected in the Δ*vps15* strain represents the basal level of Vps34 lipid kinase activity in the absence of Vps15p-mediated activation. The low levels of Vps34p PI 3-kinase activity in the Δ*vps15* strain also could be the result of the mislocalization of Vps34p as the lack of Vps15p causes the great majority of membrane-associated Vps34p to shift to the cytoplasm (Figure 2). To test directly the possible involvement of Vps15 protein kinase activity in Vps34 PI 3-kinase activation, we analyzed Vps34 lipid kinase activity in the *vps15* kinase domain point mutant strain *vps15-E200R*. Like the Δ*vps15* strain, the *vps15-E200R* strain also is extremely defective for Vps34p-associated PI 3-kinase activity (Figure 6). The decrease in Vps34 lipid kinase activity is not likely to be the result of mislocalization of Vps34p as the Vps15 kinase domain mutant is able to associate with Vps34p in a manner nearly identical to the wild-type protein (Figure 5) and both Vps15p and Vps34p show wild-type subcellular fractionation characteristics in the *vps15-E200R* strain (data not shown). These data indicate that Vps15 protein kinase activity is required for the activation of Vps34 PI 3-kinase activity.

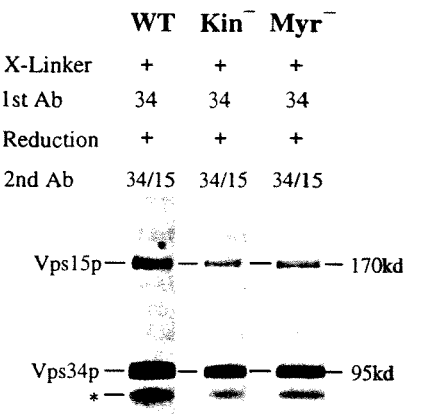

Fig. 5. Cross-linking of Vps34p to Vps15 mutant proteins. Labeled spheroplasts were osmotically lysed, treated with DSP and incubated with antisera to Vps34p. The immunoprecipitated proteins were reduced and re-immunoprecipitated with anti-Vps15p and anti-Vps34p antisera and electrophoresed on an SDS–polyacrylamide gel as described in the legend to Figure 4B. The strains used were: WT, SEY6210 harboring pPHY15E (*VPS15*, 2μ) and pJSY324.34 (*VPS34*, 2μ); Kin⁻, PHY112 containing pPHY15E-K2 (*vps15-E200R*, 2μ) and pJSY324.34; Myr⁻, PHY112 containing pPHY15E-G2A (*vps15-G2A*, 2μ) and pJSY324.34. The asterisk indicates a labeled species that non-specifically cross-reacts with anti-Vps34 antisera.

Discussion

The serine/threonine protein kinase encoded by the *VPS15* gene and the PI 3-kinase encoded by the *VPS34* gene have previously been shown to be required for the efficient sorting and

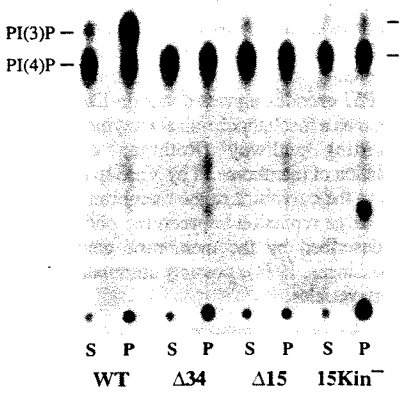

Fig. 6. PI 3-kinase activity in *vps34* and *vps15* mutant strains. Yeast cell lysates were fractionated by centrifugation at 100 000 *g* for 30 min at 4°C. The S100 and P100 fractions were assayed for PI 3-kinase activity in the presence of PI and [γ-^{32}P]ATP as described in Materials and methods. The lipids were extracted with chloroform–methanol and samples were separated on Silica gel 60 plates in a borate buffer system (Walsh *et al.*, 1991). The strains used were: SEY6210 (WT), PHY102 (Δ34), PHY112 (Δ15) and PHY112 containing pPHY15C-K2 (*vps15-E200R*, *CEN*) (15Kin⁻). The positions of the products of PI 3-kinase [PI(3)P] and PI 4-kinase [PI(4)P] are indicated.

delivery of proteins to the yeast vacuole (Robinson *et al.*, 1988; Herman and Emr, 1990; Herman *et al.*, 1991b). Mutational analyses of Vps15p and Vps34p indicate that the observed homologies are functional; alterations in residues in Vps15p and Vps34p that are conserved among protein kinases and lipid kinases, respectively, result in their inactivation (Herman *et al.*, 1991a,b; Schu *et al.*, 1993). Analysis of an allele of *vps15* that results in a temperature-conditional vacuolar protein sorting defect has indicated that the primary role of the Vps15 protein kinase is to regulate the sorting of soluble vacuolar proteins (Herman *et al.*, 1991a). We present evidence here that the Vps15 protein kinase and Vps34 lipid kinase act together as components of a membrane-associated protein complex required for regulation of the intracellular sorting of soluble vacuolar proteins.

Genetic analysis indicates that Vps15p and Vps34p functionally interact *in vivo*. The common set of terminal phenotypes exhibited by the Δ*vps15* and Δ*vps34* strains suggests that they may act together at the same step in the pathway. The fact that strains deleted for both genes show no additive or synergistic defects is also consistent with this interpretation (P.K.Herman and S.D.Emr, unpublished observations). More definitive evidence for genetic interaction between the *VPS15* and *VPS34* gene products has been provided by the observation that the overexpression of Vps34p suppresses the growth and vacuolar protein sorting defects associated with *vps15* kinase domain mutants (Figure 1). Suppression of a mutant phenotype by overproduction of another gene product argues for a functional interaction between the two proteins (reviewed in Huffaker *et al.*, 1987; Rine, 1991). Multicopy plasmid suppressors of a temperature-sensitive *sec* phenotype have permitted the identification of numerous proteins involved in the yeast secretory pathway (Salminen and Novick, 1987; Nakano and Muramatsu, 1989; Deshaies and Schekman, 1990; Newman *et al.*, 1990; Dascher *et al.*, 1991).

Several mechanisms can be proposed to explain the observed Vps34p-mediated suppression of *vps15* kinase domain mutants: (i) Vps34p may bypass the cell's requirement for Vps15p (either via a parallel pathway or by functional substitution for Vps15p); (ii) Vps15p and Vps34p may functionally interact *in vivo* (e.g. Vps15p could directly interact with Vps34p and/or regulate Vps34p activity). One prediction of a bypass mechanism is that overproduction of Vps34p will suppress Δ*vps15* as well as *vps15* kinase domain alleles. The fact that overproducing Vps34p will not suppress a Δ*vps15* strain demonstrates that overexpression of Vps34p cannot bypass the requirement for Vps15p in vacuolar protein sorting, and further suggests that the Vps34 lipid kinase cannot functionally substitute for the Vps15 protein kinase. Therefore, the genetic data indicate that Vps15p and Vps34p functionally interact in the cell. Since Vps15p is a protein kinase, one can propose that Vps34p may be acting as an upstream activator or downstream substrate or effector of the Vps15 kinase (see below). As activation of the Vps34 PI 3-kinase requires Vps15 protein kinase activity, it is possible that Vps34p suppresses a *vps15* kinase domain point mutation but not a Δ*vps15* strain because the *vps15* point mutation retains a low level of residual kinase activity sufficient to activate at least a portion of the Vps34 lipid kinase molecules. Consistent with this idea, the combination of three kinase domain point mutations into a single Vps15p molecule, effectively creating a *vps15* kinase null mutant, abolishes the suppression seen by overproducing Vps34p (J.H.Stack and S.D.Emr, unpublished observations). Because Vps15p and Vps34p have been demonstrated to form a hetero-oligomeric complex (Figure 4), Vps15p also may be functioning as a targeting subunit to direct Vps34p to the site of its membrane action. Multicopy *VPS34* suppression of *vps15* kinase domain mutants may then be the result of increasing the concentration of productive Vps15p–Vps34p complexes at the membrane face during the sorting process.

A role for phosphoinositides in regulating intracellular protein traffic

Data from mammalian cells indicate that PI 3-kinase acts at the plasma membrane to generate signals for cell proliferation (reviewed in Cantley *et al.*, 1991). The observed homology between Vps34p and the catalytic subunit of mammalian PI 3-kinase (Hiles *et al.*, 1992), together with the lipid kinase activity of Vps34p (Schu *et al.*, 1993), indicate that phosphorylation of membrane PI is critical for regulating protein traffic within the yeast secretory pathway. PI 3-kinase also has been suggested to be involved in directing internalized receptors to the mammalian lysosome (Sherr, 1991). Colony stimulating factor (CSF) receptor mutants that lack tyrosine kinase activity or the binding site for PI 3-kinase are rapidly internalized after ligand binding but fail to be degraded (Downing *et al.*, 1989; Carlberg *et al.*, 1991). In addition, it has been shown that stimulation of rat adipocytes with insulin caused a redistribution of PI 3-kinase from the cytoplasm to a low density, Golgi-enriched microsome fraction distinct from the plasma membrane, suggesting that PI 3-kinase may play a role in regulating intracellular protein trafficking events (Kelly *et al.*, 1992). These data must be interpreted carefully due

to the different systems examined and the multiple PI 3-kinase substrates known (which may have different biological effects; see Majerus, 1992 for a review), but evidence exists that suggests that PI 3-kinase may be involved in regulating protein sorting decisions.

There is precedent for the involvement of phospholipid metabolism in regulating protein traffic through the secretory pathway. Sec14p is required for Golgi function in yeast and has been shown to be a PI/phosphatidylcholine (PC) transfer protein (Bankaitis *et al.*, 1990). Genetic studies implicating phospholipid biosynthetic pathways in Sec14p function indicate that the ratio of different phospholipids in specific compartments may be important for secretory protein traffic in yeast (Cleves *et al.*, 1991). PI 4-kinase has also been suggested to be involved in the regulation of intracellular protein traffic due to its co-localization with membrane vesicles (Del Vecchio and Pilch, 1991).

Genetic and biochemical studies in mammalian and yeast cells indicate that proteins destined for the vacuole and lysosome are sorted in a late Golgi compartment (Kornfeld and Mellman, 1989; Graham and Emr, 1991). We are faced with the question of how a specific phosphorylation event on a membrane phospholipid is involved in regulating the flow of proteins to the yeast vacuole. One possibility is that Vps34p-mediated phosphorylation of membrane PI and subsequent incorporation of this modified phospholipid into transport vesicles designates these vesicles for delivery to the vacuole. Vesicle traffic has been proposed to occur between membrane-bound organelles throughout the secretory pathway and the fidelity of the pathway is dependent on the docking and fusion of such vesicles with the correct target organelle (reviewed in Pryer *et al.*, 1992; Rothman and Orci, 1992). Mechanisms involving specific membrane receptors that recognize PI 3 phosphate-tagged vesicles and mediate the docking/fusion of the vesicles with the appropriate membrane may facilitate vacuolar protein sorting in yeast.

Another model proposes that phospholipids could play a dynamic role in regulating the activity or membrane association of proteins involved in the vacuolar protein sorting pathway. It has been shown that the association of SecA with the *Escherichia coli* inner membrane and activation of its ATPase activity require acidic phospholipids (Lill *et al.*, 1990; Hendrick and Wickner, 1991). Acidic phospholipids also have been proposed to be involved in other processes such as the import of precursor proteins into mitochondria (Eilers *et al.*, 1989), DNA replication in *E.coli* (Yung and Kornberg, 1988), regulation of phospholipase C activity (Bell and Burns, 1991) and association of annexins with the plasma membrane (Lin *et al.*, 1992). These examples suggest that the ionic nature of the phospholipid head groups may be important in regulating the biochemical properties of lipid-associated proteins. This model for the role of PI 3-kinase activity in regulating vacuolar protein sorting predicts that the increased ionic character of PI upon phosphorylation of the inositol ring affects the association or activity of proteins involved in the sorting process. Vps34p-mediated phosphorylation of membrane PI may catalyze the recruitment of accessory proteins involved in the budding or transport of vesicles from the sorting compartment. Candidates for such vesicle accessory proteins include clathrin and clathrin-associated adaptors. In addition to its role in endocytosis, clathrin has been shown to be required for the sorting of both mammalian lysosomal and yeast vacuolar proteins (Kornfeld and Mellman, 1989; Seeger and Payne, 1992). Other candidates include cytoskeletal elements and molecular motors, which may be involved in vesicular trafficking between the Golgi and the vacuole. In this regard, it is of note that *VPS1* encodes a yeast dynamin-like molecule that may function as a mechanochemical enzyme in the vacuolar protein sorting pathway (Rothman *et al.*, 1990). Phosphorylation of membrane PI by Vps34p also could result in changes in the curvature of the membrane bilayer due to increased charge repulsion between the phospholipid polar heads as described by the membrane couple hypothesis (Sheetz and Singer, 1974), possibly stimulating the budding of transport vesicles.

A model for the interaction between the Vps15 protein kinase and the Vps34 lipid kinase

The genetic and biochemical data presented here demonstrate that the Vps15 protein kinase and the Vps34 lipid kinase act together within a protein complex to facilitate the sorting of yeast vacuolar proteins. We propose that Vps15p and Vps34p function as key components of a multiprotein complex required for the recognition and sorting of vacuolar proteins (Figure 7). In addition to Vps15p and Vps34p, other possible components include transmembrane receptors for vacuolar proteins and vesicle coat proteins. The Vps15p–Vps34p complex may interact with the cytoplasmic tails of receptors for vacuolar proteins (Figure 7). In a manner analogous to cell surface receptor proteins, ligand binding to vacuolar protein receptors may transduce a signal through a conformational change that promotes receptor association with and/or activation of the Vps15 protein kinase. A Vps15p-mediated protein phosphorylation event then results in the activation of the PI 3-kinase activity associated with Vps34p (Figure 6). Phosphorylation of membrane PI by Vps34p then could trigger a cascade of events that ultimately results in vesicle formation and delivery of ligand–receptor complexes to the vacuole directly or via an endosome intermediate. In such a model, Vps15p and Vps34p effectively act as components of a signal transduction complex that

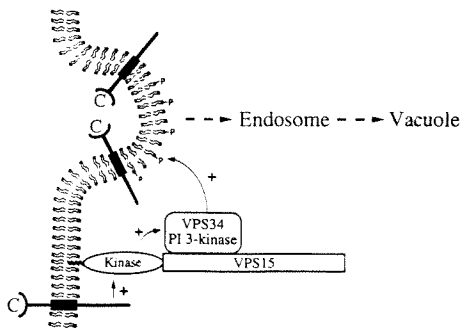

Fig. 7. Model for a yeast protein sorting complex. A transmembrane receptor for carboxypeptidase Y is shown in the sorting compartment, which may correspond to the late Golgi complex. Ligand (C) binding to the receptor activates the associated Vps15p–Vps34p complex. Activation of the Vps15p protein kinase leads to a protein phosphorylation event that results in activation of the Vps34p PI 3-kinase. Vps34p-mediated phosphorylation of membrane PI triggers a cascade of events that results in the vesicular delivery of soluble hydrolases to the vacuole, most likely involving an endosome intermediate.

transduces the signal received by specific membrane receptors into a second messenger molecule [PI (3)P] that could trigger the action of as yet unknown effector proteins (Herman *et al.*, 1992).

Consistent with such a model, our data indicate that Vps34p acts downstream of Vps15p. Vps34 PI 3-kinase activity is significantly reduced in a *vps15* kinase domain mutant, suggesting that Vps15 protein kinase activity is required for activation of the Vps34 lipid kinase. The fact that the great majority of Vps34-mediated PI 3-kinase activity is found in a pelletable fraction, presumably due to interaction of Vps34p with Vps15p, also supports a direct role for Vps15p in regulating Vps34p activity (Figure 6; Schu *et al.*, 1993). Vps15p may act directly through phosphorylation of Vps34p itself or indirectly through phosphorylation of a protein intermediate, to effect the activation of the Vps34 PI 3-kinase. We are currently attempting to purify the Vps15p–Vps34p complex to rigorously test for Vps15p-dependent phosphorylation of Vps34p as well as to identify other members of the complex.

Vps15p kinase activity also may be required for other aspects of vacuolar protein delivery such as regulating the packaging of vacuolar proteins into their specific vesicular carriers or mediating the delivery and/or fusion of transport vesicles with the endosome or the vacuole (Herman *et al.*, 1991a,b, 1992). In mammalian cells, protein phosphorylation has been implicated in the regulation of the sorting of specific transmembrane receptors, including the polymeric immunoglobulin receptor, within an early endosome (Casanova *et al.*, 1990). In addition, phosphorylation of the cytoplasmic tail of mammalian mannose-6-phosphate receptors has been suggested to stabilize their association with clathrin-associated adaptor proteins (Meresse *et al.*, 1990). Many aspects of the models presented here are readily testable and it is expected that future experiments will provide many new insights into the roles of this novel protein complex and of phosphoinositides in the general control of protein sorting and membrane trafficking events in eukaryotic cells.

Materials and methods

Strains, media and yeast genetic methods

S.cerevisiae strains used were SEY6210 (*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2–Δ9*; Robinson *et al.*, 1988), PHY102 (SEY6210 *vps34Δ1::TRP1*; Herman and Emr, 1990), PHY112 (SEY6210 *vps15Δ1::HIS3*; Herman *et al.*, 1991b) and TVY1 (SEY6210 *pep4Δ1::LEU2*; T.Vida, unpublished). Standard yeast (Sherman *et al.*, 1979) and *E.coli* (Miller, 1972) media were used and supplemented as needed. Standard yeast genetic methods were used throughout (Sherman *et al.*, 1979). Yeast cells were transformed using the alkali cation treatment method (Ito *et al.*, 1983) and transformants were selected on the appropriate SD media.

Plasmid constructions

Plasmids pPHY15E (*VPS15*, 2μ, *URA3*), pPHY15C-K2 (*vps15-E200R*, *CEN*, *URA3*), pPHY15E-K2 (*vps15-E200R*, 2μ, *URA3*), pPHY15E-G2A (*vps15-G2A*, 2μ, *TRP1*), pJSY324 (2μ, *TRP1*), pJSY324.15 (*VPS15*, 2μ, *TRP1*) and pPHYC18 (*CEN*, *URA3*) have been described previously by Herman *et al.* (1991a,b). Plasmid pJSY324.34 (*VPS34*, 2μ, *TRP1*) was constructed by subcloning the 3.9 kb *ClaI–Asp*718 fragment of pPHY34 (Herman and Emr, 1990), which contains *VPS34* in pJSY324.

Cell labeling and immunoprecipitation

For analysis of CPY processing, whole yeast cells were labeled essentially as described by Herman and Emr (1990). Cells were pulse-labeled with Trans^{35}S-label (ICN Radiochemicals) for 15 min at 30°C and chased for 30 min at 30°C by the addition of methionine and cysteine to 2 mM. The media contained bovine serum albumin (1 mg/ml) and α_2-macroglobulin (10 μg/ml; Boehringer Mannheim) to stabilize secreted proteins. Following the chase, an equal volume of cold 2 × stop buffer (2 M sorbitol, 50 mM Tris–HCl, pH 7.5, 40 mM NaF, 40 mM NaN_3 and 20 mM DTT) was added and the cultures were incubated on ice for 5 min. Zymolyase-100T (Seikagako Kogyo Co, Tokyo, Japan) was added to 20 μg/ml and the cells were incubated at 30°C for 25 min. The culture was separated into intracellular and extracellular fractions by centrifugation at 4000 *g* for 30 s and proteins were precipitated by the addition of TCA to a final concentration of 5%. Immunoprecipitation of CPY was as described previously by Herman and Emr (1990) and samples were electrophoresed on 9% SDS–polyacrylamide gels. Following electrophoresis, the gels were fixed in 50% methanol and 10% acetic acid, and treated with 1.0 M sodium salicylate and 10% glycerol, then dried and subjected to autoradiography.

Subcellular fractionation of Vps15p and Vps34p

Fractionation of Vps15p and Vps34p was performed essentially as described previously by Herman and Emr (1990). Yeast strains were grown to mid-logarithmic phase, converted to spheroplasts, labeled with Tran^{35}S-label for 30 min at 30°C and chased for 1 h at 30°C by adding methionine and cysteine to 2 mM and yeast extract (Difco) to 0.2%. The labeled spheroplasts were osmotically lysed in the presence of antipain, leupeptin, chymostatin, pepstatin (all at 2 μg/ml), aprotinin (0.1 TIU/ml), phenylmethylsulfonyl fluoride (100 μg/ml) and α_2-macroglobulin (10 μg/ml). Unlysed cells were removed by centrifugation at 750 *g* for 5 min at 4°C. The lysate was then centrifuged at 100 000 *g* for 30 min at 4°C in a Beckman TLA 100.3 rotor. The supernatant (S100) was made 5% with respect to TCA and the pellet (P100) was resuspended in 5% TCA. Immunoprecipitation of Vps15p and Vps34p was as previously described by Herman *et al.* (1991b); the TCA pellets were resuspended by heating at 70°C for 5 min in urea-cracking buffer (50 mM Tris–HCl, pH 7.2, 6 M urea and 1% SDS). This results in a substantial increase in the solubilization of Vps34p as compared with standard boiling buffer (Herman and Emr, 1990). The immunoprecipitated proteins were electrophoresed on 8% SDS–polyacrylamide gels and processed as described above.

Sucrose gradient fractionation

The yeast strain TVY1 was spheroplasted, labeled, chased and osmotically lysed as described above for subcellular fractionation. The lysate was centrifuged at 100 000 *g* for 30 min at 4°C. The resulting pellet was resuspended in 2.5 ml of 60% (w/w) sucrose using a Dounce homogenizer, loaded into the bottom of a Beckman Ultra-Clear centrifuge tube and overlayed with 2.5 ml of 35% (w/w) sucrose as described previously by Paravicini *et al.* (1992). All solutions contained protease inhibitors. The gradient was centrifuged at 170 000 *g* for 18 h at 4°C in a Beckman SW 50.1 rotor. Eleven 0.45 ml fractions were collected and made 5% with respect to TCA. Any remaining pellet was resuspended in 0.45 ml 5% TCA. The TCA-precipitated proteins were subjected to immunoprecipitation using anti-Vps15p and anti-Vps34p antisera as described above. Immunoprecipitation of Kex2p was identical to that of Vps15p and Vps34p. Antisera to Kex2p was a gift from Dr William Wickner.

Native immunoprecipitation of Vps15p and Vps34p

0.5 OD_{600} units of the yeast strain TVY1 harboring the plasmids pPHY15E (2μ, *URA3*) and pJSY324.34 (2μ, *TRP1*) were converted to spheroplasts and lysed by the addition of 0.5 ml cold Tween-20 IP buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.5, 0.1 mM EDTA and 0.5% Tween-20) in the presence of protease inhibitors. The lysate was centrifuged at 13 000 *g* for 2 min at 4°C and precleared with 50 μl of protein A–Sepharose (4% protein A–Sepharose in 10 mM Tris–HCl, pH 7.5) for 20 min at 4°C. The precleared lysate was incubated with 5 μl of antisera specific for Vps15p or Vps34p for 4 h at 4°C. Immune complexes were collected on protein A–Sepharose and washed five times with Tween-IP buffer. Immunoprecipitated proteins were electrophoresed on an 8% SDS–polyacrylamide gel and transferred to nitrocellulose at 10 V for 1 h in Tris–glycine buffer using a Bio-Rad semi-dry transfer cell. The blot was blocked in non-fat dry milk solution (5% non-fat dry milk, 140 mM NaCl, 25 mM Tris–HCl, pH 7.5 and 1 mM NaN_3) and incubated with antisera specific for Vps15p and Vps34p at a 1:1000 dilution in dry milk solution. Vps15p and Vps34p were visualized using ^{35}S-labeled protein A (Amersham) and autoradiography.

Cross-linking of yeast cell extracts

Yeast cells were grown, spheroplasted, labeled and chased as described for subcellular fractionation. Labeled spheroplasts were resuspended in XL lysis buffer (1.2 M sorbitol, 0.1 M KH_2PO_4, pH 7.5 and 5 mM EDTA) at 10–20 OD_{600} units/ml. Cells were lysed by the addition of 4 vol of H_2O. All solutions contained protease inhibitors. DSP [dithio*bis*(succinimidylpropionate); Pierce Chemicals] cross-linker, dissolved in dimethylsulfoxide (DMSO), was added to a final concentration of 200 μg/ml. Control samples

without cross-linker received DMSO alone. The extracts were incubated at room temperature for 30 min, after which the reaction was quenched by the addition of 1 M hydroxylamine to a final concentration of 20 mM. Proteins were precipitated by the addition of TCA to 5%. The TCA pellets were resuspended in urea-cracking buffer without reducing agent and processed for immunoprecipitation using anti-Vps15p or anti-Vps34p antisera as described above. Following the first immunoprecipitation, the cross-linked samples were solubilized in urea-cracking buffer with or without 2% 2-mercaptoethanol and re-immunoprecipitated with the appropriate antisera. Control experiments demonstrated that anti-Vps15p and anti-Vps34p antisera were irreversibly denatured by incubation with urea-cracking buffer in the presence or absence of reducing agent (not shown). The final samples were solubilized in urea-cracking buffer containing 2% 2-mercaptoethanol and electrophoresed on 8% polyacrylamide gels.

PI 3-kinase assays

Yeast spheroplasts were resuspended in 0.1 M KCl, 15 mM HEPES, pH 7.5, 3 mM EGTA and 10% glycerol at 15–20 OD_{600}/ml and vortexed in the presence of 0.25 mm glass beads and protease inhibitors. The lysates were centrifuged at 750 *g* for 5 min and the resulting supernatant was spun at 100 000 *g* for 30 min at 4°C to generate S100 and P100 fractions. The pellet was resuspended in a volume of lysis buffer equal to the supernatant and S100 and P100 fractions were frozen in a dry ice–ethanol bath and stored at −80°C until use. Approximately 0.05 OD_{600} equivalents (<4 μg protein) were assayed for PI 3-kinase activity as described by Whitman *et al.* (1988) and Schu *et al.* (1993). The 50 μl reactions were performed in 20 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 0.2 mg/ml sonicated PI, 60 μM ATP and 0.2 mCi/ml[γ-^{32}P]ATP. The reactions were incubated at 25°C for 5 min and were terminated by the addition of 80 μl 1 M HCl. The lipids were extracted with 160 μl chloroform–methanol (1:1) and the organic phase was dried down and stored at −80°C. Labeled samples dissolved in chloroform were spotted onto Silica gel 60 TLC plates (Merck) and developed in a borate buffer system (Walsh *et al.*, 1991). Labeled species were detected by autoradiography.

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Chapter 6:

Vps34p Required for Yeast Vacuolar Protein Sorting is a Multiple Specificity Kinase That Exhibits Both Protein Kinase and Phosphatidylinositol-Specific PI 3-Kinase Activities

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Vps34p Required for Yeast Vacuolar Protein Sorting is a Multiple Specificity Kinase That Exhibits Both Protein Kinase and Phosphatidylinositol-Specific PI 3-Kinase Activities

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Running title: Vps34p is a phosphatidylinositol-specific PI 3-kinase

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Summary

The Vps15p protein kinase and the Vps34p PI 3-kinase have been shown to function as a membrane-associated complex which facilitates the delivery of proteins to the vacuole in yeast. Biochemical characterization of the autophosphorylation reaction catalyzed by Vps15p demonstrates that it is a functional serine/threonine protein kinase. In addition, we show that the Vps34 PI 3-kinase undergoes an autophosphorylation event both *in vivo* and *in vitro* indicating that it represents a novel multiple specificity kinase capable of phosphorylating both protein and lipid substrates. Vps34p is phosphorylated predominately on serine *in vivo* and is able to phosphorylate serine, threonine and tyrosine residues *in vitro*. Mutant Vps34 proteins containing alterations in conserved amino acids in the lipid kinase domain are severely defective for both PI 3-kinase activity and autophosphorylation. Characterization of the PI 3-kinase activity of Vps34p demonstrates that it, unlike the mammalian p110 PI 3-kinase, is highly resistant to the PI 3-kinase inhibitors wortmannin and LY294002. We also find that Vps34p is a phosphatidylinositol-specific 3-kinase, as it is able to utilize phosphatidylinositol (PtdIns) but not PtdIns(4)P or PtdIns(4,5)P_2 as substrates in an *in vitro* PI kinase reaction. The substrate specificity, wortmannin resistance, and other biochemical characteristics of its PtdIns 3-kinase activity suggest that Vps34p is quite similar to a PtdIns-specific 3-kinase activity recently characterized from mammalian cells. These data indicate the existence of a family of PI 3-kinases composed of p110-like PI 3-kinases and Vps34p-like PtdIns-specific 3-kinases. On the basis of the role for Vps34p in vacuolar protein sorting,

we propose that production by PI and PtdIns 3-kinases of a specific phosphoinositide, PtdIns(3)P, is involved in regulating intracellular protein sorting reactions in eukaryotic cells.

Introduction

Genetic selections in the yeast *Saccharomyces cerevisiae* have resulted in the isolation of a large number of mutants specifically defective in the delivery of proteins to the lysosome-like vacuole (Klionsky et al., 1990; Raymond et al., 1992). Analyses of these *vps* mutants has revealed components of the cellular machinery that are responsible for the active diversion of proteins from the secretory pathway to the vacuole. The products of the *VPS15* and *VPS34* genes have been demonstrated to function together as members of a membrane-associated protein complex (Stack et al., 1993). The *VPS15* gene encodes a 1455 amino acid protein whose N-terminal ~300 residues show significant similarity to the serine/threonine family of protein kinases (Herman et al., 1991a). Site-directed mutagenesis of the *VPS15* gene has suggested that Vps15p protein kinase activity is required for the sorting of vacuolar proteins (Herman et al., 1991a; Herman et al., 1991b). Analysis of a temperature-conditional allele of *VPS15* has further indicated that Vps15p plays a direct role in the sorting of vacuolar proteins (Herman et al., 1991b). Vps34p shares significant sequence similarity with the p110 catalytic subunit of mammalian phosphoinositide 3-kinase (PI 3-kinase[1]; Herman et al., 1990; Hiles et al., 1992). Point mutations altering residues in Vps34p that are highly conserved between the lipid kinase domains of p110 and Vps34p result in the missorting of vacuolar proteins, suggesting that Vps34p PI 3-kinase activity is important for vacuolar protein delivery. Vps34p has been shown to possess phosphatidylinositol 3-kinase activity *in vitro*, and yeast strains containing mutations in the *VPS34* gene are highly defective

for this activity (Schu et al., 1993). Collectively, these data indicate that *VPS34* encodes a yeast PI 3-kinase that is required for the sorting of vacuolar proteins.

Vps15p and Vps34p have been demonstrated to functionally and physically interact *in vivo*. Overproduction of Vps34p will suppress the growth and protein sorting defects associated with *vps15* kinase domain mutant strains (Stack et al., 1993). Furthermore, Vps15p and Vps34p form a protein complex which is associated with an intracellular membrane fraction possibly corresponding to a late Golgi compartment. Chemical cross-linking and native immunoprecipitation experiments have shown a direct interaction between Vps15p and Vps34p. Vps15p protein kinase activity also appears to be required for activation of Vps34p as strains containing *vps15* kinase domain mutants are very defective for PtdIns 3-kinase activity (Stack et al., 1993). These data indicate that Vps15p and Vps34p act together within a membrane-associated hetero-oligomeric protein complex to facilitate delivery of proteins to the vacuole.

PI 3-kinase activity was initially characterized in mammalian cells due to its association with activated receptor and non-receptor tyrosine kinases (Cantley et al., 1991). This association with activated growth factor receptors and the correlation of the production of 3'-phosphorylated phosphoinositides with growth factor stimulation led to the suggestion that PI 3-kinase may be involved in signaling cell proliferation. PI 3-kinase activity also has been implicated in the regulation of growth factor-induced membrane ruffling events (Kotani et al., 1994; Wennstrom et al., 1994), histamine secretion (Yano et al., 1993), activation of neutrophils (Arcaro and Wymann, 1993; Okada et al., 1994; Thelen et al., 1994) and

translocation of glucose transporters to the cell surface (Cheatham et al., 1994; Hara et al., 1994; Okada et al., 1994). Determination of a role for PI 3-kinase in mammalian cells is complicated by the fact that this activity appears to be generated by a family of PI 3-kinase enzymes. At least three biochemically distinct PI 3-kinases have been described from mammalian cells: the p110/p85 heterodimer associates with tyrosine-phosphorylated proteins through SH2 domains in the p85 targeting subunit (McGlade et al., 1992; Soltoff et al., 1992), a PI 3-kinase activated by G protein βγ subunits has been isolated from platelets and myeloid-derived cells (Stephens et al., 1994b; Thomason et al., 1994) and a PtdIns-specific 3-kinase has been characterized from bovine adrenoreticulosa cells (Stephens et al., 1994a). In a manner perhaps analogous to the participation of Vps34p in vacuolar protein sorting in yeast, evidence exists of a role for PI 3-kinase in regulating protein sorting reactions in mammalian cells. Work with mutant colony stimulating factor (CSF) and platelet-derived growth factor (PDGF) receptors has suggested that PI 3-kinase may be involved in the delivery of cell surface receptors to the lysosome for degradation (Downing et al., 1989; Carlberg et al., 1991; Joly et al., 1994). Together, these studies have raised the possibility that PI 3-kinases represent a family of multifunctional enzymes that are able to perform diverse and essential cellular roles.

In work presented here, we investigate the biochemical characteristics of both the Vps15p protein kinase and the Vps34p lipid kinase. We find that Vps34p is able to catalyze the transfer of phosphate from ATP to both lipid (phosphatidylinositol) and protein (itself) substrates. Vps34p is shown to have a strict substrate specificity for PtdIns

and is extremely insensitive to inhibitors of mammalian PI 3-kinase such as wortmannin. These biochemical characteristics indicate that Vps34p has functional properties distinct from the mammalian p110/p85 enzyme and suggest instead that the Vps34p PtdIns 3-kinase may be closely related to a PtdIns-specific 3-kinase activity characterized from mammalian cells (Stephens et al., 1994a). These data lead us to propose that formation of PtdIns(3)P through the action of PI and PtdIns 3-kinases may function to regulate intracellular protein trafficking decisions in eukaryotic cells.

Experimental Procedures

Strains, Plasmids, Media and Yeast Genetic Methods

S. cerevisiae strains used were SEY6210 (*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*; Robinson et al., 1988), PHY102 (SEY6210 *vps34Δ1::TRP1*; Herman and Emr, 1990) and PHY112 (SEY6210 *vps15Δ1::HIS3*; Herman et al., 1991a). Plasmids pPHY15E (*VPS15*, 2μ, *URA3*), pJSY324.15 (*VPS15*, 2μ, *TRP1*) and plasmids containing *VPS15* point mutations were described previously (Herman et al., 1991a; Herman et al., 1991b). Plasmid pJSY324.34 (*VPS34*, 2μ, *TRP1*) and plasmids containing *VPS34* point mutations were described previously (Herman et al., 1991b; Schu et al., 1993) or generated by site-directed mutagenesis as described (Herman et al., 1991b). Standard yeast (Sherman et al., 1979) and *E. coli* (Miller, 1972) media were used and supplemented as needed. Standard yeast genetic methods were used throughout (Sherman et al., 1979). Yeast cells were transformed using the alkali cation treatment method (Ito et al., 1983) and transformants were selected on the appropriate SD media.

Cell Labeling, Immunoprecipitation and Phosphoamino Acid Analysis

Yeast cells were grown and labeled with ^{32}P-orthophosphate as described previously (Herman et al., 1991a). Briefly, cells were grown to early log phase and resuspended in LPSM media at a concentration of 5 OD_{600} units/ml. $^{32}PO_4$ (Amersham) was added to 1 mCi/ml and the cells were incubated for 30-60 min at 30°C. The labeling was terminated by the

addition of trichloroacetic acid to a final concentration of 5%. The TCA pellets were resuspended in urea-cracking buffer (50 mM Tris-HCl, pH 7.2, 6M urea, 1% SDS) and subjected to immunoprecipitation under denaturing conditions using antisera specific for Vps15p or Vps34p as described previously (Herman et al., 1991a). The immunoprecipitated labeled proteins were separated by SDS-PAGE on 8% gels that were then processed for autoradiography.

For phosphoamino acid analysis, immunoprecipitated ^{32}P-labeled Vps15p or Vps34p was electrophoresed on 8% SDS-polyacrylamide gels and transferred to PVDF membranes (Immobilon, Millipore) at 10 V for 1 hour in Tris-glycine buffer using a Bio-Rad semi-dry transfer cell. The labeled proteins were localized on the filter by autoradiography and cut out. The excised bands were hydrolyzed in 5.7 M HCl (constant boiling HCl, Sigma) at 110°C for 120 min. The hydrolysate was lyophilized several times from water and subjected to two-dimensional thin layer electrophoresis on a Hunter TLE system (CBS Scientific, Del Mar, CA) as described (Boyle et al., 1991).

Native Immunoprecipitation of Vps15p and Vps34p and Protein Kinase Assays

Yeast strains harboring the appropriate plasmids were converted to spheroplasts and lysed at 5 OD_{600} units/ml by the addition of cold TBS (140 mM NaCl, 25 mM Tris-HCl, pH 7.4) in the presence of protease inhibitors (2 µg/ml antipain, leupeptin, chymostatin and pepstatin, 0.1 TIU/ml aprotinin, 100 µg/ml phenylmethylsulfonyl fluoride, and 10 µg/ml α_2-macroglobulin). The lysate was centrifuged at 13,000 x g for 2 min at 4°C

and precleared with 50 µl of Protein A-Sepharose (4% Protein A-Sepharose in 10 mM Tris-HCl, pH 7.5) for 15 min at 4°C. The precleared lysate was incubated with 1 µl/OD of antisera specific for Vps15p or Vps34p for 2-4 hr at 4°C. Immune complexes were collected on Protein A-Sepharose and washed 6-8 times with TBS. Immunoprecipitated proteins were then subjected to *in vitro* kinase assays.

Immunoprecipitated proteins were washed twice with PK reaction buffer (20 mM HEPES, pH 7.4, 10 mM $MnCl_2$) and resuspended in 35 µl of reaction buffer containing 0.5 mCi/ml [γ–^{32}P]ATP. The reactions were incubated for 30 min at 30°C and terminated by heating at 65°C for 5 min in 60 µl of urea-cracking buffer with 1% 2-mercaptoethanol. The labeled proteins were subjected to an additional round of immunoprecipitation prior to electrophoresis and autoradiography.

Partial Purification of Vps34p and Vps15p-Vps34p Complexes

For purification of Vps34p, a yeast strain deleted for the *VPS15* gene and harboring the *VPS34* gene on a multicopy plasmid was used. The yeast were converted to spheroplasts and resuspended in 0.5 M NaCl, 20 mM Tris-HCl, pH 7.4, 1 mM EDTA in the presence of protease inhibitors. The cells were lysed by vortexing with acid-washed 0.2 mm glass beads. The lysate was cleared by centrifugation at 100,000 g for 45 min at 4°C. Vps34p was precipitated from the cleared lysate by the addition of solid ammonium sulfate. The 40-45% ammonium sulfate pellet was resuspended in 50 mM Tris, pH 7.5 and desalted on a Fast Desalting HR 10/10 column (Pharmacia) on an FPLC system. Vps34p separated on a

Mono Q HR 5/5 column (Pharmacia) using a linear salt gradient eluted at approximately 75 mM NaCl. The fractions were collected into BSA (0.5 mg/ml final conc.) and glycerol (10% final conc.) and frozen in a dry ice-ethanol bath. Fractions were assayed for Vps34p by both PI kinase assay and western blotting using antisera specific for Vps34p.

For purification of Vps15p-Vps34p complexes, a strain containing both the *VPS15* and *VPS34* genes on multicopy plasmids was used. Purification of Vps15p-Vps34p was essentially identical to that described above for Vps34p; cleared lysates were precipitated with ammonium sulfate and the 25-30% fraction was desalted and run on a Mono Q column. The Vps15p-Vps34p complex eluted at approximately 200 mM NaCl. The active fractions were assayed by both western blotting using antisera specific for Vps15p and Vps34p and PI kinase assay.

PI 3-Kinase Assays

Yeast cell lysates were generated as described previously (Stack et al., 1993) except that they were centrifuged at 2000 g for 10 min and the supernatant was used in the assays. Immunoprecipitated Vps34p was isolated as described above. Approximately 0.05 OD_{600} equivalents (<4 μg protein) of lysate or 0.2-0.5 OD_{600} equivalents of immunoprecipitated Vps34p were assayed for PI 3-kinase activity as described (Whitman et al., 1988; Schu et al., 1993). Addition of inhibitors or enzymes prior to the assay is described below. The 50 μl reactions were performed in 20 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 0.2 mg/ml sonicated PtdIns, 60 μM ATP and 0.2 mCi/ml [γ-^{32}P]ATP. The reactions were incubated at 25°C for 5 min and were terminated by the addition of 80 μl 1 M HCl. The lipids were extracted

with 160 µl chloroform/methanol (1:1) and the organic phase was dried down and stored at -80°C. The reaction products were dissolved in chloroform and spotted onto Silica gel 60 TLC plates (Merck). The plates were developed in a borate buffer system (Walsh et al., 1991) and labeled products were detected by autoradiography. Quantitation of the labeled species was performed either by use of a Phosphorimager (Molecular Dynamics) or by scraping the phospholipids from the TLC plates and quantitating by liquid scintillation counting. Both techniques gave essentially identical results.

Effect of PI 3-kinase inhibitors and various compounds. Wortmannin (Sigma) or LY294002 (a kind gift of Dr. Chris Vlahos, Lilly Research Labs) was diluted into water from 10 mM (wortmannin) or 10 mg/ml (LY294002) stocks in DMSO and added to the reaction mixture prior to the addition of ATP. The appropriate concentration of NP-40 (Sigma) was added to the reaction mixture containing phosphatidylinositol, $MgCl_2$ and buffer prior to sonication. The lysates or immunoprecipitates were incubated with inhibitor for 5 min at 25°C after which the reactions were initiated by the addition of ATP and performed as described above.

ATP treatment of Vps34p. Immunoprecipitated Vps34p was incubated in a 50 µl volume of 10 mM ATP, 20 mM HEPES pH 7.4, 10 mM $MnCl_2$ for the indicated times at 30°C. The immunoprecipitates were then washed twice with TBS and twice with 20 mM HEPES, pH7.4, 10 mM $MgCl_2$ prior to performing a standard PI kinase reaction.

Potato acid phosphatase (PAP) treatment of Vps34p. One unit of ammonium sulfate precipitated PAP (Boerhinger Mannheim) was

desalted on a Sephadex G-25 column and added to immunoprecipitated Vps34p in a 50 µl volume of 20 mM MES, pH 6.0, 1 mM $MgCl_2$, 0.5 mM DTT. The dephosphorylation reaction was incubated at 30°C for 20 min. The immunoprecipitates were washed twice with TBS and twice with 20 mM HEPES, pH 7.4, 10 mM $MgCl_2$ prior to performing a standard PI kinase reaction. To assess the degree of dephosphorylation of Vps34p by PAP, immunoprecipitated Vps34p labeled in an *in vitro* protein kinase assay was incubated with PAP under the conditions described above and analyzed by SDS-PAGE and autoradiography.

Substrate specificity of Vps34p. Partially purified preparations of Vps34p or Vps15p-Vps34p complexes were incubated with the appropriate substrate (PtdIns, PtdIns(4)P, or PtdIns(4,5)P_2; Sigma) in a standard PI kinase assay with the addition of 10 µg of phosphatidylserine (Sigma). The reaction products were separated on potassium oxalate-impregnated Silica gel 60 plates developed in a 1-propanol:2 M acetic acid (65:35) buffer (Auger et al., 1992). Standards for PtdIns, PIP and PIP_2 were visualized by staining with iodine vapor.

Results

Vps15p is a Serine/Threonine Protein Kinase

The homology between Vps15p and protein kinases suggested that protein phosphorylation reactions are required for the localization of vacuolar proteins in yeast (Herman et al., 1991a). Mutagenesis of *VPS15* that altered residues conserved among protein kinases resulted in its functional inactivation; point mutations corresponding to conserved residues in the Vps15 kinase domain led to the missorting and secretion of vacuolar proteins in a manner indistinguishable to strains deleted for the *VPS15* gene (Herman et al., 1991a; Herman et al., 1991b). Additional evidence for a functional protein kinase domain in Vps15p came from *in vivo* labeling of yeast cells with $^{32}PO_4$. These experiments demonstrated that Vps15p is phosphorylated *in vivo* in a reaction that required a wild-type Vps15p kinase domain; *vps15* kinase domain mutant alleles that were found to be non-functional for vacuolar protein sorting were also defective for *in vivo* phosphorylation of Vps15p (Herman et al., 1991a; Herman et al., 1991b). These data suggest that Vps15p is able to undergo an autophosphorylation event *in vivo* and indicate that an intact Vps15p protein kinase domain is required for vacuolar protein sorting.

The sequence of Vps15p suggests that it encodes a serine/threonine protein kinase. Sequence analysis of a large number of protein kinases has identified several regions which appear to be indicators of substrate specificity (Hanks and Quinn, 1991). The sequence of Vps15p in kinase subdomains VI and VIII suggests that Vps15p exhibits a preference for serine and/or threonine residues. We utilized the autophosphorylation

reaction of Vps15p to characterize its substrate specificity. Yeast cells were labeled with ^{32}P-orthophosphate, lysed, and Vps15p was immunoprecipitated under denaturing conditions using antisera specific for Vps15p. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by autoradiography detected a single labeled band of ~170 kDa corresponding to Vps15p (Fig. 1A). To determine the identity of the labeled amino acid, ^{32}P-labeled immunoprecipitates were separated by SDS-PAGE, and proteins were transferred to polyvinylidine difluoride (PVDF) membranes and subjected to acid hydrolysis. The hydrolysates were then separated by two-dimensional thin layer electrophoresis on cellulose TLC plates. Phosphoamino acid analysis of *in vivo* ^{32}P-labeled Vps15p showed that it is labeled almost exclusively on serine with a very small amount of phosphothreonine present (Fig. 1B).

To test whether Vps15p is able to undergo an autophosphorylation event in an *in vitro* reaction, yeast cells were lysed and incubated under non-denaturing conditions with antisera specific for Vps15p. Immune complexes were collected on protein A-Sepharose beads and washed extensively in non-denaturing buffer. The immunoprecipitated Vps15p was then incubated with [γ–^{32}P]ATP and divalent cation at 30°C for 30 min and separated by SDS-PAGE. A labeled species of ~170 kDa corresponding to Vps15p was detected, indicating that Vps15p is able to undergo an autophosphorylation event *in vitro* (Fig. 1C). The *in vitro* phosphorylation of Vps15p absolutely required divalent cation, with Mn^{+2} resulting in the highest degree of autophosphorylation (Fig. 1C). Phosphoamino acid analysis of *in vitro*-labeled Vps15p showed that most of the labeled phosphate was incorporated into phosphoserine with a

lesser amount of phosphothreonine present (Fig. 1D). Together with the *in vivo* phosphorylation experiments, these results demonstrate that Vps15p is a functional protein kinase of the serine/threonine family.

Extensive mutational analysis of the N-terminus of Vps15p using site-directed mutagenesis has established that vacuolar protein sorting, growth at 37°C and *in vivo* phosphorylation of Vps15p require an intact Vps15p kinase domain. We tested several mutant Vps15 proteins for *in vitro* phosphorylation as outlined above. The alleles tested contain mutations that correspond to residues present in several distinct protein kinase subdomains as defined by Hanks et al. (1991). K149D represents an alteration in the conserved DIKTEN motif in subdomain IV, D165R in the conserved DFA motif in subdomain V and E200R in the conserved APE motif in subdomain VI (see Fig. 2A). These mutant alleles were expressed in a Δ*vps15* strain, the proteins were immunoprecipitated under native conditions with anti-Vps15p antisera and subjected to an *in vitro* kinase assay. In agreement with the functional assays described above (Herman et al., 1991a; Herman et al., 1991b), these mutant proteins exhibit significantly reduced Vps15p autophosphorylation levels *in vitro* (Fig. 2B). The decreased phosphate incorporation into the mutant proteins is not due to decreased protein levels as Western blot analysis showed that they were present at approximately wild-type levels (data not shown). These results argue against the possibility that the *in vitro* phosphorylation of wild-type Vps15p is due to a contaminating kinase that is coprecipitated and artifactually phosphorylates Vps15p during the reaction. Collectively, these data show that Vps15p is an active serine/threonine protein kinase and, together with the functional analysis of Vps15p mutants, indicate that

protein phosphorylation is required for the efficient sorting of vacuolar proteins in yeast.

The Vps34 PI 3-Kinase Possesses Intrinsic Protein Kinase Activity

While investigating the protein kinase activity of Vps15p, we noted that native immunoprecipitations of yeast extracts using antisera specific for Vps15p followed by *in vitro* kinase assays showed two prominently ^{32}P-labeled species. One labeled species was approximately 170 kDa and comigrated with ^{35}S-labeled Vps15p and therefore represents the Vps15p protein kinase activity characterized in the previous section. The other ^{32}P-labeled species was approximately 95 kDa. The fact that Vps15p and Vps34p have been shown to be present as a complex that can be coimmunoprecipitated under native conditions (Stack et al., 1993) suggested that the two ^{32}P-labeled species may represent Vps15p and Vps34p. The identity of the 95 kDa species as Vps34p was confirmed when antisera specific for Vps34p was found to immunoprecipitate the ^{32}P-labeled 95 kDa species and the 95 kDa ^{32}P-labeled band was absent in immunoprecipitations from Δ*vps34* strains (data not shown). As Vps15p and Vps34p are present in a complex and Vps15p functions to activate Vps34 PI 3-kinase activity (Stack et al., 1993), we next attempted to determine if Vps34p was phosphorylated in a Vps15p-dependent manner. To achieve this, Vps34p was immunoprecipitated from a Δ*vps15* strain and *in vitro* protein kinase assays were performed. Surprisingly, Vps34p was phosphorylated to approximately the same extent in native immunoprecipitations from both wild-type and Δ*vps15* strains (data not shown), suggesting that Vps34p phosphorylation is not the result of

Vps15p protein kinase activity. Additional *in vitro* kinase experiments, including denaturing immunoprecipitation of Vps34p followed by renaturation of the protein (where Vps34p represents the sole ^{32}P-labeled species), further suggested that Vps34p may possess an intrinsic protein kinase activity (data not shown). This interpretation is consistent with observations from mammalian cells, where the p110 catalytic subunit of PI 3-kinase has been shown to be both a lipid kinase and a protein kinase (Carpenter et al., 1993; Dhand et al., 1994).

In order to determine if Vps34p is a phosphoprotein *in vivo*, strains deleted for the *VPS15* gene were labeled with ^{32}P-orthophosphate and Vps34p was immunoprecipitated from the labeled extracts using antisera specific for Vps34p. These experiments showed that Vps34p is indeed phosphorylated *in vivo* in the absence of Vps15p (Fig. 3A). Phosphoamino acid analysis of *in vivo* ^{32}P-labeled immunopreciptated Vps34p found that Vps34p is phosphorylated predominately on serine with a small amount of phosphothreonine present (Fig. 3B). This result is similar to that found with the p110 catalytic subunit of mammalian PI 3-kinase expressed in insect cells, where *in vivo* labeling with ^{32}P-orthophosphate showed that p110 is phosphorylated mainly on serine (Dhand et al., 1994).

To test whether Vps34p could catalyze an autophosphorylation event *in vitro*, Vps34p was immunoprecipitated from Δ*vps15* yeast strains under non-denaturing conditions using antisera specific for Vps34p and then incubated with [γ–^{32}P]ATP in an *in vitro* protein kinase assay. These experiments showed that Vps34p kinase activity *in vitro* requires divalent cation and is essentially Mn^{+2}-dependent (Fig. 3C). While incorporation

of phosphate into Vps34p *in vitro* in the presence of Mg^{+2} could be detected, it was approximately tenfold lower than in the presence of Mn^{+2}. This cation requirement is again very similar to that determined for the p110 subunit of mammalian PI 3-kinase either expressed in insect cells (Dhand et al., 1994) or purified from rat liver (Carpenter et al., 1993). Phosphoamino acid analysis of Vps34p labeled *in vitro* showed the presence of phosphoserine, phosphothreonine and phosphotyrosine (Fig. 3D). The ability to phosphorylate both tyrosine and serine/threonine residues, while very uncommon, has been reported for several "dual specificity" protein kinases (Lindberg et al., 1992). The relevance of phosphorylation of both classes of amino acids *in vitro* by Vps34p is unclear and at this time we cannot rule out the possibility that phosphotyrosine in Vps34p may represent an artifact of the *in vitro* kinase assay.

The data on Vps34p phosphorylation both *in vivo* and *in vitro* suggests that Vps34p may be undergoing an autophosphorylation event and thus would be a member of a novel class of kinase with the ability to phosphorylate both lipid and protein substrates. The only known member of this class is the p110 catalytic subunit of mammalian PI 3-kinase. Collectively, the data presented above support the notion that Vps34p is both a lipid and protein kinase; however, the possibility of a contaminating protein kinase phosphorylating Vps34p in these experiments cannot be ruled out. To test this possibility, site-directed mutagenesis was used to construct mutant alleles of *VPS34* which contain altered residues within the putative lipid kinase domain and have previously been shown to be extremely defective for vacuolar protein

sorting and PI 3-kinase activity (Fig. 4A; Schu et al., 1993). Interestingly, these motifs in Vps34p are shared with both mammalian PI 3-kinase and protein kinases and represent sequences that are most likely involved in binding nucleotide phosphates and in phosphotransferase activity (Knighton et al., 1991). Mutant Vps34 proteins were immunoprecipitated under non-denaturing conditions from Δ*vps34* strains containing the mutant *vps34* alleles. The Vps34p immunoprecipitates were incubated with [γ-^{32}P]ATP and Mn^{+2}. The mutant Vps34 proteins which are defective for PI 3-kinase activity were found also to be defective in the phosphorylation of Vps34p *in vitro* (Fig. 4B). In addition, these mutant proteins also exhibit decreased levels of *in vivo* phosphorylation of Vps34p (data not shown). The decreased levels of phosphate incorporation into the mutant proteins is not the result of decreased protein expression levels as they are made at near wild-type levels (Schu et al., 1993). Altogether, these data indicate that Vps34p is able to function both as a lipid kinase in the phosphorylation of phosphatidylinositol and as a protein kinase in an autophosphorylation reaction.

Effect of PI 3-kinase Inhibitors on Vps34p Lipid and Protein Kinase Activities

The fungal metabolite wortmannin is a potent inhibitor of mammalian PI 3-kinase (Arcaro and Wymann, 1993). Wortmannin appears to bind specifically and irreversibly to mammalian PI 3-kinase (Yano et al., 1993; Thelen et al., 1994) and inhibits p110-like PI 3-kinase in the low nanomolar range with IC_{50}=1-5 nM (Arcaro and Wymann, 1993; Yano et al., 1993; Okada et al., 1994). At these concentrations, wortmannin is quite specific for PI 3-kinase as PtdIns 4-kinase is unaffected at 100 nM

(Yano et al., 1993). Mammalian cells are permeable to wortmannin and this inhibitor has been used to demonstrate a role for PI 3-kinase in regulating processes such as membrane ruffling, histamine secretion and glucose transporter translocation (Yano et al., 1993; Kotani et al., 1994; Okada et al., 1994; Wennstrom et al., 1994). We tested wortmannin on yeast cells and found it had no effect on CPY sorting or cell growth (data not shown), suggesting the possibility that the drug may not be able to enter yeast cells. To directly examine the effects of wortmannin on Vps34p PI 3-kinase activity, yeast extracts from a wild-type strain were incubated in PI kinase reaction buffer containing varying concentrations of wortmannin for five minutes prior to initiating the reaction by adding ATP. The reactions were performed at 25°C for 5 minutes after which the reaction products were extracted and lipids separated by thin layer chromatography on Silica gel 60 plates developed in a borate buffer system (Walsh et al., 1991). Vps34p was found to be quite insensitive to wortmannin at concentrations effective on mammalian p110-like PI 3-kinases as Vps34p PI 3-kinase activity was essentially unaffected at 100 nM (Fig. 5A; see also Fig. 9). Vps34p lipid kinase activity was inhibited by wortmannin in the low micromolar range; however, the high concentrations of wortmannin used make it difficult to interpret this inhibition in a functional manner. While it is clear that wortmannin in the low micromolar range did not inhibit yeast PtdIns 4-kinase (Fig. 5A), it has been reported that micromolar concentrations of wortmannin will inhibit some protein kinases (i.e., myosin light chain kinase) but have no effect on others (Yano et al., 1993). The requirement of high concentrations of wortmannin for Vps34p inhibition does not appear to be

due to a degrading or neutralizing activity in yeast extracts, as essentially identical results have also been obtained from immunoisolated and partially purified Vps34p (data not shown). The wortmannin resistance of Vps34p observed here is consistent with the results of a preliminary study of the PI 3-kinase activities of Vps34p and p110 expressed in *S. pombe* (Woscholski et al., 1994).

The effect of wortmannin on Vps34p autophosphorylation *in vitro* was also tested. Extracts from a *Δvps15* strain containing *VPS34* on a multicopy plasmid were incubated with antisera specific for Vps34p. Immune complexes were then collected on protein A-Sepharose and washed under non-denaturing conditions. The immunoprecipitated Vps34p was incubated for 5 min in protein kinase reaction buffer containing Mn^{+2} and varying concentrations of wortmannin. The kinase reaction was initiated by the addition of [γ–^{32}P]ATP and incubated at 30°C for 30 minutes. The reaction products were separated by SDS-PAGE. The profile of incorporation of phosphate into Vps34p was very similar to the sensitivity of Vps34p PI 3-kinase activity. Vps34p autophosphorylation was insensitive to wortmannin concentrations below the micromolar range and is inhibited by micromolar wortmannin (Fig. 5B). These data suggest that Vps34p autophosphorylation and PI 3-kinase activity are manifestations of the same catalytic activity and further suggest the possibility that Vps34p autophosphorylation may regulate PI 3-kinase activity (however, see below).

Vps34p is also resistant to a second inhibitor of mammalian PI 3-kinase. LY294002 is an analog of the bioflavinoid quercetin, which had been previously shown to inhibit several lipid and protein kinases.

LY294002 was found to be a selective, reversible inhibitor of PI 3-kinase with an IC_{50}=1.4 μM (Vlahos et al., 1994). LY294002 at a concentration of 100 μM completely inhibited p110-like mammalian PI 3-kinase while PtdIns 4-kinase and several protein kinases were unaffected. We tested whether Vps34p PI 3-kinase activity is sensitive to LY294002 by immunoprecipitating Vps34p from Δ*vps15* yeast cell extracts under non-denaturing conditions. Immunoprecipitated Vps34p was incubated for 5 minutes at 25°C in PI kinase reaction buffer containing varying concentrations of LY294002. PI kinase reactions were initiated by the addition of ATP and performed for 5 minutes at 25°C. Labeled lipids were extracted and separated by TLC as described above. As was seen for wortmannin, Vps34p PI 3-kinase activity is relatively insensitive to the inhibitor LY294002; concentrations of LY294002 10-20 fold higher than the IC_{50} for mammalian PI 3-kinase are required to have any significant inhibitory effect on Vps34p PI 3-kinase activity (Fig. 6A; see also Fig. 9B). Indeed, the IC_{50} for Vps34p is approximately 50 μM (Fig. 9B). These results are strikingly similar to the effects of wortmannin and indicate that Vps34p PI 3-kinase activity is resistant to PI 3-kinase inhibitors such as LY294002 and wortmannin. The effects of LY294002 on Vps34p autophosphorylation *in vitro* also demonstrates the insensitivity of Vps34p to PI 3-kinase inhibitors; incorporation of phosphate into immunoisolated Vps34p is unaffected by concentrations of LY294002 that significantly inhibit mammalian p110-like PI 3-kinase activity and relatively high concentrations are required to inhibit Vps34p autophosphorylation *in vitro* (Fig. 6B). Altogether, the resistance to known PI 3-kinase inhibitors suggests that Vps34p may have different

functional properties from mammalian p110-like PI 3-kinases and further suggests a similarity to the mammalian PtdIns-specific 3-kinase which is also resistant to wortmannin (Stephens et al., 1994a).

The Role of Vps34p Autophosphorylation in Regulating PI 3-kinase Activity

The observations that Vps34p mutants that are defective for PI 3-kinase activity are also defective for Vps34p autophosphorylation (Fig. 4B) and that inhibitors which affect PI 3-kinase activity also inhibit Vps34p autophosphorylation suggest the possibility that autophosphorylation of Vps34p may regulate its PI 3-kinase activity. Indeed, it has been shown that phosphorylation of the p85 subunit of PI 3-kinase by the p110 catalytic subunit causes a decrease in PI 3-kinase activity (Carpenter et al., 1993; Dhand et al., 1994); however, no functional relationship between catalytic subunit autophosphorylation and PI 3-kinase activity has been investigated. In order to examine this possibility, Vps34p was immunoprecipitated under native conditions from yeast cell extracts, collected onto protein A-Sepharose beads, washed, and subjected to *in vitro* phosphorylation or dephosphorylation prior to performing an *in vitro* PI kinase assay. To test the effect of phosphorylation of Vps34p on PI 3-kinase activity, immunoprecipitated Vps34p was incubated in the presence of Mn^{+2} and 10 mM unlabeled ATP at 30°C for varying lengths of time. These conditions favor the autophosphorylation of Vps34p (Fig. 3C). Following this incubation, excess ATP and Mn^{+2} was washed away and a standard PI kinase assay was performed for 5 minutes at 25°C. The labeled lipids were separated by TLC and detected by autoradiography. It was

found that incubation of Vps34p under conditions that favor autophosphorylation resulted in no significant change in PI 3-kinase activity (Fig. 7A). It does not appear that this was the result of Vps34p being maximally phosphorylated prior to incubation with ATP because dephosphorylation of Vps34p followed by phosphorylation with ATP prior to PI kinase assay also had no effect (data not shown; see below). To test directly the effect of dephosphorylation on Vps34p PI 3-kinase activity, immunoprecipitated Vps34p was incubated with potato acid phosphatase for 15 minutes at 30°C, washed and then subjected to an *in vitro* PI kinase assay. These experiments showed that dephosphorylation of Vps34p had no apparent effect on PI 3-kinase activity (Fig. 7B). Potato acid phosphatase was able to at least partially dephosphorylate Vps34p, as ^{32}P-labeled Vps34p incubated under identical conditions showed a significant decrease in ^{32}P-label upon phosphatase treatment (Fig. 7C). Essentially identical results were obtained with calf intestine alkaline phosphatase and protein phosphatase 2A (data not shown), suggesting that dephosphorylation of Vps34p under these conditions has no significant effect on Vps34p PI 3-kinase activity *in vitro*.

Vps34p is a Phosphatidylinositol-Specific PI 3-kinase

PI 3-kinase from mammalian cells is capable of phosphorylating several forms of phosphatidylinositol at the D-3 position of the inositol ring. The p110/p85 PI 3-kinase will act on PtdIns to form PtdIns(3)P, on PtdIns(4)P to form PtdIns(3,4)P_2, and on PtdIns(4,5)P_2 to form PtdIns(3,4,5)P_3 (Carpenter and Cantley, 1990; Soltoff et al., 1992). The time course of appearance of these phosphoinositides in the cell following

growth factor stimulation is quite different for the individual forms (Cantley et al., 1991) and suggests that they may perform distinct functions *in vivo*. A biochemical basis for this notion was provided by the existence of a PtdIns-specific 3-kinase in mammalian cells (Stephens et al., 1994a), suggesting that distinct PI 3-kinase enzymes may be responsible for generation of the different phosphoinositides. As such, it is important to characterize the activities of known PI 3-kinases, especially when a physiological function can be assigned, such as the role for Vps34p in vacuolar protein sorting. Initial experiments in *S. cerevisiae* and with Vps34p expressed in *S. pombe* suggested that Vps34p was not able to utilize more highly phosphorylated forms of PtdIns, such as PtdIns(4)P or PtdIns(4,5)P_2, in an *in vitro* PI kinase assay (Auger et al., 1989; Kodaki et al., 1994). In addition, *in vivo* labeling of yeast cells with ^{3}H-inositol followed by HPLC analysis of deacylated lipids to determine the head group structure readily detected PtdIns(3)P, PtdIns(4)P and PtdIns(4,5)P_2; however, more highly phosphorylated forms of PtdIns(3)P could not be unambiguously determined (Auger et al., 1989; Hawkins et al., 1993; Schu et al., 1993).

To accurately assess the substrate specificity of Vps34p, partially purified preparations of Vps34p were subjected to an *in vitro* PI kinase assay using the substrates PtdIns, PtdIns(4)P or PtdIns(4,5)P_2 in a carrier of phosphatidylserine. Labeled lipids were extracted and separated on oxalate-treated silica gel 60 TLC plates developed in a 1-propanol-acetic acid buffer system (Auger et al., 1992). Standards for PtdIns, PtdInsP (PIP) and PtdInsP_2 (PIP_2) were visualized by staining the developed TLC plates with I_2 vapor. This analysis clearly showed that Vps34p was able to utilize

PtdIns but not PtdIns(4)P or PtdIns(4,5)P_2 as a substrate *in vitro* (Fig. 8). As the active, functional form of Vps34p *in vivo* appears to be in a complex with Vps15p (Stack et al., 1993), it is possible that Vps15p may serve to regulate the substrate specificity of Vps34p in addition to stimulating its PtdIns 3-kinase activity. To test this possibility, we assessed the substrate specificity of Vps34p when present in a complex with Vps15p. Partially purified preparations of the Vps34p-Vps15p complex were subjected to *in vitro* PI kinase assays using PtdIns, PtdIns(4)P or PtdIns(4,5)P_2 as substrates as described above. Analysis of the reaction products showed that Vps34p retained its strict substrate specificity for PtdIns when present in a complex with Vps15p (Fig. 8). Together, these data indicate that Vps34p is a PtdIns-specific 3-kinase and suggest a role for PtdIns(3)P in regulating intracellular membrane trafficking.

Biochemical Properties of Vps34p PI 3-Kinase Activity

Cellular PI kinase activities can be distinguished by the effects of various inhibitors and chemicals. The detergent NP-40 has been used to differentiate between PI 3-kinase and PtdIns 4-kinase activities in mammalian cells. NP-40 inhibits PI 3-kinase while PtdIns 4-kinase is activated by NP-40 (Carpenter and Cantley, 1990). PI 3-kinase and PtdIns 4-kinase also are differentially affected when the PI kinase assays are performed in the presence of adenosine. PtdIns 4-kinase is strongly inhibited by adenosine while PI 3-kinase is largely unaffected (Carpenter and Cantley, 1990). In addition to being used to distinguish between PI 3-kinase and PtdIns 4-kinase, NP-40, adenosine and wortmannin have characteristic effects on different PI 3-kinase activities isolated from

mammalian cells. PI 3-kinase, represented by the p110/p85 heterodimer, is much more sensitive to inhibition by NP-40 than is the PtdIns-specific 3-kinase. In fact, the PtdIns-specific 3-kinase is modestly activated at low concentrations of NP-40 while it is inhibited at higher concentrations (Stephens et al., 1994a). Both PI 3-kinase and PtdIns 3-kinase are quite insensitive to adenosine inhibition and the PtdIns 3-kinase appears to be completely unaffected by adenosine concentrations up to 100 μM (Stephens et al., 1994a). Finally, while p110/p85 is very sensitive to wortmannin (IC_{50}=~3 nM), the mammalian PtdIns 3-kinase is at least 100-fold more resistant to the drug (Stephens et al., 1994a).

As we have shown that Vps34p is a PtdIns-specific 3-kinase (Fig. 8); we determined its sensitivity to these compounds in order to allow comparison with the distinct mammalian PI 3-kinase activities. Partially purified preparations of Vps34p were incubated with the various compounds for 5 min prior to performing a standard PI kinase assay. As indicated earlier (Figs. 5A, 6A), Vps34p is very resistant to the PI 3-kinase inhibitors wortmannin and LY294002 (Fig. 9A,B). Based on these data, we have calculated an IC_{50}= ~3 μM for wortmannin and IC_{50}= ~50 μM for LY294002. The fact that p110/p85 exhibits an IC_{50}= ~3nM for wortmannin (Arcaro and Wymann, 1993; Yano et al., 1993) and IC_{50}=1-2 μM for LY294002 (Vlahos et al., 1994) illustrates the differences between Vps34p and p110/p85 and the similarities between Vps34p and the mammalian PtdIns 3-kinase. Vps34p also exhibits insensitivity to adenosine similar to PtdIns 3-kinase (Fig. 9C). Finally, while similar to the mammalian PtdIns-specific 3-kinase in that it is stimulated at low concentrations of NP-40, Vps34p appears to be activated to a greater extent by NP-40 as Vps34p

shows a several-fold increase in PI kinase activity at modest concentrations of the detergent (Fig. 9D). It is unclear at this time if the effects of NP-40 on PI kinases are due to direct binding of detergent molecules to the enzyme or to altered presentation of substrate. Taken together with the substrate specificity experiments (Fig. 8), these data indicate that Vps34p represents a PtdIns 3-kinase distinct from p110/p85 and is quite similar to the PtdIns 3-kinase of Stephens et al. (1994a) and further suggests that Vps34p and the mammalian PtdIns 3-kinase may perform similar functions *in vivo*.

Discussion

Previous work has established that the *VPS15* and *VPS34* genes encode homologs of a protein kinase and a PI 3-kinase, respectively, that are required for the efficient sorting of proteins to the vacuole in yeast. Vps15p and Vps34p have been shown to form a membrane-associated complex and a functional Vps15 protein kinase domain is required for the activation of the Vps34p PtdIns 3-kinase (Stack et al., 1993). Studies with Vps34p have demonstrated that the homology between Vps34p and the p110 catalytic subunit of mammalian PI 3-kinase is functional; Vps34p has been shown to possess PtdIns 3-kinase activity and this activity is required for efficient vacuolar protein localization (Schu et al., 1993). In this study, we characterize the protein kinase activity of Vps15p and show that Vps34p is a member of a unique class of kinases able to phosphorylate both lipid and protein substrates. Finally, the similarities between the biochemical characteristics of Vps34p and mammalian PtdIns-specific 3-kinase suggest that a family of distinct PI 3-kinases exists in mammalian cells and that Vps34p-like PtdIns 3-kinases may play a similar role in specifically regulating protein sorting decisions in higher organisms.

The predicted sequence of Vps15p contains all the catalytic subdomains of protein kinases with the possible exception of the glycine-rich nucleotide-binding motif (Herman et al., 1991a). *In vivo* phosphorylation assays and site-directed mutagenesis has suggested that Vps15p is a protein kinase and that Vps15p kinase activity is required for vacuolar protein sorting (Herman et al., 1991a; Herman et al., 1991b). We have shown here that Vps15p is capable of catalyzing a specific

autophosphorylation event *in vitro*. The fact that Vps15p kinase domain point mutants are defective for vacuolar protein sorting suggests that Vps15p protein kinase activity is functionally relevant. The observations that Vps15p and Vps34p form a complex *in vivo* and that strains containing Vps15p kinase domain point mutants are defective for Vps34p PtdIns 3-kinase activity suggest that Vps15p may phosphorylate and thereby activate Vps34p. That Vps34p is able to undergo an autophosphorylation event *in vivo* and *in vitro* makes detection of Vps15p-dependent phosphorylation of Vps34p difficult. It appears that most, if not all, of the phosphate incorporated into Vps34p is a result of its autophosphorylation activity; Vps34p phosphorylation *in vivo* and *in vitro* is not significantly different between wild-type and *Δvps15* strains (J.H.S. and S.D.E., unpublished observations) and Vps34p kinase domain point mutants are not significantly phosphorylated *in vivo* or *in vitro* (Fig. 6B). Vps15p kinase activity may function to activate Vps34p by a mechanism distinct from direct modification of Vps34p. One possibility is that Vps15p protein kinase activity is required for an autophosphorylation event which leads to the association of Vps15p with Vps34p. This association of autophosphorylated Vps15p with Vps34p may then trigger a conformational change in Vps34p which results in stimulation of PtdIns 3-kinase activity. While the precise mechanism of activation of Vps34p by Vps15p remains to be determined, it is possible that Vps15p protein kinase activity acts independently of Vps34p phosphorylation to stimulate PtdIns 3-kinase activity. The question of whether Vps15p directly phosphorylates Vps34p can be unambiguously determined once the autophosphorylation sites on Vps34p are mapped and mutated.

The demonstration that Vps34p is able to phosphorylate both phosphatidylinositol (Schu et al., 1993) and itself (Fig. 3) places it in a novel class of multiple specificity kinases which have the ability to catalyze the transfer of phosphate from ATP to both lipid and protein substrates. As the only other known member of this class is the p110 catalytic subunit of mammalian PI 3-kinase (Carpenter et al., 1993; Dhand et al., 1994), it will be interesting to learn whether this is a general characteristic of lipid kinases or specifically of PI 3-kinases. From a structural and mechanistic viewpoint, it would appear that the ability to accommodate both lipid and protein substrates reflects considerable flexibility in the catalytic active site of PI 3-kinases; however, one must consider the strict stereospecificity of the PI 3-kinase reaction in that it modifies only the D-3 position of the inositol ring. The fact that alterations in the single identified kinase domain of both Vps34p and p110 affect both PI 3-kinase and protein kinase activities suggests that a single active site is responsible for both activities (Fig. 4B; Schu et al., 1993; Dhand et al., 1994). The similar inhibitory effects of wortmannin and LY294002 on both activities is also consistent with a single catalytic domain.

Phosphorylation of the p85 subunit by the p110 catalytic subunit of mammalian PI 3-kinase has been shown to result in a decrease in PI 3-kinase activity that can be reversed by protein phosphatase 2A, suggesting a functional link between p85 phosphorylation and PI 3-kinase activity (Carpenter et al., 1993; Dhand et al., 1994). The catalytic subunit of PI 3-kinase has also been shown to phosphorylate the insulin receptor substrate IRS-1 on serine residues (Lam et al., 1994). Carpenter et al. (1993) described phosphorylation of the p110 subunit; however, no functional

relationship between p110 phosphorylation and PI 3-kinase activity was indicated. We have attempted to directly test whether Vps34p autophosphorylation has an effect on PtdIns 3-kinase activity. Incubation of Vps34p either under conditions favoring Vps34p autophosphorylation or in the presence of protein phosphatase (Fig. 7A,B) had no apparent effect on PtdIns 3-kinase activity. This observation does not rule out the possibility that, in a manner analogous to mammalian p110, Vps34p phosphorylates proteins other than itself and this transphosphorylation event in turn regulates PtdIns 3-kinase activity. A good candidate for such a substrate of Vps34p would be Vps15p. Strains containing *vps15* kinase domain point mutants are defective for PtdIns 3-kinase activity, suggesting that Vps15p kinase activity is required for Vps34p PtdIns 3-kinase activation (Stack et al., 1993). If Vps34p phosphorylates Vps15p, the result of the respective kinase activities may be a complex regulatory circuit composed of protein kinase reactions that serve to regulate vacuolar protein sorting by modulating PtdIns 3-kinase activity.

The identification of Vps34p as a yeast PtdIns 3-kinase and mutational analysis of Vps34p (Schu et al., 1993) indicate that PtdIns 3-kinase activity regulates membrane vesicle trafficking in yeast and suggest the possibility that PI 3-kinase activity may function similarly in mammalian cells. Mammalian PI 3-kinase, represented by the p110/p85 complex, has been shown to associate with activated receptor tyrosine kinases (Soltoff et al., 1992). A recent report of a PI 3-kinase activity distinct from p110/p85 that is activated by G protein βγ subunits (Stephens et al., 1994b; Thomason et al., 1994) indicates that PI 3-kinases may associate with numerous types of cell surface receptor proteins. PI 3-kinase

activity in mammalian cells may be involved in regulating the intracellular fate of internalized cell surface receptors. Receptors for PDGF have been shown to be internalized as a complex with p85/p110 PI 3-kinase (Kapeller et al., 1993). Mutant PDGF receptors specifically lacking the binding site for PI 3-kinase fail to accumulate intracellularly, suggesting a role for PI 3-kinase activity in the normal endocytic trafficking of cell surface receptors (Joly et al., 1994). In addition, it has been shown that mutant CSF receptors that do not associate with PI 3-kinase are internalized but fail to be delivered to the lysosome for degradation (Downing et al., 1989; Carlberg et al., 1991). PI 3-kinase activity may therefore be involved in the sorting step for mammalian cell surface receptors at which the decision is made to recycle to the cell surface or be diverted to the lysosome for turnover. A role for PI 3-kinase in regulating intracellular membrane trafficking may also be indicated by the observation that PI 3-kinase activity is required for the translocation to the cell surface of vesicles containing glucose transporters following insulin stimulation (Hara et al., 1994; Okada et al., 1994). In addition, it has been shown that insulin stimulation of adipocytes resulted in a redistribution of PI 3-kinase from the cytoplasm to an intracellular membrane fraction distinct from the plasma membrane, further suggesting the involvement of PI 3-kinase in glucose transporter translocation (Kelley et al., 1992).

Determining a role for PI 3-kinase in mammalian cells is complicated by the fact that it acts upon multiple substrates. Mammalian PI 3-kinases of the p110/p85 type are capable of producing PtdIns(3)P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 (Soltoff et al., 1992). It is likely that these different products do not have identical effects *in vivo*. Formation of

PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 is stimulated by growth factor addition to cells while PtdIns(3)P levels remain relatively constant (Auger et al., 1989). This suggests that PtdIns(3)P may perform a function distinct from PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3. Evidence exists that PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 may act as messengers in signaling cell proliferation as a result of growth factor receptor activation (Cantley et al., 1991; Fantl et al., 1992; Chung et al., 1994) and in mediating membrane ruffling and cytoskeletal rearrangement events triggered by growth factor receptor stimulation (Kotani et al., 1994; Wennstrom et al., 1994; Wymann and Arcaro, 1994). The role for Vps34p in vacuolar protein sorting in yeast suggests the possibility that PtdIns(3)P formed through the action of PI 3-kinase(s) at specific intracellular sites may function in mammalian cells in the trafficking of cell surface receptors and possibly at the Golgi in regulating lysosomal protein sorting.

The notion that different phosphorylated forms of phosphatidylinositol may have different cellular functions in mammalian cells is supported by recent reports of multiple distinct PI 3-kinase activities with very different substrate specificities. These distinct PI 3-kinase activities imply that multiple PI 3-kinase enzymes with different functional properties exist in mammalian cells. Indeed, several p110-like PI 3-kinases have been described that are coupled to tyrosine kinases through SH2 domains in the p85 subunit (Hiles et al., 1992; Hu et al., 1993), a G protein regulated PI 3-kinase has been reported (Stephens et al., 1994b; Thomason et al., 1994) and a PtdIns-specific 3-kinase has been recently characterized (Stephens et al., 1994a). It is possible that a family of PI 3-kinases may exist in mammalian cells that serve to regulate diverse

cellular functions. Our work here has shown that Vps34p is a PtdIns-specific 3-kinase that is highly resistant to PI 3-kinase inhibitors such as wortmannin and LY294002. The mammalian PtdIns-specific 3-kinase activity has biochemical characteristics strikingly similar to Vps34p (Fig. 9; Stephens et al. 1994a). This may indicate that this mammalian PtdIns 3-kinase is the functional homolog of Vps34p or that it is a member of a family of Vps34p-like mammalian PI 3-kinases. One can speculate that these PtdIns-specific 3-kinases may function in regulating intracellular protein trafficking rather than in signaling cell proliferation due to their strict substrate specificity. It will be interesting to learn the intracellular distribution of mammalian PtdIns 3-kinases and the cloning of such molecules may find a protein more closely related to Vps34p than to p110. Studies with PI 3-kinases in yeast and mammalian cells may well reveal a class of lipid kinases whose members perform diverse and essential cellular roles such as regulating signal transduction, cytoskeletal rearrangements and intracellular protein trafficking.

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Footnote

[1] The abbreviations used are: PI 3-kinase, phosphoinositide 3-kinase (utilizes PtdIns, PtdIns(4)P and PtdIns(4,5)P_2 as substrates); PtdIns 3-kinase, phosphatidylinositol 3-kinase (utilizes PtdIns as substrate); PtdIns, phosphatidylinositol; PtdIns(3)P, phosphatidylinositol 3-phosphate; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PtdIns 4-kinase, phosphatidylinositol 4-kinase; PAGE, polyacrylamide gel electrophoresis; IC_{50}, the concentration at which 50% inhibition occurs.

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Figure 1. Analysis of Vps15p labeled with ^{32}P-orthophosphate. **A.** *In vivo* ^{32}P-labeling of Vps15p. Wild-type yeast cells containing the *VPS15* gene on a multicopy plasmid were labeled with $^{32}PO_4$ and Vps15p was immunoprecipitated and analyzed by SDS-PAGE and autoradiography. The position of Vps15p is indicated. **B.** Phosphoamino acid analysis of Vps15p labeled *in vivo*. Immunoprecipitated ^{32}P-labeled Vps15p was separated by SDS-PAGE and transferred to PVDF membranes. Vps15p was located by autoradiography and the excised band was subjected to acid hydrolysis. The hydrolysates were separated by two dimensional thin layer electrophoresis on cellulose TLC plates. The first dimension was run at pH 1.9 and the second dimension was run at pH 3.5. The positions of the phosphoamino acids were determined by ninhydrin staining of unlabeled standards. **C.** Analysis of *in vitro* ^{32}P-labeled Vps15p. Vps15p was immunoprecipitated from yeast cell extracts under non-denaturing conditions using Vps15p-specific antisera. The immunoprecipitates were washed and incubated at 30°C for 30 min with [γ–^{32}P]ATP in the presence of the indicated divalent cation as described in Experimental Procedures. The labeled proteins were run on SDS-PAGE and detected by autoradiography. The position of Vps15p is indicated. **D.** Phosphoamino acid anlysis of *in vitro* ^{32}P-labeled Vps15p. Native immunoprecipitates of Vps15p were labeled as described above and separated by SDS-PAGE. The proteins were transferred to PVDF filters and subjected to acid hydrolysis and thin layer electrophoresis as described above. The positions of the phosphoamino acids are indicated.

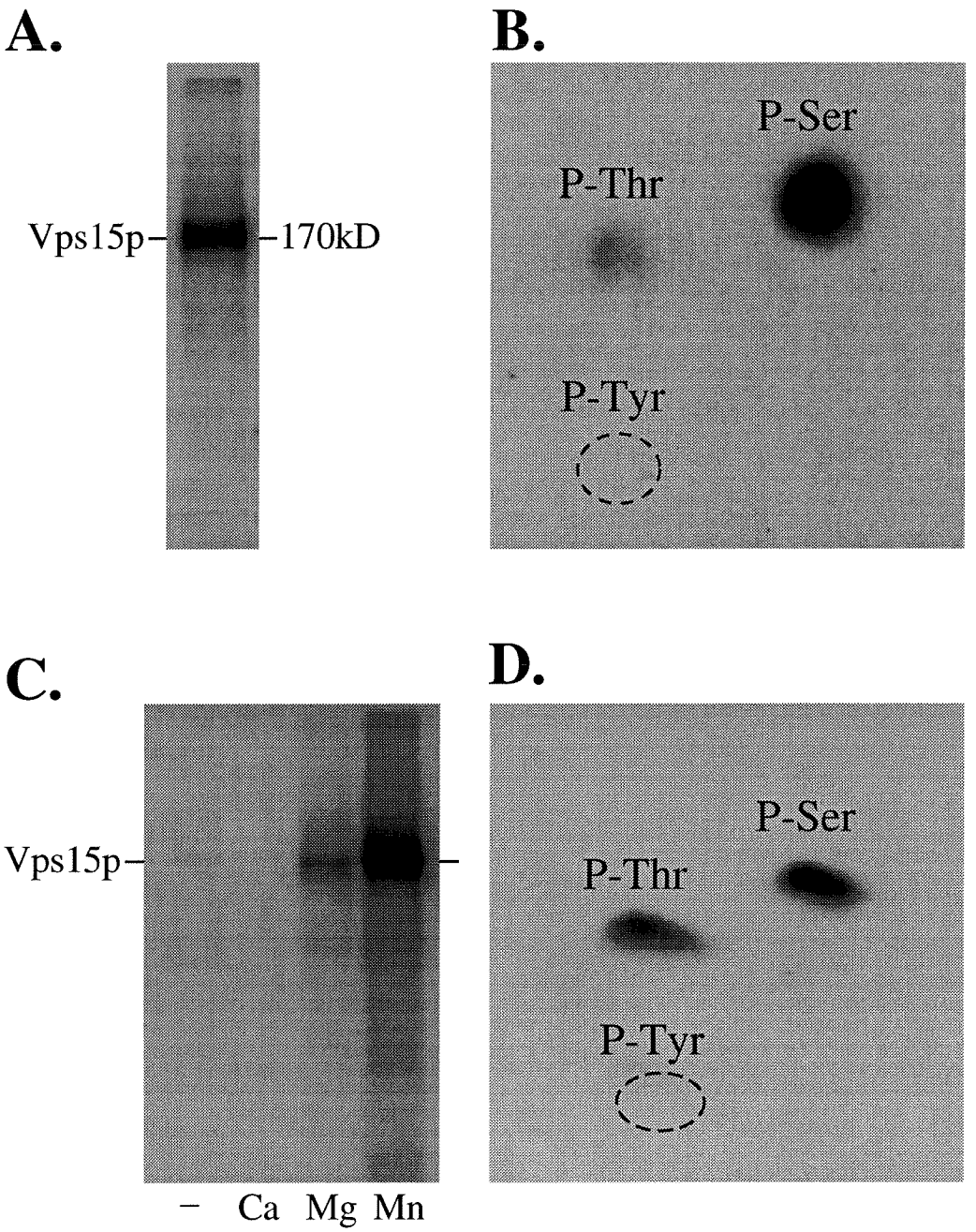

A.
Vps15p
170kD
B.
P-Ser
P-Thr
P-Tyr
C.
Vps15p
– Ca Mg Mn
D.
P-Ser
P-Thr
P-Tyr

Figure 2. Mutations altering conserved residues in the kinase domain of Vps15p abolish protein kinase activity. **A**. Sequence comparison between Vps15p and a consensus serine/threonine protein kinase. The numbers and asterisks refer to amino acids altered by site-directed mutagenesis of the *VPS15* gene to generate mutant Vps15 proteins which were analyzed by *in vitro* kinase assay in (B). In the comparisons, identities are indicated by a solid line and conservative changes by two dots. Gaps in the amino acid sequence are indicted by the numbers in parentheses. Adapted from Ref. 4. **B**. Labeling of mutant Vps15 proteins with [γ–^{32}P]ATP *in vitro*. Wild-type (WT) or mutant Vps15 proteins were isolated by native immunoprecipitation and labeled in an *in vitro* protein kinase reaction in the presence of 10 mM $MnCl_2$ as described in Experimental Procedures. The labeled proteins were analyzed by SDS-PAGE and autoradiography.

A.

```
                149                 165                 200
                 *                   *                   *
Vps15p:  DIKTEN-(11)-DFA-(30)-APE
         |:|  ||       ||:       |||
Prot. Kinase: DLKPEN-(11)-DFG-(25)-APE
```

B.

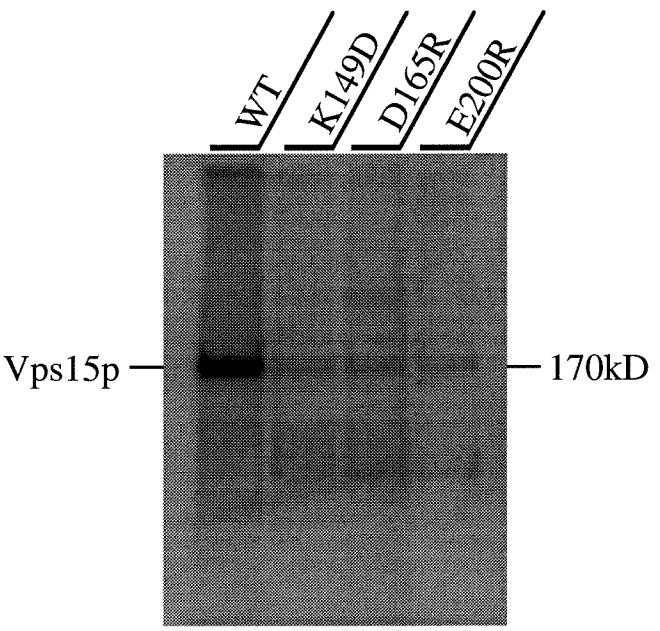


Figure 3. The Vps34p PI 3-kinase has protein kinase activity. **A.** Vps34p is a phosphoprotein *in vivo*. A Δ*vps15* strain containing the *VPS34* gene on a multicopy plasmid was labeled with $^{32}PO_4$ and Vps34p was immunoprecipitated under denaturing conditions. The immunoprecipitates were run on SDS-PAGE and labeled species detected by autoradiography. The position of Vps34p is indicated. **B.** Phosphoamino acid analysis of *in vivo* labeled Vps34p. Immunoprecipitated *in vivo* labeled Vps34p was separated by SDS-PAGE, and the proteins were transferred to PVDF membranes. Labeled Vps34p was localized by autoradiography, excised and subjected to acid hydrolysis and thin layer electrophoresis as described in Figure 1. **C.** Vps34p is labeled in an *in vitro* protein kinase reaction. Vps34p was immunoprecipitated under native conditions from a Δ*vps15* strain containing *VPS34* on a multicopy plasmid. The immunoprecipitates were incubated with [γ-^{32}P]ATP and the indicated divalent cation for 30 min at 30°C. Labeled Vps34p was detected by SDS-PAGE followed by autoradiography. **D.** Phosphoamino acid analysis of *in vitro* labeled Vps34p. Vps34p was immunoprecipitated under non-denaturing conditions as described above and subjected to an *in vitro* protein kinase assay in the presence of [γ-^{32}P]ATP and 10 mM $MnCl_2$. Labeled proteins were analyzed by SDS-PAGE and transferred to a PVDF filter. Labeled Vps34p was excised and subjected to acid hydrolysis and thin layer electrophoresis as described in Figure 1.

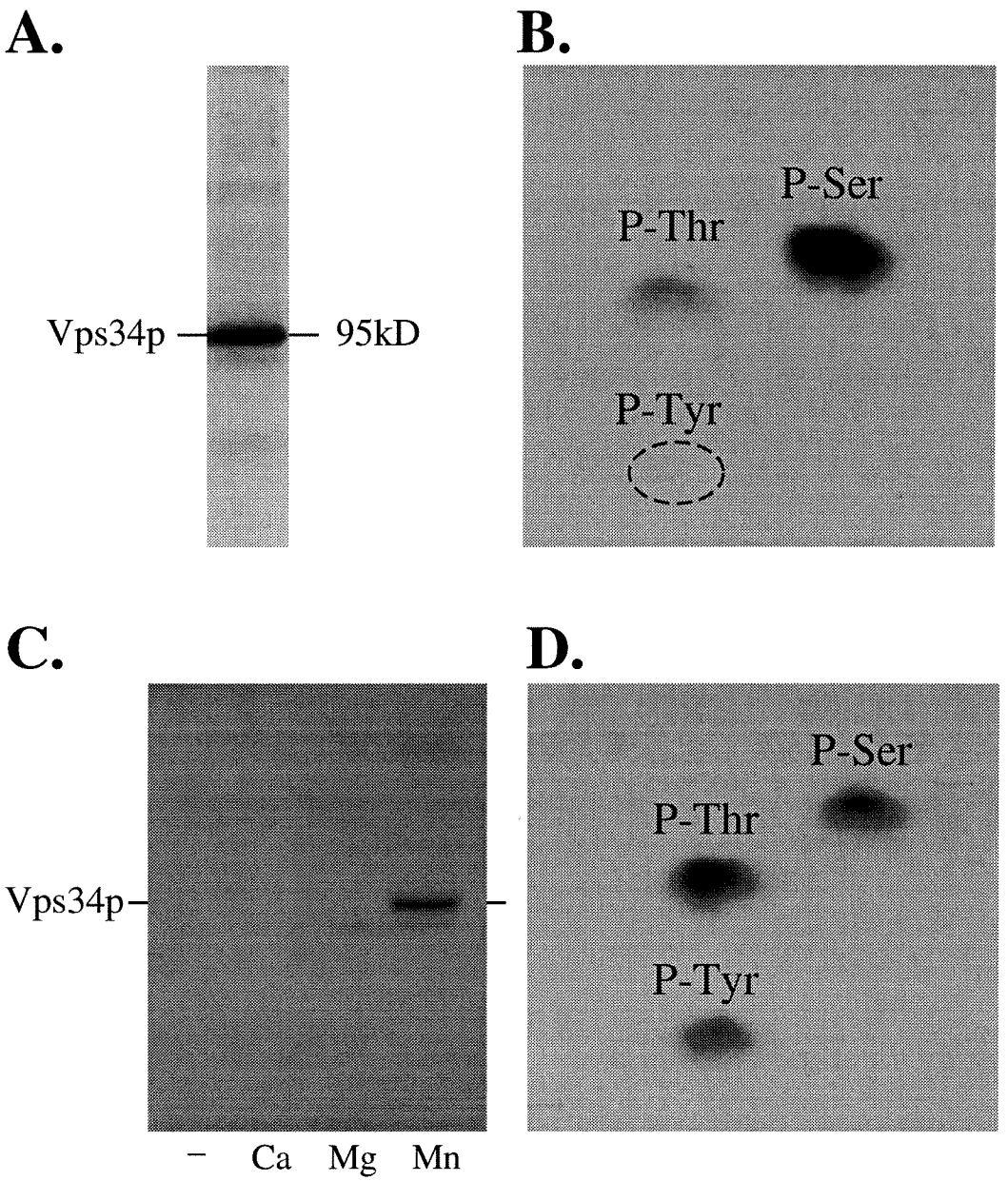

A.
Vps34p
95kD
B.
P-Ser
P-Thr
P-Tyr
C.
Vps34p
–
Ca
Mg
Mn
D.
P-Ser
P-Thr
P-Tyr

Figure 4. Mutations altering conserved residues in the lipid kinase domain of Vps34p abolish protein kinase activity. **A**. Sequence alignment of Vps34p with the p110 catalytic subunit of mammalian PI 3-kinase (Hiles et al., 1992) and a consensus sequence for serine/threonine protein kinases over the region proposed to be involved in ATP binding and phosphotransferase activity. Identities are indicated by a solid bar and conserved residues by two dots. The numbers in parentheses refer to the number of residues between the displayed domains. The asterisks and numbers indicate residues altered by site-directed mutagenesis of *VPS34*; mutant proteins were analyzed for *in vitro* protein kinase activity in (B). Adapted from Ref. 54. **B**. *In vitro* protein kinase activity of mutant Vps34p molecules. The indicated alleles were generated by site-directed mutagenesis and introduced into a Δ*vps34* strain on a multicopy plasmid. None of the indicated mutant alelles are able to complement the vacuolar protein sorting defect of the Δ*vps34* strain (Schu et al., 1993). To assay *in vitro* protein kinase activity, wild-type or mutant Vps34 proteins were immunoprecipitated under native conditions and incubated at 30°C for 30 min in the presence of [γ–^{32}P]ATP and 10 mM $MnCl_2$. The labeled proteins were analyzed by SDS-PAGE and autoradiography.

A.

```
                                                    731  735 736        749
                                                     *    **            *
Vps34p:  LATGPQIGAI--(11)--LSK--(45)---DRHLDNL-(11)-DFG
         |: |   :| |           |          |||   |:       |||
PI 3-Kin:LSIGDCVGLI--(12)--QCK--(50)---DRHNSNI-(11)-DFG
         |: |    | :          |          | :   |:       |||
Prot. Kin:LGxGxx-GxV-(9-12)-AxK-(90-95)-DxKxxNL-(11)-DFG
```

B.

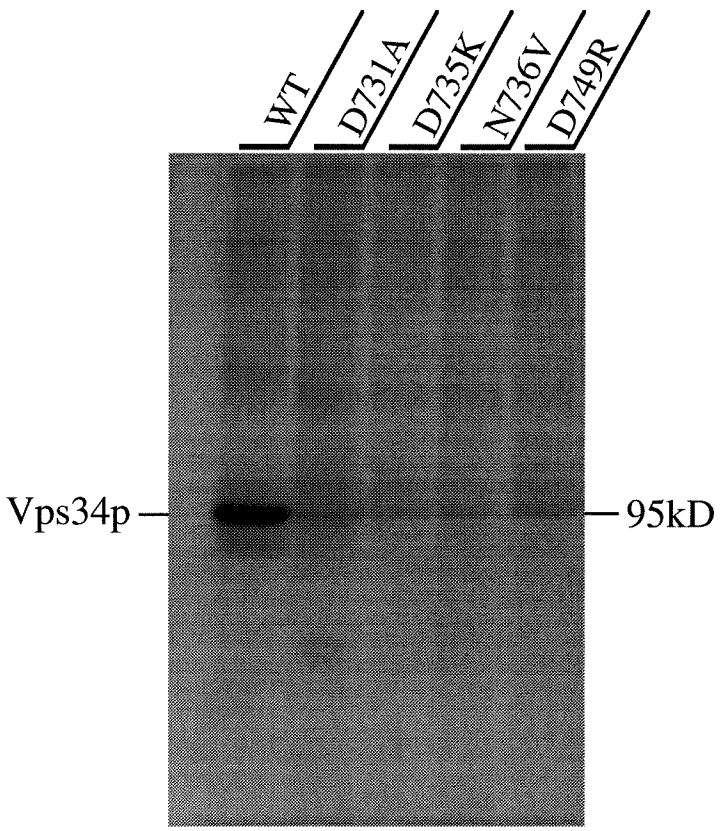
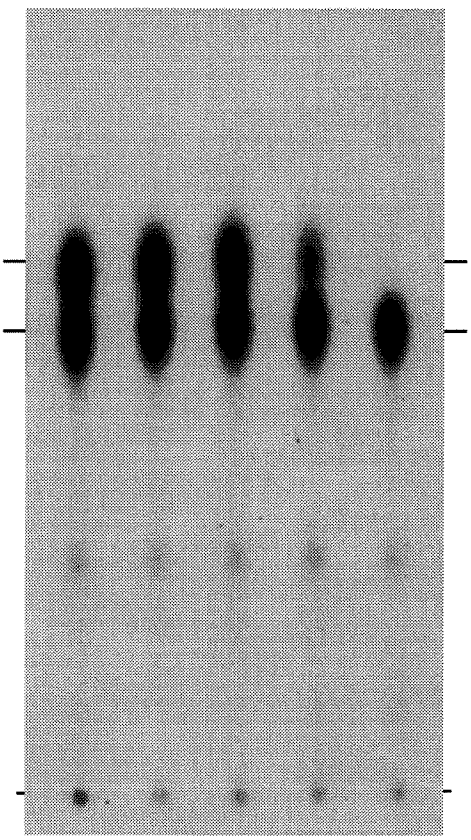


Figure 5. Vps34p PI 3-kinase and protein kinase activities are relatively insensitive to the PI 3-kinase inhibitor wortmannin. **A**. PI 3-kinase activity in the presence of wortmannin. Extracts from a wild-type yeast strain were incubated with the indicated concentration of wortmannin for 5 min at 25°C prior to performing an *in vitro* PI 3-kinase assay. The assays contained PtdIns and were initiated by the addition of ATP. After incubation at 25°C for 5 min, the reaction products were extracted with chloroform:methanol and analyzed by thin layer chromatography on Silica gel 60 plates developed in a borate buffer system (Walsh et al., 1991). The position of the products of PtdIns 3-kinase [PI(3)P] and PtdIns 4-kinase [PI(4)P] are indicated. **B**. Wortmannin effect on the protein kinase activity of Vps34p. Vps34p was immunoprecipitated under non-denaturing conditions from a Δ*vps15* strain containing *VPS34* on a multicopy plasmid. The immune complexes were collected onto protein A-Sepharose beads and washed as described in Experimental Procedures. The immunoprecipitates were incubated with the indicated concentration of wortmannin for 5 min at 25°C in 20 mM HEPES, pH 7.4, 10 mM $MnCl_2$. The reactions were initiated by the addition of [γ–^{32}P]ATP and incubated at 30°C for 30 min. The labeled proteins were analyzed by SDS-PAGE followed by autoradiography.

A.


PI(3)P
PI(4)P
[wortmannin] 0 0.01 0.1 3.0 10.0 μM

B.

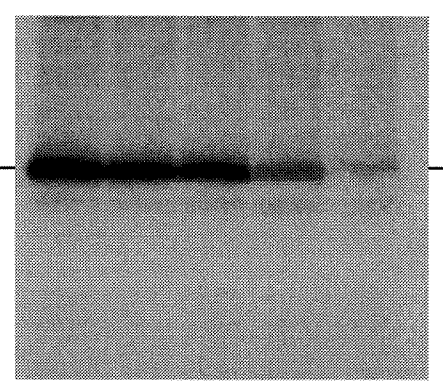
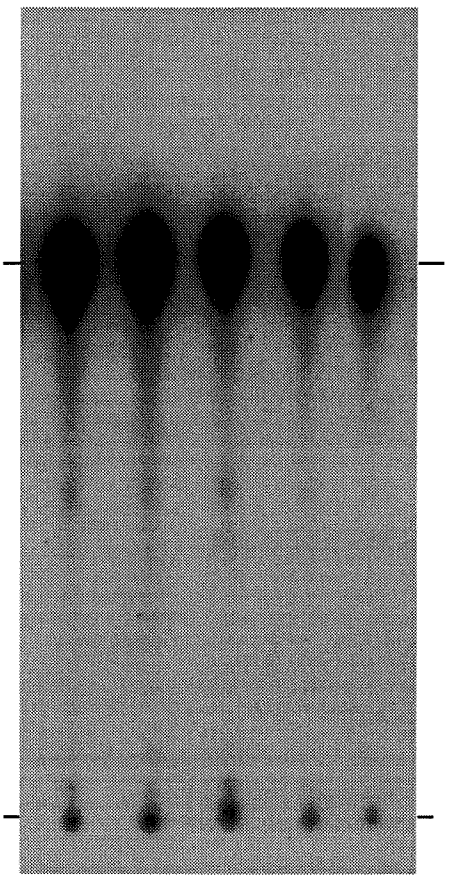

Vps34p
[wortmannin] 0 0.01 0.1 3.0 10.0 μM

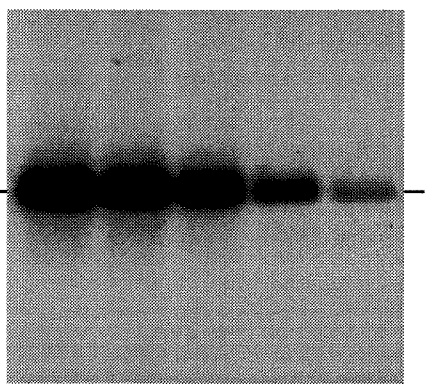
Figure 6. The PI 3-kinase and protein kinase activities of Vps34p are relatively insensitive to the PI 3-kinase inhibitor LY294002. **A**. The effect of LY294002 on Vps34p *in vitro* PI 3-kinase activity. Vps34p was immunoprecipitated under native conditions from a Δ*vps15* strain containing *VPS34* on a multicopy plasmid. The immunoprecipitates were incubated with the indicated concentration of LY294002 for 5 min at 25°C in PI kinase assay buffer lacking ATP. The reactions were initiated by the addition of ATP and were incubated at 25°C for 5 min. The labeled lipids were extracted with chloroform:methanol and separated by TLC on Silica gel 60 plates as described in Figure 5A. The product of Vps34p [PI(3)P] is indicated. **B**. The effect of LY294002 on Vps34p protein kinase activity. Vps34p was immunoprecipitated under native conditions, incubated with the indicated concentrations of LY294002 and subjected to an *in vitro* protein kinase assay as described in Figure 5B. Labeled Vps34p was analyzed by SDS-PAGE and autoradiography.

A.



[LY294002] 0 2 10 50 200 μM

B.



[LY294002] 0 2 10 50 200 μM

Figure 7. Vps34p autophosphorylation does not significantly affect PI 3-kinase activity. **A**. The effect of Vps34p phosphorylation on PI 3-kinase activity. Vps34p was isolated by native imunoprecipitation from a Δ*vps15* strain containing *VPS34* on a multicopy plasmid. The immunoprecipitates were incubated with 10 mM unlabeled ATP for the indicated times in the presence of 10 mM $MnCl_2$ to promote the autophosphorylation of Vps34p. The samples were then washed to remove excess ATP and subjected to a standard PI 3-kinase assay as described in Experimental Procedures. The labeled lipids were extracted and separated by TLC on Silica gel 60 plates (Walsh et al., 1991). **B**. The effect of dephosphorylation of Vps34p on PI 3-kinase activity. Immunoprecipitated Vps34p was incubated with potato acid phosphatase (PAP) for 15 min at 30°C. The phosphatase was then washed away and the dephosphorylated Vps34p was subjected to a PI 3-kinase asssay. The reaction products were separated by TLC as described in Figure 5A. **C**. Dephosphorylation of Vps34p by potato acid phosphatase. Immunoprecipitated Vps34p was ^{32}P-labeled in an *in vitro* protein kinase assay and incubated in the presence or absence of PAP under conditions identical to those described in Figure 7B. Vps34p was analyzed by SDS-PAGE and autoradiography.

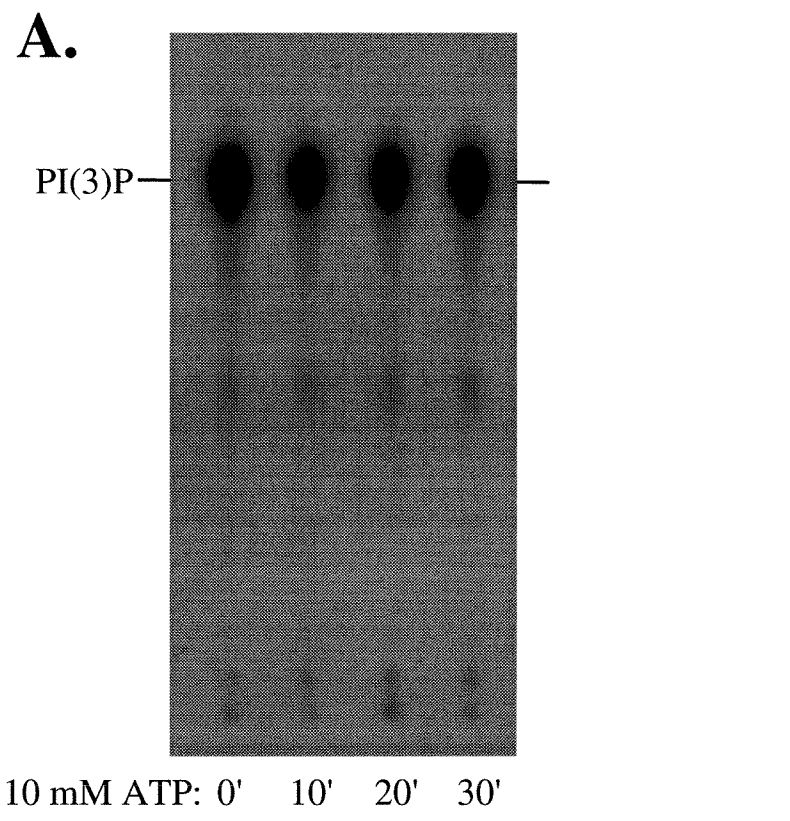

A.
PI(3)P
10 mM ATP: 0' 10' 20' 30'
B.
PI(3)P
PAP: – +
C.
Vps34p
PAP: – +

Figure 8. Vps34p is a phosphatidylinositol-specific PI 3-kinase. Substrate specificity of Vps34p. Partially purified preparations of Vps34p alone (Vps34p) or the Vps15p-Vps34p complex (Vps34p+Vps15p) were incubated with different phosphoinositide substrates in a PI kinase assay in the presence of phosphatidylserine carrier. Substrates tested were phosphatidylinositol (PI), phosphatidylinositol (4) phosphate (PIP), and phosphatidylinositol (4,5) bisphosphate (PIP_2). The reaction products were extracted and separated on oxalate-treated Silica gel 60 plates developed in a propanol:acetic acid system (Auger et al., 1992). The Vps34p alone reactions contain approximately 20-fold more enzyme than the Vps34p-Vps15p reactions; however, all reactions were performed under conditions yielding linear incorporation of label into substrate. The positions of the standards were determined by iodine vapor staining of unlabeled phosphoinositides.

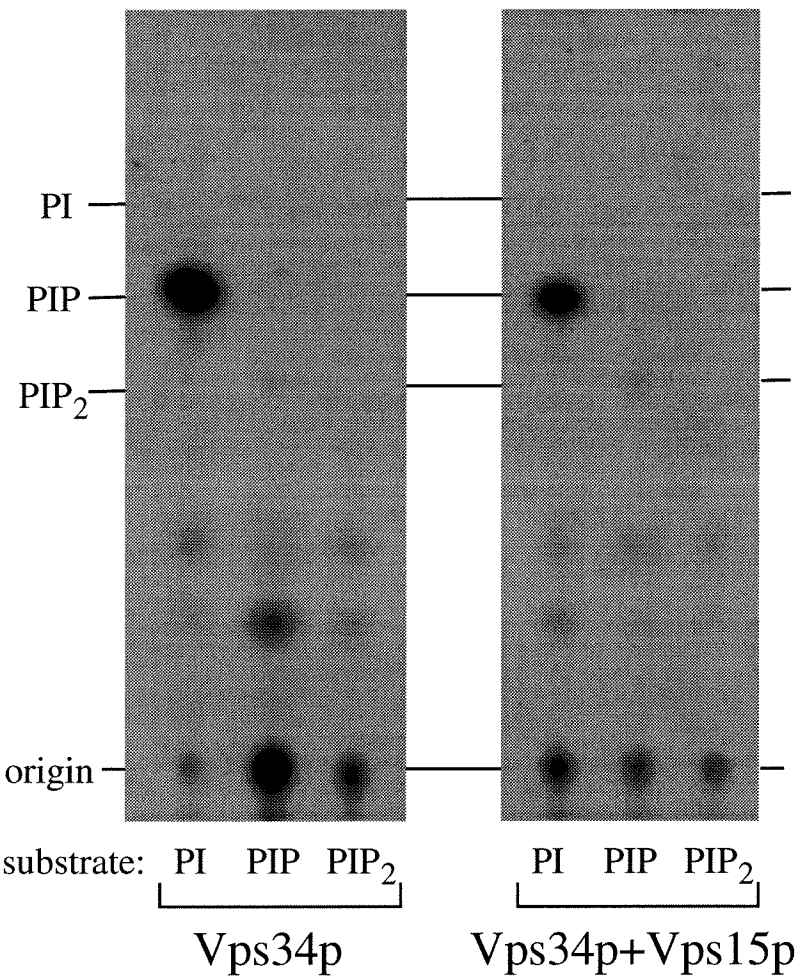
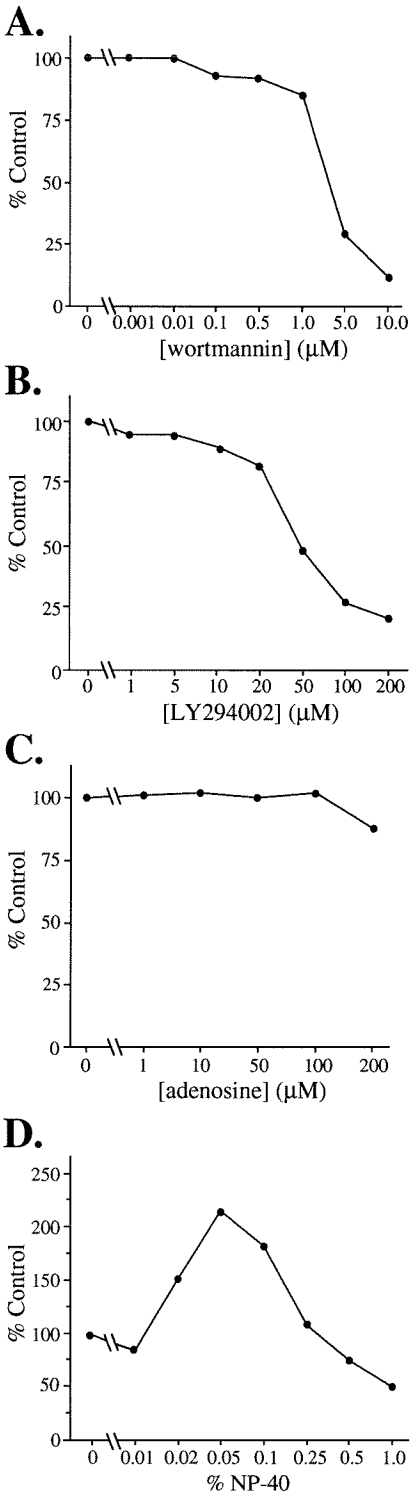

PI
PIP
PIP2
origin
substrate: PI PIP PIP2
PI PIP PIP2
Vps34p
Vps34p+Vps15p

Figure 9. Biochemical properties of Vps34p PtdIns 3-kinase activity. **A**. Inhibition of Vps34p PtdIns 3-kinase activity in the presence of wortmannin. Partially purified preparations of Vps34p were incubated in wortmannin for 5 min prior to performing a PI kinase assay as described in Figure 5A. The reaction products were analyzed by TLC on Silica gel 60 plates developed in a borate buffer system (Walsh et al., 1991). The incorporation of $^{32}PO_4$ into PtdIns was quantitated either by use of a Phosphorimager or by scraping the labeled lipids from the plate and quantitating by liquid scintillation counting. **B**. Inhibition of Vps34p PtdIns 3-kinase activity by LY294002. Partially purified preparations of Vps34p were incubated with various concentrations of LY294002 for 5 min prior to performing a PI kinase assay. Analysis of the labeled phospholipids was as described above. **C**. Effect of adenosine on Vps34p PI 3-kinase activity. Partially purified preparations of Vps34p were incubated with adenosine and subjected to a PI kinase assay as described above. Analysis of the reaction products was as described above. **D**. Effect of the detergent NP-40 on Vps34p PI 3-kinase activity. The assay mixture including PtdIns and NP-40 but excluding enzyme was sonicated together prior to addition of Vps34p and ATP. The products of the PI kinase assay were analyzed as described above.


A.
% Control
100
75
50
25
0
0 0.001 0.01 0.1 0.5 1.0 5.0 10.0
[wortmannin] (μM)
B.
% Control
100
75
50
25
0
0 1 5 10 20 50 100 200
[LY294002] (μM)
C.
% Control
100
75
50
25
0
0 1 10 50 100 200
[adenosine] (μM)
D.
% Control
250
200
150
100
50
0
0 0.01 0.02 0.05 0.1 0.25 0.5 1.0
% NP-40

Chapter 7:

Molecular and Genetic Dissection of the Regulatory Interactions Between the Vps15 Protein Kinase and the Vps34 PI 3-kinase and Demonstration of a Direct Role for PI 3-Kinase Activity in Yeast Vacuolar Protein Sorting

Submitted to the Journal of Cell Biology

Molecular and Genetic Dissection of the Regulatory Interactions Between the Vps15 Protein Kinase and the Vps34 PI 3-kinase and Demonstration of a Direct Role for PI 3-Kinase Activity in Yeast Vacuolar Protein Sorting

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Running title: Vps15p and Vps34p in yeast protein sorting

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Abstract

A membrane-associated complex of the Vps15 protein kinase and the Vps34 phosphatidylinositol 3-kinase (PtdIns 3-kinase) is required for the delivery of proteins to the yeast vacuole. Association with Vps15p recruits Vps34p to the membrane and results in the stimulation of Vps34p PtdIns 3-kinase activity. Analysis of a temperature-conditional allele of *VPS34* demonstrates that inactivation of Vps34p leads to the immediate missorting of vacuolar proteins. This rapid block in vacuolar protein sorting appears to be the result of loss of PtdIns 3-kinase activity as cellular PtdIns(3)P levels decrease dramatically in *vps34^{tsf}* cells incubated at the non-permissive temperature. Mutations altering highly conserved residues in the lipid kinase domain of Vps34p lead to a dominant-negative phenotype due to titration of activating Vps15 proteins. In contrast, catalytically inactive Vps15p mutants do not produce a dominant mutant phenotype because they are unable to associate with Vps34p in a wild-type manner. These data indicate that Vps15p protein kinase activity is required for the association with and subsequent activation of Vps34p. This regulatory relationship between Vps15p and Vps34p is also demonstrated by use of a temperature-conditional allele of *vps15* in which a shift to the non-permissive temperature leads to a decrease in PtdIns(3)P levels in a manner similar to that observed in the *vps34^{tsf}* strain. Finally, analysis of the defects in cellular PtdIns(3)P levels in various *vps15* and *vps34* mutant strains has led to additional insights into the importance of

PtdIns(3)P localization and the roles for Vps15p and Vps34p in vacuolar protein sorting.

Introduction

The accurate and efficient delivery of proteins to intracellular organelles is essential to establish and maintain the functional integrity of these compartments. Proteins destined for the mammalian lysosome or the yeast vacuole are transported through the early stages of the secretory pathway from the endoplasmic reticulum to the Golgi complex (Kornfeld and Mellman, 1989; Klionsky et al., 1990). In a late Golgi compartment, lysosomal/vacuolar proteins are sorted away from proteins headed to the cell surface in a process that requires a functional lysosomal/vacuolar targeting signal. In mammalian cells, the major lysosomal sorting signal is the mannose-6-phosphate modification present on soluble lysosomal proteins (Kornfeld and Mellman, 1989; Kornfeld, 1992). Lysosomal proteins containing phosphomannosyl residues bind to specific mannose-6-phosphate receptors in the late Golgi and are delivered to the lysosome via an endosomal intermediate. In contrast, the delivery of proteins to the yeast vacuole does not involve modification of carbohydrate residues. Instead, the targeting signal is found within the amino acid sequence of vacuolar proteins. For example, the sorting signal for the vacuolar hydrolase carboxypeptidase Y (CPY) has been mapped to a short peptide sequence at its N-terminus (Johnson et al., 1987; Valls et al., 1987; Valls et al., 1990). The recent identification of a transmembrane sorting receptor for CPY indicates that, like lysosomal protein sorting, the delivery of proteins to the vacuole in yeast is a receptor-mediated process (Marcusson et al., 1994).

Genetic selections in *Saccharomyces cerevisiae* have identified a large number of mutants that are specifically defective in vacuolar protein sorting (Bankaitis et al., 1986; Rothman and Stevens, 1986; Robinson et al., 1988; Rothman et al., 1989). Instead of delivering proteins to the vacuole, these *vps* (*v*acuolar *p*rotein *s*orting defective) mutants missort and secrete vacuolar proteins such as CPY, proteinase A (PrA) and proteinase B (PrB) as their Golgi-modified precursors. Genetic analyses of the *vps* mutants has indicated that they define >40 complementation groups, indicating that the efficient delivery of proteins to the vacuole is a complex process (Klionsky et al., 1990; Raymond et al., 1992). Characterization of the products of the *VPS* genes has provided considerable insight into the molecules and mechanisms involved in the regulated delivery of proteins to the vacuole. Analyses of the *VPS15* and *VPS34* genes have indicated that they encode homologs of a serine/threonine protein kinase and a phosphatidylinositol 3-kinase (PtdIns 3-kinase), respectively, suggesting that protein and phospholipid phosphorylation events are required for vacuolar protein sorting (Herman and Emr, 1990; Herman et al., 1991a; Hiles et al., 1992).

Mutations in the *VPS15* gene that alter residues in the Vps15 protein (Vps15p) that are highly conserved among protein kinases result in its functional inactivation. These mutations eliminate Vps15p protein kinase activity and the mutant strains missort multiple vacuolar proteins (Herman et al., 1991a; Herman et al., 1991b; Stack and Emr, 1994). In addition, truncation of as little as 30 amino acids from the C-terminus of Vps15p results in a temperature-conditional vacuolar protein sorting defect (Herman et al., 1991b). A shift to the non-permissive temperature

in *vps15ΔC30* cells causes an immediate but reversible defect in the sorting of soluble vacuolar proteins. The extremely rapid onset of the sorting defect in the *vps15ΔC30* strain indicates that Vps15p is directly involved in the delivery of soluble proteins to the vacuole.

The product of the *VPS34* gene shares extensive sequence similarity with the p110 catalytic subunit of mammalian phosphoinositide 3-kinase (PI 3-kinase; Herman and Emr, 1990; Hiles et al., 1992). In mammalian cells, PI 3-kinase phosphorylates membrane PtdIns and its more highly phosphorylated derivatives [PtdIns(4)P and PtdIns(4,5)P_2] and these products have been postulated to serve as second messenger molecules important in regulating cell growth and proliferation (Auger et al., 1989; Cantley et al., 1991; Soltoff et al., 1992). *S. cerevisiae* has been shown to contain PtdIns 3-kinase activity and strains deleted for the *VPS34* gene are extremely defective for this activity (Auger et al., 1989; Schu et al., 1993). Alteration of residues in the lipid kinase domain of Vps34p that are conserved between Vps34p and p110 results in severe defects in both PtdIns 3-kinase activity and vacuolar protein sorting (Schu et al., 1993). These data indicate that Vps34p is a functional PtdIns 3-kinase and suggest that Vps34p-mediated phosphorylation of membrane PtdIns is required for vacuolar protein sorting in yeast. Biochemical characterization of Vps34p PI 3-kinase activity has shown that, unlike mammalian p110, it is only able to utilize PtdIns as a substrate and is inactive toward PtdIns(4)P and PtdIns(4,5)P_2 (Stack and Emr, 1994). The substrate specificity and other biochemical properties of its PI 3-kinase activity suggest that Vps34p may be similar to a PtdIns-specific 3-kinase activity recently characterized from mammalian cells (Stack and Emr, 1994; Stephens et al., 1994). On the basis

of the role for Vps34p in vacuolar protein sorting, we have proposed that production of a specific phosphoinositide, PtdIns(3)P, is involved in regulating intracellular protein sorting reactions in eukaryotic cells (Stack and Emr, 1994).

Vps15p and Vps34p have been shown by genetic and biochemical criteria to interact as a membrane-associated complex. Overproduction of Vps34p will suppress the vacuolar protein sorting defects of *vps15* protein kinase domain mutants suggesting a functional interaction between the two proteins (Stack et al., 1993). Sucrose gradient and chemical cross-linking experiments demonstrated that Vps15p and Vps34p form a complex that is associated with the cytoplasmic face of an intracellular membrane fraction most likely corresponding to a late Golgi compartment. In addition to recruiting Vps34p to the membrane, Vps15p also serves to activate Vps34p as PtdIns 3-kinase activity is defective in *vps15* mutant strains (Stack et al., 1993). Therefore, Vps15p and Vps34p appear to act within a membrane-associated complex to facilitate the delivery of proteins to the vacuole in yeast. In this study, we took a genetic approach in order to investigate the regulatory role for Vps15p in the activation of Vps34p. It was found that catalytically inactive forms of Vps34p will act in a dominant-negative manner by titrating functional Vps15 proteins, leading to defects in vacuolar protein sorting and PtdIns 3-kinase activity. Analysis of kinase-defective Vps15p mutants has shown that Vps15p protein kinase activity is required for the association with and subsequent activation of Vps34p. In addition, we have generated a temperature-conditional allele of *VPS34* which demonstrates that Vps34p PtdIns 3-kinase activity is directly involved in vacuolar protein sorting.

Finally, analysis of the *in vivo* levels of the product of Vps34p, PtdIns(3)P, has provided insight into the role for Vps15p in activation of Vps34p and on the functional significance of cellular PtdIns(3)P levels in vacuolar protein sorting.

Materials and Methods

Strains, Plasmids, Media and Yeast Genetic Methods

S. cerevisiae strains used were SEY6210 (*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*; Robinson et al., 1988), PHY102 (SEY6210 *vps34Δ1::TRP1*; Herman and Emr, 1990), BHY10 (SEY6210 *leu2-3,112*::pBHY11[*CPY-Inv LEU2*]; Horazdovsky et al., 1994), KYT214 (BHY10 *vps34Δ1::HIS3*) and PHY112 (SEY6210 *vps15Δ1::HIS3*; Herman et al., 1991a). Plasmids containing *VPS15* and *VPS34* point mutations were described previously (Herman et al., 1991a; Herman et al., 1991b; Schu et al., 1993). Standard yeast (Sherman et al., 1979) and *E. coli* (Miller, 1972) media were used and supplemented as needed. Standard yeast genetic methods were used throughout (Sherman et al., 1979). Yeast cells were transformed using the alkali cation treatment method (Ito et al., 1983) and transformants were selected on the appropriate synthetic glucose media.

PCR Mutagenesis and Screening for *vps34*^{tsf} allele

The temperature-conditional allele of *VPS34* was generated by random polymerase chain reaction (PCR) mutagenesis (Muhlrad et al., 1992). A 3′ portion of the *VPS34* gene was synthesized in the presence of $MnCl_2$ and limiting dATP in order to decrease the fidelity of Taq polymerase (Perkin-Elmer). The oligonucleotide primers used in this reaction annealed 100 nucleotides upstream of the 5′ SpeI site in *VPS34* and 100 nucleotides downstream of the 3′ SpeI site in *VPS34* and amplified an 800 base pair fragment. Standard reaction conditions were used with the modifications

of 0.1 mM $MnCl_2$ and 50 mM dATP. An acceptor plasmid was constructed by digesting a low copy number plasmid (*VPS34 CEN URA3*) containing the *VPS34* gene with SpeI to create a deletion slightly smaller than the mutagenized DNA (Fig. 1A). Equimolar amounts of the gel-purified acceptor plasmid and mutagenized DNA were cotransformed into KYT214 (*Δvps34, CPY-Inv*). Transformants were selected on minimal yeast plates, replica plated to YP-fructose plates and incubated at 26°C (permissive temperature) or 37°C (non-permissive temperature). Screening for *vps34^{tsf}* mutants was accomplished using an overlay assay to detect invertase enzymatic activity (Paravicini et al., 1992). Mutants which only secreted the CPY-invertase fusion protein at the non-permissive temperature were selected. Plasmids containing candidate *vps34^{tsf}* alleles were isolated from the strain and retransformed into PHY102 (*Δvps34*) and rescreened by a pulse-chase experiment to assess CPY sorting at the permissive and non-permissive temperatures.

Cell Labeling and Immunoprecipitation

For analysis of CPY processing, whole yeast cells were labeled essentially as described (Herman and Emr, 1990). Cells were pulse-labeled with Expres^{35}S-label (NEN Radiochemicals) for 10 minutes at 30°C and chased for 30 min at 30°C by the addition of methionine and cysteine to 2 mM. The media contained bovine serum albumin (1 mg/ml) and α_2-macroglobulin (10 μg/ml; Boehringer Mannheim) to stabilize secreted proteins. Following the chase, an equal volume of cold 2X stop buffer (2 M sorbitol, 50 mM Tris-HCl, pH 7.5, 40 mM NaF, 40 mM NaN_3, 20 mM DTT) was added and the cultures were incubated on ice for 5 min. Zymolyase-

100T (Seikagako Kogyo Co; Tokyo, Japan) was added to 20 μg/ml and the cells were incubated at 30°C for 25 min. The culture was separated into intracellular and extracellular fractions by centrifugation at 4000 x g for 30 sec and the proteins were precipitated by the addition of trichloroacetic acid (TCA) to a final concentration of 5%. Immunoprecipitation of CPY and ALP was as described previously (Herman and Emr, 1990) and samples were electrophoresed on 9% SDS-polyacrylamide gels. Following electrophoresis, the gels were fixed in 40% methanol, 10% acetic acid and treated with 1.0 M sodium salicylate, 1% glycerol, then dried and subjected to autoradiography.

In Vivo Labeling and HPLC Analysis of Phosphoinositides

For analysis of cellular phosphoinositides, yeast cells were grown for ~16 hr at 26°C in minimal media lacking inositol and including 5μCi/ml [^{3}H]-*myo*-inositol (18.8 Ci/mmol; Amersham). For temperature-shift experiments, the labeled cells were centrifuged and resuspended in YPD media that had been pre-warmed to the appropriate temperature. Samples were collected by rapid centrifugation of the cells and resuspended in 0.5 ml 1.0 M HCl. 1.0 ml of chloroform:methanol (1:1) was added and the cells were lysed by vortexing vigorously in the presence of glass beads. The organic phase was dried down and the labeled lipids were deacylated essentially as described (Serunian et al., 1991). The pellet was resuspended in 1.0 ml methylamine reagent (0.428 ml 25% methylamine, 0.457 ml methanol, 0.114 ml n-butanol) and incubated at 53°C for 50 min. The deacylated lipids were dried down in a Speed-vac and lyophilized several times from water. The resulting pellet was resuspended in 0.3 ml of water

and extracted with 0.3 ml of butanol:ether:ethyl formate (20:4:1) to remove the acyl groups. The aqueous phase was dried down and resuspended in 50 µl of water. The resulting glycerophosphoinositols were separated by HPLC on a Beckman System Gold using a 25 cm Partisil 5 SAX column (Whatman). The column was developed with a gradient of $(NH_4)_2PO_4$, pH 3.8 generated as follows: 10 mM for 5 min, 10 mM to 125 mM over 40 min, and 125 mM to 1.0 M over 10 min; the flow rate was 1.0 ml/min. The column was calibrated using ^{32}P-labeled glycerophosphoinositols generated in an *in vitro* PI 3-kinase assay. In addition, each sample was spiked with unlabeled AMP and ADP and their elution was monitored with a UV absorbance detector in order to assess column performance. 0.3 ml fractions were collected and counted in a Beckman LC6000IC scintillation counter using Cytoscint (ICN Radiochemicals) scintillation fluid.

PtdIns 3-Kinase Assays

Yeast spheroplasts were resuspended in 0.1 M KCl, 15 mM HEPES, pH 7.5, 3 mM EGTA, 10% glycerol at 15-20 OD_{600}/ml and vortexed in the presence of 0.25 mm glass beads and protease inhibitors. The lysates were centrifuged at 750 x g for 5 min at 4°C to generate a crude lysate. The lysate was frozen in a dry ice-ethanol bath and stored at -80°C until use. Approximately 0.05 OD_{600} equivalents (<4 µg protein) were assayed for PtdIns 3-kinase activity as described (Whitman et al., 1988; Schu et al., 1993). The 50 µl reactions were performed in 20 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 0.2 mg/ml sonicated PtdIns, 60 µM ATP and 0.2 mCi/ml $\gamma[^{32}P]$-ATP. The reactions were incubated at 25°C for 5 min and were terminated

by the addition of 80 µl 1 M HCl. The lipids were extracted with 160 µl chloroform/methanol (1:1) and the organic phase was dried down and stored at -80°C. Labeled samples dissolved in chloroform were spotted onto Silica gel 60 TLC plates (Merck) and developed in a borate buffer system (Walsh et al., 1991). Labeled species were detected by autoradiography.

Cross-Linking of Yeast Cell Extracts

Immunoprecipitation and cross-linking of yeast extracts was as previously described (Stack et al., 1993). Yeast strains were grown to mid-logarithmic phase, converted to spheroplasts, labeled with Expres^{35}S-label for 30 min at 30°C, and chased for one hour at 30°C by adding methionine and cysteine to 2 mM and yeast extract to 0.2%. Labeled spheroplasts were resuspended in XL lysis buffer (1.2 M sorbitol, 0.1 M KH_2PO_4, pH 7.5, 5 mM EDTA) at 10-20 OD_{600} units/ml. Cells were lysed by the addition of 4 volumes of H_2O. All solutions contained the protease inhibitors antipain, leupeptin, chymostatin, pepstatin (all at 2 µg/ml), aprotinin (0.1 TIU/ml), phenylmethylsulfonyl fluoride (100 µg/ml), and α_2-macroglobulin (10 µg/ml). DSP [dithio*bis*(succinimidylpropionate); Pierce Chemicals] cross-linker, dissolved in dimethylsulfoxide (DMSO), was added to a final concentration of 200µg/ml. Control samples without cross-linker received DMSO alone. The extracts were incubated at room temperature for 30 min after which the reaction was quenched by the addition of 1 M hydroxylamine to a final concentration of 20 mM. Proteins were precipitated by the addition of TCA to 5%. The TCA pellets were resuspended in urea-cracking buffer (50 mM Tris-HCl, pH 7.2, 6 M urea,

1% SDS) without reducing agent and processed for immunoprecipitation using anti-Vps15p antisera. Following the first immunoprecipitation, the cross-linked samples were solubilized in urea-cracking buffer with or without 2% 2-mercaptoethanol and re-immunoprecipitated with the appropriate antisera. Control experiments demonstrated that anti-Vps15p antisera was irreversibly denatured by incubation with urea-cracking buffer in the presence or absence of reducing agent (not shown). The final samples were solubilized in urea-cracking buffer containing 2% 2-mercaptoethanol and electrophoresed on 8%-polyacrylamide gels.

Results

A temperature-sensitive allele of *VPS34* exhibits a rapid defect in the sorting of soluble vacuolar proteins

Mutational analyses of Vps34p have suggested that PtdIns 3-kinase activity is involved in the delivery of proteins to the vacuole (Schu et al., 1993). We generated an allele of *VPS34* that is temperature-sensitive for protein sorting in order to test a role for Vps34p PtdIns 3-kinase activity in vacuolar protein delivery. The *VPS34* gene was mutagenized by polymerase chain reaction (PCR) amplification under error-prone conditions. Primers were chosen that would amplify a region of *VPS34* that corresponds to approximately the C-terminal one-third of Vps34p which contains the regions of highest sequence similarity to mammalian PI 3-kinase, including the putative lipid kinase domain. The mutagenized DNA was introduced into a yeast strain by use of a "gapped" plasmid repair method (Fig. 1A). In this technique, the amplified mutagenized DNA is cotransformed into yeast together with a low copy number vector containing the *VPS34* gene which contains a deletion corresponding to a region slightly smaller than the amplified DNA. The active recombination system of yeast will efficiently repair the gapped plasmid using the overlapping mutagenized DNA to generate an intact, mutagenized *VPS34* gene on a low copy number plasmid. Plasmids that recircularize without incorporating the mutagenized DNA will generate *vps34* null alleles due to the lack of the gapped region (data not shown). The amplified mutagenized DNA and the gapped plasmid were transformed into a Δ*vps34* strain which contains a CPY-invertase fusion

construct whose product has been shown to be correctly sorted to the vacuole in wild-type strains and is missorted to the cell surface in *vps* mutant strains (Bankaitis et al., 1986; Johnson et al., 1987). A colorimetric assay based on secreted invertase activity (Paravicini et al., 1992) was used to screen for yeast transformants which exhibited wild-type sorting of the CPY-Inv fusion at 26°C and missorted the fusion to the cell surface at 37°C. Analysis of 50,000 yeast transformants yielded 15 candidate *vps34^{tsf}* (tsf: temperature-sensitive for function) alleles. The plasmids containing several of these putative *vps34^{tsf}* alleles were isolated and reintroduced into a Δ*vps34* strain. These mutant alleles were characterized for vacuolar protein sorting by analyzing wild-type CPY. The yeast strains were preincubated for 5 min at permissive (26°C) or non-permissive (37°C) temperature, pulse-labeled with Expres^{35}S-label for 10 min and chased with unlabeled methionine and cysteine for 30 min at the respective temperatures. The labeled cultures were separated into pellet and supernatant fractions and then subjected to immunoprecipitation using CPY-specific antisera. In wild-type cells incubated at either 26°C or 37°C, >95% of the newly synthesized CPY was present inside the cell as the 61 kd mature form indicating delivery to and processing in the vacuole (Fig. 1B). In contrast, a Δ*vps34* strain missorts and secretes CPY as the Golgi-modified p2 precursor at either temperature (Herman and Emr, 1990). The *vps34^{tsf}* allele incubated at 26°C is very similar to wild-type cells, as the great majority of CPY is present inside the cell as the mature vacuolar form (Fig. 1B). Preincubation for 5 min at 37°C resulted in the functional inactivation of this mutant Vps34 protein; *vps34^{tsf}* cells missort and secrete p2CPY at 37°C in a manner similar to a Δ*vps34* strain. The

temperature-conditional sorting phenotype of *vps34^{tsf}* cells was not restricted to CPY. Analysis of two other soluble (lumenal) vacuolar hydrolases, proteinase A (PrA) and proteinase B (PrB), showed that they were also selectively missorted and secreted at the non-permissive temperature (data not shown). The fate of the vacuolar membrane protein alkaline phosphatase (ALP) was analyzed to determine if the sorting defect of *vps34^{tsf}* cells is restricted to soluble vacuolar proteins. A temperature-shift pulse-chase experiment similar to that used for CPY showed that *vps34^{tsf}* cells matured ALP at both 26 °C and 37°C in a manner indistinguishable from wild-type cells (Fig. 1C). The efficient processing of ALP in *vps34^{tsf}* cells indicates that this membrane protein has been delivered to the vacuole as the proteolytic event generating mALP is known to require vacuolar PrA (Klionsky and Emr, 1989). In addition, the processing of ALP indicates that the vacuoles of *vps34^{tsf}* cells are competent for proteolytic processing at 37°C. Collectively, these data demonstrate that *vps34^{tsf}* cells exhibit pleiotropic, temperature-sensitive defects in the delivery of soluble but not membrane vacuolar proteins.

vps34^{tsf} allele is temperature-sensitive for PtdIns 3-kinase activity

The rapid onset of the CPY sorting defect of the *vps34^{tsf}* allele argues that Vps34p is directly involved in the delivery of proteins to the vacuole. Vps34p has been shown to possess PtdIns 3-kinase activity and mutations altering residues in the lipid kinase domain are defective for CPY localization suggesting a role for PtdIns 3-kinase activity in vacuolar protein sorting (Schu et al., 1993). Vps34p also appears to represent the major, if not sole, PI 3-kinase activity in yeast as *in vivo* labeling with [^{3}H]-

inositol shows that Δ*vps34* strains lack detectable PtdIns(3)P (Schu et al., 1993). To determine if Vps34p PtdIns 3-kinase activity is directly involved in vacuolar protein sorting, we examined PtdIns(3)P levels in the *vps34*^{tsf} allele incubated at the non-permissive temperature. To accomplish this, *vps34*^{tsf} cells were labeled to steady state with ^{3}H-*myo*-inositol. The labeled cells were then shifted to 37°C and samples were taken at 0, 10, 30 and 60 min following temperature shift. The cells were lysed and lipids extracted by vortexing in acidified chloroform:methanol in the presence of glass beads. The extracted lipids were deacylated with methylamine and acyl groups removed by organic extraction. The deacylated lipids were separated by HPLC using an elution profile that optimizes the separation between the deacylated derivatives of PtdIns(3)P and PtdIns(4)P. These analyses demonstrated that shifting *vps34*^{tsf} cells to the non-permissive temperature of 37°C resulted in a rapid decrease in cellular PtdIns(3)P levels (Fig. 2). These data indicate that *vps34*^{tsf} cells are conditional for both CPY sorting and PtdIns 3-kinase activity and suggest that the CPY sorting defect observed when *vps34*^{tsf} cells are shifted to the non-permissive temperature is the result of loss of Vps34p PtdIns 3-kinase activity. Furthermore, for the first time, these data directly implicate PI 3-kinase activity in the sorting of proteins in eukaryotic cells.

vps15^{tsf} allele results in temperature-conditional Vps34p PtdIns 3-kinase activity

The preceding sections show that Vps34p PtdIns 3-kinase activity is required for the sorting of vacuolar proteins. Vps34p has been shown to be present *in vivo* in a complex with the Vps15 protein kinase. Vps15p

serves to recruit Vps34p to the site of its membrane substrate and Vps15p is required for the activation of the Vps34 PtdIns 3-kinase as *vps15* mutants are very defective for PtdIns 3-kinase activity *in vitro* (Stack et al., 1993). These data have indicated that the functionally active form of Vps34 is in association with Vps15p.

It has been shown that deletions from the C-terminus of Vps15p of as little as 30 amino acids result in selective temperature-sensitive defects in the sorting of soluble vacuolar proteins (Herman et al., 1991b). The similarity in phenotypes of the *vps15ΔC30* and *vps34^{tsf}* alleles and the requirement of Vps15p for stimulation of Vps34p PtdIns 3-kinase activity suggest that the *vps15ΔC30 tsf* allele may be specifically defective in the activation of Vps34p. To test this possibility, we labeled *vps15ΔC30* cells with [^{3}H]-inositol and shifted them to the non-permissive temperature of 38°C for 30 min. The labeled lipids were then extracted, deacylated and separated by HPLC. We found that incubation of the *vps15ΔC30* strain at 38°C resulted in a significant decrease in cellular PtdIns(3)P levels in a manner very similar to that obtained with the *vps34^{tsf}* allele (Fig. 3). The correlation between CPY sorting and PtdIns(3)P levels in both the *vps15ΔC30* and *vps34^{tsf}* alleles further demonstrates the involvement of PtdIns 3-kinase activity in the sorting of vacuolar proteins. In addition, the analysis of the *vps15ΔC30* allele indicates that the loss of Vps15p function results in the subsequent inactivation of the Vps34 PtdIns 3-kinase and provides further evidence of the regulatory relationship between Vps15p and Vps34p.

Catalytically inactive Vps34p results in a dominant negative mutant phenotype

Studies of Vps34p lipid kinase domain mutants (Schu et al., 1993) and the *vps34*^{tsf} allele (Fig. 1) demonstrate that PtdIns 3-kinase activity is required for vacuolar protein sorting. Therefore, we performed a thorough genetic analysis of the phenotypic consequences of loss of Vps34p PtdIns 3-kinase activity. In order to test for an effect due to overproducing kinase-defective Vps34 proteins, we transformed wild-type and Δ*vps34* strains with multicopy plasmids containing mutant alleles of *VPS34*. The alleles tested contained mutations altering highly conserved residues in the lipid kinase domain of Vps34p and have been previously shown to be extremely defective for both CPY sorting and *in vitro* PtdIns 3-kinase activity when present on a low copy plasmid in a Δ*vps34* strain (Schu et al., 1993). Overproduction of the mutant proteins was unable to even partially complement the CPY sorting defects associated with *vps34* mutant cells; a Δ*vps34* strain harboring either the *N736K* or the *D749E* *vps34* alleles on a multicopy plasmid missorted CPY as the Golgi-modified p2 precursor (Fig. 4A). These data demonstrate that these mutants proteins are completely non-functional for vacuolar protein sorting. These proteins are also extremely defective for PtdIns 3-kinase activity *in vitro*. Extracts from a Δ*vps34* strain containing either mutant allele on a multicopy plasmid were assayed by incubating with Mg^{2+}, phosphatidylinositol and γ[^{32}P]-ATP. The reactions were incubated for 5 min at 25°C after which the reaction products were extracted and analyzed by thin layer chromatography on Silica gel 60 plates developed in a borate buffer system (Walsh et al., 1991). Extracts from a Δ*vps34* strain containing

either the *N736K* or the *D749E* allele on a multicopy plasmid were completely defective for PtdIns 3-kinase activity *in vitro* and were indistinguishable from extracts of Δ*vps34* cells (Fig. 4B). This result is particularly striking given the 20-30-fold overexpression of the mutant proteins (data not shown) and emphasizes that they are catalytically inactive.

To test for a possible dominant negative effect of overproducing catalytically inactive Vps34 proteins, we transformed the *N736K* or *D749E* alleles on multicopy plasmids into a wild-type strain. CPY was analyzed by a pulse-chase experiment to assay for vacuolar protein sorting in these strains. It has been previously shown that overproducing wild-type Vps34p had no dominant mutant effects; CPY was present inside the cell as the mCPY form in a wild-type strain containing the *VPS34* gene on a multicopy plasmid (Herman and Emr, 1990). In contrast, overproducing the catalytically inactive N736K or D749E Vps34p mutants in a wild-type strain resulted in a dominant mutant phenotype as approximately 50% of newly synthesized CPY was missorted and secreted from the cell as the p2 precursor (Fig. 4A). This phenotype appears to be due to dominant interference with the function of wild-type Vps34p present in this strain because overproduction of wild-type Vps34p suppressed the dominant mutant phenotype (data not shown). As Vps34p has been shown to exist in a complex with Vps15p and Vps15p appears to regulate Vps34p PtdIns 3-kinase activity (Stack et al., 1993), we tested whether the dominant mutant effect involves Vps15p. Indeed, the dominant interference also appears to involve Vps15p as overproduction of Vps15p suppressed the dominant-

negative phenotype associated with strains overproducing catalytically inactive forms of Vps34p (data not shown; also, see below).

Cellular PtdIns 3-kinase activity has been shown to correlate tightly with CPY sorting (Schu et al., 1993; Figs. 1-3). Therefore, we examined *in vitro* PtdIns 3-kinase activity in extracts prepared from wild-type strains overproducing the N736K and D749E forms of Vps34p. TLC analysis of the reaction products showed that PtdIns 3-kinase activity was significantly decreased relative to a wild-type strain (Fig. 4B). These data suggest that the dominant CPY missorting phenotype observed in wild-type strains overproducing catalytically inactive Vps34p is the result of a decrease in the PtdIns 3-kinase activity associated with wild-type Vps34p. This interpretation is also consistent with an involvement of Vps15p in the dominant mutant phenotype because of the role for Vps15p in regulating Vps34p PtdIns 3-kinase activity.

Kinase-defective Vps15p does not produce a dominant mutant phenotype

The results with the N736K and D749E Vps34p mutants demonstrated that the presence of a catalytically inactive form of Vps34p acts in a dominant mutant manner. The functional relationship between Vps15p and Vps34p suggests that a catalytically-impaired Vps15p may also produce a dominant-negative phenotype. Mutations in the *VPS15* gene corresponding to residues highly conserved among protein kinases result in severe defects in vacuolar protein sorting and Vps15p protein kinase activity (Herman et al., 1991a; Herman et al., 1991b; Stack and Emr, 1994). Therefore, these mutant alleles are good candidates to test for a dominant mutant phenotype similar to that exhibited by the Vps34p mutants.

The *D165R* and *E200R* alleles of *VPS15* have been previously shown to be severely defective for CPY sorting when present on a low copy number plasmid (Herman et al., 1991a). We tested whether overproduction of the mutant proteins would complement the CPY sorting defect of a Δ*vps15* strain. It was found that Δ*vps15* cells containing either the *D165R* or the *E200R* allele on a multicopy plasmid had a vacuolar protein sorting defect identical to a Δ*vps15* strain; Δ*vps15* cells overproducing either mutant Vps15 protein missorted and secreted >95% of newly synthesized CPY as the p2 precursor (Fig. 5A). These data indicate that the mutant proteins are unable to even partially complement the CPY sorting defect of Δ*vps15* cells even when overproduced 20-30-fold.

Defects in PtdIns 3-kinase activity of a Δ*vps15* strain containing the *vps15-E200R* allele on a low copy number plasmid have led to the suggestion that Vps15p in general and Vps15p protein kinase activity in particular are required for activation of the Vps34 PtdIns 3-kinase (Stack et al., 1993). We tested whether overproduction of the mutant Vps15 proteins could stimulate Vps34p in an *in vitro* PtdIns 3-kinase assay. Extracts from a Δ*vps15* strain harboring either the *D165R* or the *E200R* allele on a multicopy plasmid were subjected to an *in vitro* PtdIns 3-kinase assay. While PtdIns 3-kinase activity in these strains was readily detectable, it was still significantly lower than the wild-type strain (Fig. 5B). Furthermore, the severe CPY sorting defect exhibited by these strains (Fig. 5A) suggests that this activity is not functional for the localization of vacuolar proteins.

The non-functional *D165R* and *E200R* alleles were introduced into a wild-type strain to determine if they resulted in any dominant mutant

phenotypes. It has been previously shown that overproduction of wild-type Vps15p had no dominant interfering phenotype on vacuolar protein sorting; CPY was present as the mature, vacuolar form in a strain containing the wild-type *VPS15* gene on a multicopy plasmid (Herman et al., 1991a). Overproduction of either mutant Vps15 protein also did not result in a dominant mutant phenotype as >95% of newly synthesized CPY was present inside the cell as the mature form (Fig. 5A). Examination of the *in vitro* PtdIns 3-kinase activity in extracts derived from these strains showed that they also exhibited PtdIns 3-kinase levels indistinguishable from a wild-type strain (Fig. 5B). Collectively, these data indicate that, in contrast to catalytically inactive Vps34p mutants, overproduction of kinase-defective forms of Vps15p does not result in a dominant mutant phenotype.

An intact Vps15p protein kinase domain is required for association with and activation of Vps34p

The functional and physical interaction observed between Vps15p and Vps34p suggests the possibility that overproduction of a non-functional form of either protein in a wild-type strain may titrate away its partner and lead to a dominant mutant phenotype. One prediction of such a model is that the mutant protein should be able to associate with its partner with wild-type or near wild-type efficiency. We used chemical cross-linking to determine the ability of mutant Vps15 or Vps34 proteins to form a complex with its wild-type partner. The homobifunctional cross-linker dithio*bis*(succinimidylpropionate) (DSP) contains a disulfide bond between the reactive groups; therefore, treatment with reducing

agent prior to electrophoresis allows resolution of the individual components of the cross-linked complex. In these experiments, yeast spheroplasts were labeled with Expres^{35}S-label for 30 min and chased for 60 min at 30°C to approximate steady-state conditions. The labeled spheroplasts were gently lysed in a hypotonic buffer and the lysate was treated with DSP. Following treatment with cross-linker, the proteins were precipitated with TCA and subjected to quantitative immunoprecipitation under denaturing but not reducing conditions using antisera specific for Vps15p. The immunoprecipitated cross-linked proteins were then incubated in a buffer containing 2-mercaptoethanol and re-immunoprecipitated under denaturing conditions with antisera specific for Vps15p and Vps34p. This should result in the precipitation of all cellular Vps15p and only the fraction of Vps34p that is associated with Vps15p. Alternatively, the anti-Vps15p antisera-treated cross-linked samples were re-immunoprecipitated with antisera specific for Vps34p without cleaving the cross-linker with reducing agent. The remaining immunoprecipitated Vps15p and Vps34p represents only the portion of the two proteins present within a cross-linkable complex.

Use of these cross-linking and immunoprecipitation techniques has shown that Vps15p and Vps34p can be co-immunoprecipitated from cross-linked extracts derived from a wild-type strain (Stack et al., 1993; Fig. 6A). These same techniques were applied to a *Δvps34* strain containing either the *N736K* or the *D749E* mutant *vps34* allele on a low copy number plasmid. These analyses showed that the mutant proteins encoded by these alleles were able to associate with Vps15p in a manner indistinguishable from wild-type Vps34p (Fig. 6A). The N736K or D749E

mutant Vps34 proteins can also be co-immunoprecipitated with Vps15p in native immunoprecipitations of uncross-linked yeast extracts (data not shown). These data suggest that the defects in PtdIns 3-kinase activity exhibited by these mutant proteins are the result of defective catalytic activity rather than the inability to associate with activating Vps15p.

A preliminary examination of Vps15p-Vps34p complex formation using overproduced mutant proteins suggested that the Vps15p E200R mutant was able to associate with Vps34p (Stack et al., 1993). Chemical cross-linking experiments identical to those described above were used to test the ability of the D165R and E200R Vps15p mutants to form a complex with Vps34p. A Δ*vps15* strain containing either of the *vps15* mutant alleles on a low copy number plasmid was labeled, lysed and treated with DSP cross-linker. Immunoprecipitations of the cross-linked extracts showed that both the D165R and E200R Vps15p mutants were highly defective for association with Vps34p (Fig. 6B). The inability of these mutants to form a complex with Vps34p was verified in native immunoprecipitations from yeast extracts (data not shown). These results indicate that Vps15 protein kinase domain mutants are unable to associate with Vps34p and suggest that the sorting defects in *vps15* kinase domain mutant strains are due to the inability of the mutant Vps15 proteins to associate with and activate Vps34p.

In vivo analysis of PtdIns(3)P, the product of the Vps34 PtdIns 3-kinase

Mammalian PI 3-kinase has been shown to be able to utilize several different forms of phosphatidylinositol as a substrate. The p110/p85 heterodimer can use PtdIns, PtdIns(4)P or PtdIns(4,5)P_2 as a substrate in an

in vitro PI 3-kinase assay (Carpenter and Cantley, 1990; Soltoff et al., 1992). In contrast, Vps34p is only active toward PtdIns and is unable to act upon PtdIns(4)P or PtdIns(4,5)P_2 (Stack and Emr, 1994). *In vivo* labeling of yeast cells with [^{3}H]-inositol has shown that wild-type yeast strains contain abundant levels of the product of Vps34p, PtdIns(3)P, while Δ*vps34* strains contain very little, if any, PtdIns(3)P (Schu et al., 1993; Fig. 7). These data indicate that Vps34p is a phosphatidylinositol-specific PtdIns 3-kinase that represents the major, it not sole, PtdIns 3-kinase activity in yeast cells. Our analyses with Vps34p mutants have demonstrated that PtdIns 3-kinase activity is required for the sorting of yeast vacuolar proteins (Schu et al., 1993; Fig. 1). Therefore, we analyzed PtdIns(3)P levels in different mutant strains in order to investigate the relationship between cellular PtdIns(3)P levels and vacuolar protein sorting.

Various yeast strains were labeled with [^{3}H]-inositol, the labeled lipids were extracted and deacylated and the deacylated products were then separated by HPLC. Due to the fact that previous work has been unable to detect PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3 in yeast (Auger et al., 1989; Hawkins et al., 1993; Schu et al., 1993; D.B.D. and S.D.E., unpublished results), we concentrated on the region of the gradient where PtdIns(3)P and PtdIns(4)P elute. As has been shown previously (Auger et al., 1989; Schu et al., 1993), wild-type yeast cells contained levels of PtdIns(3)P equal to or exceeding PtdIns(4)P (Fig. 7). Strains deleted for *VPS34* contained wild-type levels of PtdIns(4)P; however, the region of the gradient where PtdIns(3)P elutes only exhibited background levels of counts (Fig. 7; Schu et al., 1993). *In vivo* labeling and HPLC analysis of phosphoinositides from a Δ*vps15* strain showed that PtdIns(3)P levels in this strain, while detectable, were

extremely low (Fig. 7). A Δ*vps15* strain harboring the *vps15E200R* kinase domain mutant allele on a low copy number plasmid also showed low levels of PtdIns(3)P, albeit at higher levels than the Δ*vps15* strain (Fig. 7). These data are consistent with the low levels of *in vitro* PtdIns 3-kinase activity observed in extracts from *vps15* mutant strains (Stack et al., 1993), and support the notion that Vps15p is required for the direct activation of the Vps34 PtdIns 3-kinase. We have previously shown that overproduction of Vps34p will suppress the growth and CPY sorting defects of the *vps15E200R* strain but not a Δ*vps15* strain (Stack et al., 1993). Therefore, we examined the levels of PtdIns(3)P in Δ*vps15* and *vps15E200R* strains containing the *VPS34* gene on a multicopy plasmid. Overproduction of Vps34p in either *vps15* mutant strain resulted in a substantial increase in levels of PtdIns(3)P (Fig. 7). This suggests that Vps34p has a low but detectable basal level of activity in the absence of Vps15p activation (also, see below).

In order to correlate PtdIns(3)P levels with CPY sorting, we needed to normalize PtdIns(3)P levels between the various strains. We decided to use the ratio of PtdIns(3)P to PtdIns(4)P because PtdIns(4)P levels do not appear to be affected by mutations in either *VPS15* or *VPS34* and the two phosphoinositides should have very similar extraction properties as they only differ in the position of the phosphate group on the inositol ring. The PtdIns(3)P and PtdIns(4)P values were calculated by determining the number of counts in the respective peaks. In wild-type yeast, very little of CPY is missorted and the PtdIns(3)P/PtdIns(4)P ratio was determined to be 1.30 (Table 1). In contrast, both Δ*vps34* and Δ*vps15* strains missort p2CPY and the PtdIns(3)P/PtdIns(4)P ratios were very nearly zero.

Overproduction of Vps34p in a Δ*vps15* strain resulted in a substantial increase in the levels of PtdIns(3)P (Fig. 7). The PtdIns(3)P/PtdIns(4)P ratio in this strain was approximately 30-fold higher than the ratio found in a Δ*vps15* strain (Table 1). This increase is strikingly similar to the 20-30-fold increase in Vps34p protein levels due to expression from the multicopy plasmid. These data suggest that Vps34p has a distinct basal level of activity in the absence of Vps15p. This notion is supported by the observation that Vps34p immunoisolated from a Δ*vps15* strain has readily detectable PtdIns 3-kinase activity (Stack and Emr, 1994). Despite the ~30-fold increase in PtdIns(3)P levels in the Δ*vps15* strain due to the overproduction of Vp34p, this strain missorts CPY in a manner identical to a Δ*vps15* strain (Table 1; Stack et al., 1993). The *vps15E200R* kinase domain mutant strain contained readily detectable levels of PtdIns(3)P (PtdIns(3)P/PtdIns(4)P=0.14) and overproduction of Vps34p in this strain resulted in a ~4-fold increase in the PtdIns(3)P/PtdIns(4)P ratio. Interestingly, the overproduction of Vps34p suppresses the CPY sorting defect of a *vps15E200R* strain; approximately 50% of CPY is found as the mature, vacuolar form. Comparison of the CPY sorting data and PtdIns(3)P/PtdIns(4)P ratio of Δ*vps15* and *vps15E200R* strains overproducing Vps34p suggests either that a threshold level of PtdIns(3)P must be obtained to allow sorting of CPY or that the PtdIns(3)P produced in the Δ*vps15*-2μ *VPS34* strain is not functional for vacuolar protein sorting (see below). Collectively, analyses of PtdIns(3)P levels in *vps15* and *vps34* mutant strains have demonstrated the direct involvement of PtdIns 3-kinase activity in vacuolar protein sorting and illustrated the regulatory relationship between Vps15p and Vps34p.

Discussion

Previous work has established that a membrane-associated complex of the Vps15 protein kinase and the Vps34 PtdIns 3-kinase is required for the delivery of proteins to the vacuole in yeast (Stack et al., 1993). Mutational analyses have demonstrated that alteration of residues in Vps15p and Vps34p that are conserved among protein kinases and lipid kinases, respectively, result in the functional inactivation of the proteins (Herman et al., 1991a; Herman et al., 1991b; Schu et al., 1993). In addition to a role in recruiting Vps34p to the membrane, Vps15p has also been shown to be required for activation of the Vps34 PtdIns 3-kinase (Stack et al., 1993). In the present work, we extend these mutational studies of Vps15p and Vps34p and demonstrate the direct involvement of PtdIns 3-kinase activity in the sorting of vacuolar proteins. In addition, genetic and biochemical analyses show that an intact Vps15p protein kinase domain is necessary for the association with and subsequent activation of the Vps34 PtdIns 3-kinase.

A role for PtdIns 3-kinase activity in the regulation of intracellular protein trafficking

Vps34p has been shown to possess PtdIns 3-kinase activity while strains deleted for *VPS34* or containing *vps34* lipid kinase domain mutations are extremely defective in this activity. The fact that mutant *vps34* strains that are defective for PtdIns 3-kinase activity also exhibit severe defects in vacuolar protein sorting suggests that PtdIns 3-kinase

activity is required for the localization of vacuolar proteins (Schu et al., 1993). Our work here on a temperature-conditional allele of *VPS34* indicates that Vps34p plays a direct role in the sorting of soluble vacuolar proteins. The extremely rapid CPY sorting defect exhibited by *vps34^{tsf}* cells shifted to the non-permissive temperature suggests that the product of this mutant allele is quickly inactivated at 37°C and argues that the CPY missorting phenotype is not a secondary consequence of loss of Vps34p function. The fact that the vacuolar membrane protein ALP is matured normally in *vps34^{tsf}* cells at the non-permissive temperature suggests that Vps34p is specifically required for the sorting of soluble vacuolar proteins. As Vps15p and Vps34p act together to facilitate vacuolar protein sorting, this result is consistent with analysis of a *tsf* allele of *vps15* which also shows a selective block in the sorting of soluble but not membrane vacuolar proteins (Herman et al., 1991b). Finally, the rapid decrease in cellular PtdIns(3)P levels in the *vps34^{tsf}* allele shifted to the non-permissive temperature indicates that this strain is also temperature-sensitive for PtdIns 3-kinase activity and strongly suggests that the CPY sorting defect is the direct result of the loss of PtdIns 3-kinase activity. Therefore, work with this *vps34* allele directly implicates PtdIns 3-kinase activity in the sorting of soluble vacuolar hydrolases. In addition, the decrease in PtdIns(3)P levels upon inactivation of Vps34p also indicates the presence in yeast of a phosphatase capable of dephosphorylating PtdIns(3)P. Indeed, progress has been made toward purifying an activity from yeast which has characteristics of a PtdIns(3)P phosphatase (H. Hama, S.D. Emr; unpublished observations). This suggests that vacuolar protein sorting in yeast may involve a cycle of specific phosphorylation and

dephosphorylation of phosphatidylinositol at the D-3 position of the inositol head group.

The demonstration of a direct role for PtdIns 3-kinase in regulating vacuolar protein sorting in yeast suggests the possibility that PI 3-kinase may perform a similar role in higher eukaryotic cells. In addition to a possible role in signaling cell proliferation (Cantley et al., 1991; Fantl et al., 1992; Soltoff et al., 1992), PI 3-kinase also has been recently implicated in the intracellular trafficking of cell surface growth factor receptors. Mutant colony stimulating factor (CSF) receptors that are unable to associate with PI 3-kinase are internalized but fail to be delivered to the lysosome for degradation (Downing et al., 1989; Carlberg et al., 1991). Corvera and co-workers have shown that platelet-derived growth factor (PDGF) receptors are internalized as a complex with PI 3-kinase and mutant PDGF receptors specifically lacking the binding site for PI 3-kinase fail to accumulate intracellularly (Kapeller et al., 1993; Joly et al., 1994). These data suggest a role for PI 3-kinase in the normal endocytic trafficking of cell surface receptors and further suggest that PI 3-kinase activity in mammalian cells may be involved in the sorting step for internalized receptors at which the decision is made whether to recycle back to the cell surface or to be diverted to the lysosome for degradation.

In mammalian cells, the p110/p85 PI 3-kinase heterodimer is able to utilize multiple forms of PtdIns as substrates to produce PtdIns(3)P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 (Carpenter and Cantley, 1990; Soltoff et al., 1992). The notion that these phosphoinositides have different effects *in vivo* is suggested by the fact that formation of PtdInsP_2 and PtdInsP_3 are stimulated by growth factor addition while PtdIns(3)P levels remain

relatively constant (Auger et al., 1989). Indeed, it has been suggested that $PtdInsP_2$ and $PtdInsP_3$ formed by PI 3-kinase may act as intracellular second messengers to signal cell proliferation in response to growth factor stimulation (Auger et al., 1989; Cantley et al., 1991; Fantl et al., 1992).

We have recently proposed that production of PtdIns(3)P is specifically involved in regulating intracellular protein sorting pathways (Stack and Emr, 1994). This prediction is based upon the observation that Vps34p is a phosphatidylinositol-specific 3-kinase. The possible involvement of mammalian PI 3-kinase in endocytic trafficking of receptors suggest that production of PtdIns(3)P may function in mammalian cells in a manner similar to yeast. In addition, a PtdIns 3-kinase activity from mammalian cells that is distinct from p110/p85 has been recently characterized and has several biochemical properties, including substrate specificity for PtdIns, which suggest that it may represent a Vps34p-like PtdIns 3-kinase (Stack and Emr, 1994; Stephens et al., 1994). This raises the possibility that a Vps34p-like PtdIns 3-kinase may function in mammalian cells to regulate the delivery of proteins to the lysosome in manner directly analogous to the role for Vps34p in vacuolar protein sorting. Collectively, our data on Vps34p have established a role for PtdIns 3-kinase activity in regulating intracellular protein trafficking in yeast and suggest the possibility that the regulation of membrane trafficking may be a function common to PI 3-kinases in all eukaryotes.

Regulatory interaction between the Vps15 protein kinase and the Vps34 PtdIns 3-kinase

Several lines of evidence suggest that association with Vps15p serves to recruit Vps34p to the membrane site of its phospholipid substrate and results in the stimulation of Vps34 PtdIns 3-kinase activity (Stack et al., 1993). We have shown that the *vps15*^{tsf} allele is temperature-conditional for both CPY sorting (Herman et al., 1991b) and PtdIns 3-kinase activity (Fig. 2). These results are further indication that Vps15p is required for Vps34 PtdIns 3-kinase activity and indicate that the loss of Vps15p function immediately results in a decrease in PtdIns 3-kinase activity. These data indicate that Vps15p and Vps34p act together to facilitate the delivery of proteins to the vacuole and are consistent with a model in which Vps34p acts downstream of Vps15p.

The generation of dominant negative mutations has been described as a method to investigate the interactions between gene products (Herskowitz, 1987). We found that overexpression of a catalytically non-functional mutant Vps34 protein results in a dominant-negative phenotype as CPY is missorted in a wild-type strain overproducing these Vps34p mutants. PtdIns 3-kinase activity is also substantially decreased in these strains suggesting the possibility that overexpression of these mutant Vps34 proteins is titrating an activator of wild-type Vps34p. The fact that this mutant phenotype can be suppressed by overexpression of Vps15p suggests that the limiting activity is Vps15p. The observation that catalytically inactive forms of Vps34p are able to interact with Vps15p in a wild-type manner suggests a model in which interaction of Vps15p with catalytically inactive Vps34p mutants sequesters Vps15p from wild-type Vps34p. This results in a mutant phenotype because functional Vps34p is not able to be translocated from the cytoplasm to the membrane nor be

catalytically activated by Vps15p. Collectively, these data indicate that association of Vps34p with Vps15p is absolutely required for the efficient localization of vacuolar proteins.

Overproduction of catalytically inactive forms of Vps15p does not lead to a dominant mutant phenotype suggesting that there is a fundamental difference between catalytically defective mutants of Vps15p and Vps34p. A biochemical basis for this difference was revealed when it was found that Vps15p mutants which are defective in protein kinase activity are also unable to associate with Vps34p in a wild-type manner. This suggests that, in contrast to catalytically-inactive Vps34p, overproduction of kinase-defective forms of Vps15p are unable to titrate its partner (Vps34p) and do not result in dominant interference in these strains. In addition, these observation indicate that an intact Vps15p protein kinase domain is required for association with Vps34p and the subsequent stimulation of PtdIns 3-kinase activity. While it is possible that alteration of residues in the kinase domain of Vps15p abolish a binding site for Vps34p, the fact that alterations in several different residues in the kinase domain of Vps15p lead to an identical phenotype suggests that Vps15p protein kinase activity is required for association with Vps34p. One possibility is that Vps15p directly phosphorylates Vps34p resulting in the stabilization of transient Vps15p-Vps34p complexes. At present, we have no evidence that Vps15p phosphorylates Vps34p as phosphate incorporation into Vps34p is not substantially different between wild-type and Δ*vps15* strains (J.H.S. and S.D.E., unpublished observations). Another possibility is that Vps15p autophosphorylation leads to its association with Vps34p. The association

of autophosphorylated Vps15p with Vps34p may then result in a conformational change in Vps34p which stimulates PtdIns 3-kinase activity. This mechanism would be analogous to activation of mammalian p85/p110 PI 3-kinase following association with autophosphorylated receptor tyrosine kinases; p85/p110 PI 3-kinase activity *in vitro* is increased upon incubation with tyrosine-phosphorylated peptides corresponding to its binding site on various molecules (Carpenter et al., 1993; Giorgetti et al., 1993; Pleiman et al., 1994). An unambiguous test of this model involves the mapping and mutagenesis of Vps15p autophosphorylation site(s).

The observation that Vps15p kinase domain mutants are unable to associate with Vps34p in a wild-type manner also presents a molecular explanation for the observation that overproduction of Vps34p will suppress the vacuolar protein sorting defects of *vps15* protein kinase domain mutants (Stack et al., 1993). In this situation, the decreased affinity of Vps15p kinase domain mutants for Vps34p can be partially overcome by the 20-30-fold overproduction of Vps34p. Therefore, the increased concentration of Vps34p will allow formation of sufficient Vps15p-Vps34p complexes such that the severe vacuolar protein sorting defects of *vps15* kinase domain mutants are partially suppressed. Altogether, the data presented here are strong evidence for the absolute requirement of a functional and stable complex between Vps15p and Vps34p for the sorting of vacuolar proteins. In addition, we have shown that formation of this functional and stable complex between Vps15p and Vps34p requires Vps15p protein kinase activity.

The analysis of *in vivo* PtdIns(3)P levels provides further evidence of a role for Vps15p in regulating the Vps34 PtdIns 3-kinase. The fact that yeast strains deleted for *VPS34* contain essentially no PtdIns(3)P when vegetatively growing cells are labeled suggests that Vps34p may represent the sole source of PtdIns(3)P in yeast cells. Alternatively, if other PI 3-kinases are present in yeast, their activities may be confined to very short periods in the cell cycle or they may be active only during specialized phases of yeast cell growth, such as sporulation or germination. The extremely low levels of PtdIns(3)P in strains deleted for *VPS15* indicate that Vps34p is essentially non-functional in the absence of Vps15p. The severe vacuolar protein sorting defect exhibited by Δ*vps15* strains is consistent with this notion. The significant levels of PtdIns(3)P found in Δ*vps15* strains overproducing Vps34p is seemingly at odds with the severe CPY sorting defect exhibited by this strain. This apparent contradiction is also illustrated by comparison with the *vps15E200R* kinase domain mutant strain overproducing Vps34p. This strain contains approximately 2-fold more cellular PtdIns(3)P than does the Δ*vps15* strain overproducing Vps34p, yet 50% of CPY is correctly delivered to the vacuole in a *vps15E200R* strain overproducing Vps34p while the Δ*vps15* strain overproducing Vps34p is completely defective for CPY sorting. This suggests that the PtdIns(3)P produced in the Δ*vps15* strain is non-functional for vacuolar protein sorting. Several explanations for this observation can be proposed. One possibility is that efficient vacuolar protein sorting requires a certain threshold level of PtdIns(3)P and Δ*vps15* cells overproducing Vps34p do not attain this level. Another possibility is that PtdIns(3)P produced in the Δ*vps15* strain overexpressing Vps34p is

not present in a manner or location that allows it to participate in vacuolar protein sorting. In the absence of Vps15p, Vps34p is found entirely within a soluble, cytoplasmic fraction of yeast cells (Stack et al., 1993). This suggests that in Δ*vps15* cells, Vps34p PtdIns 3-kinase activity and PtdIns(3)P are not directed to the intracellular membrane site of vacuolar protein sorting and a sorting defect results. The fact that *vps15E200R* cells overproducing Vps34p correctly sort ~50% of CPY suggests that at least a portion of PtdIns(3)P produced in this strain is correctly localized. We are currently attempting a direct test of the model by intracellular fractionation of PtdIns(3)P in various yeast strains.

The requirement for correct localization of PtdIns(3)P is an important feature of models we have proposed to explain how PtdIns(3)P may facilitate the vesicular delivery of proteins to the vacuole (Stack et al., 1993). One possibility is that PtdIns(3)P serves as a molecular tag such that vesicles which incorporate it are designated for delivery to the vacuole or an endosomal intermediate. Another model is that phophorylation of membrane PtdIns may recruit or activate proteins involved in the budding or transport of vesicles from the sorting compartment. Possible transport accessory proteins include coat proteins such as clathrin. A final model suggests that the increase in charge repulsion between the polar head groups of PtdIns upon phosphorylation may lead to changes in membrane curvature and possibly stimulate the budding of transport vesicles. Genetic epistasis experiments between *vps15* or *vps34* mutants and *vps* mutants that accumulate vesicles (e.g., *vps21* and *vps45*; Refs. (Cowles et al., 1994; Horazdovsky et al., 1994) may help distinguish between roles for PtdIns(3)P in vesicle formation and targeting. The work

presented here has important implications on the function and regulation of PI 3-kinases in eukaryotic cells and we anticipate that further application of genetic and biochemical approaches in yeast will lead to additional insights into the roles for the Vps15p-Vps34p complex and PtdIns(3)P in the vacuolar protein sorting pathway.

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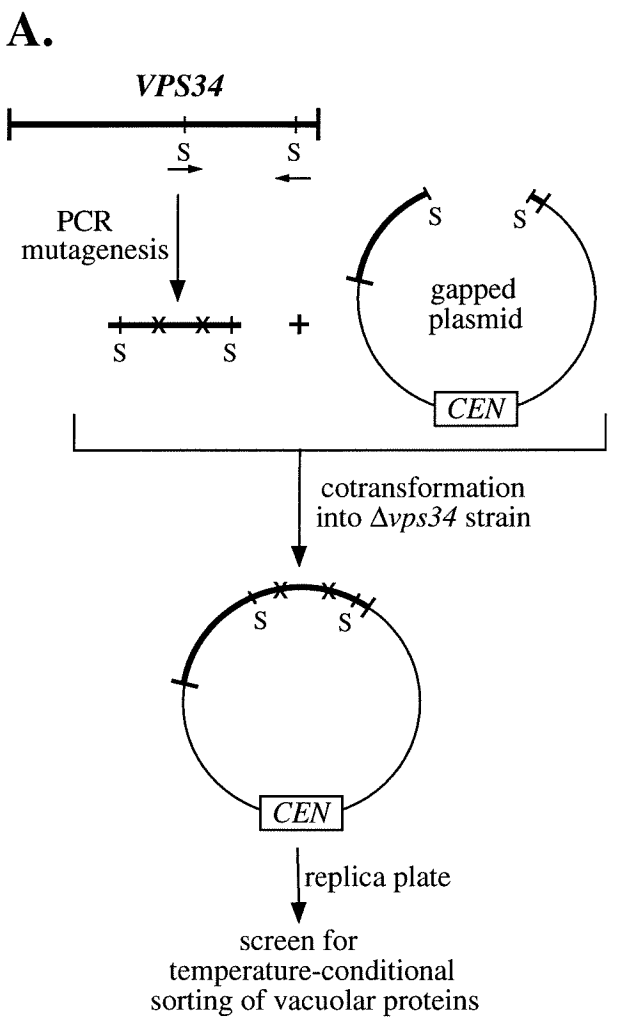
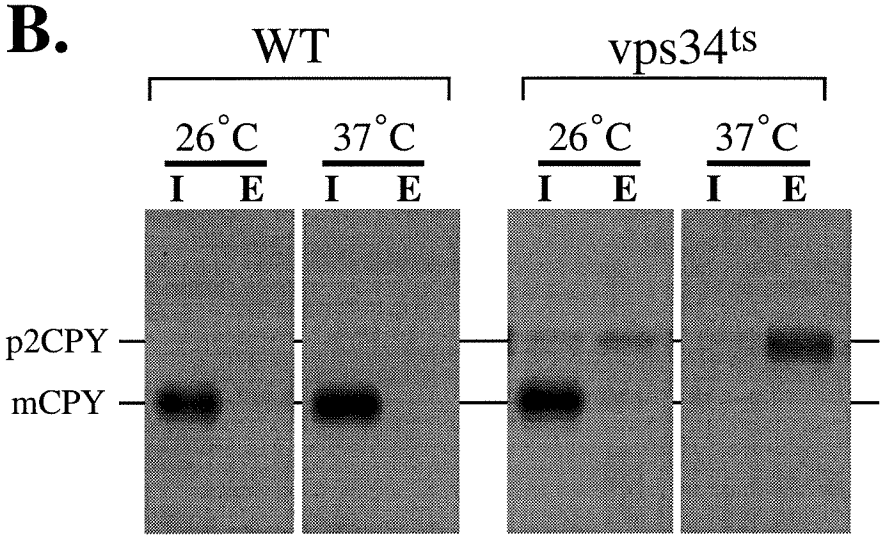
Figure 1. Analysis of a temperature-conditional allele of *VPS34*. **A**. Plasmid gap repair technique for generation of *vps34^{tsf}* allele. A region corresponding to approximately the 3' one-third of the *VPS34* gene was mutagenized by amplification using PCR performed under error-prone conditions. An acceptor plasmid for the mutagenized DNA was produced by digesting a low copy number plasmid containing the *VPS34* gene with the restriction enzyme SpeI which introduced a deletion in the *VPS34* gene which was slightly smaller than the PCR-mutagenized DNA. The gapped plasmid and the mutagenized DNA were cotransformed into *Δvps34* strain. The recombination system of yeast will efficiently repair the gapped region of the plasmid using the mutagenized DNA. The transformants were screened at 26°C and 37°C for sorting of a CPY-invertase fusion protein. **B**. Temperature-conditional CPY sorting phenotype of *vps34^{tsf}* cells. Yeast cells were pre-incubated at 26°C or 37°C for 5 min prior to addition of label. The cells were labeled with Expres^{35}S-label for 10 min and unlabeled methionine and cysteine were added for a 30 min chase. The label and chase were performed at the same temperature as the pre-incubation. Following the chase, the cells were converted to spheroplasts and separated into pellet (I, intracellular) and supernatant (E, extracellular) fractions. Quantitative immunoprecipitation of CPY from each fraction was performed and the immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis. The positions of p2CPY and mCPY are indicated. The strains examined were SEY6210 (WT) and the *vps34^{tsf}* allele (vps34^{ts}). **C**. Sorting and processing of the vacuolar membrane protein alkaline phosphatase (ALP). Cells were labeled and chased as described in (A)

except that a sample was taken after the labeling period in order to generate precursor (pALP) and mature (mALP) forms of ALP.


A.
VPS34
S
S
PCR
mutagenesis
S
S
+
S
S
gapped
plasmid
CEN
cotransformation
into Δvps34 strain
S
S
CEN
replica plate
screen for
temperature-conditional
sorting of vacuolar proteins


B.
WT
vps34ts
26°C
37°C
26°C
37°C
I E I E I E I E
p2CPY
mCPY

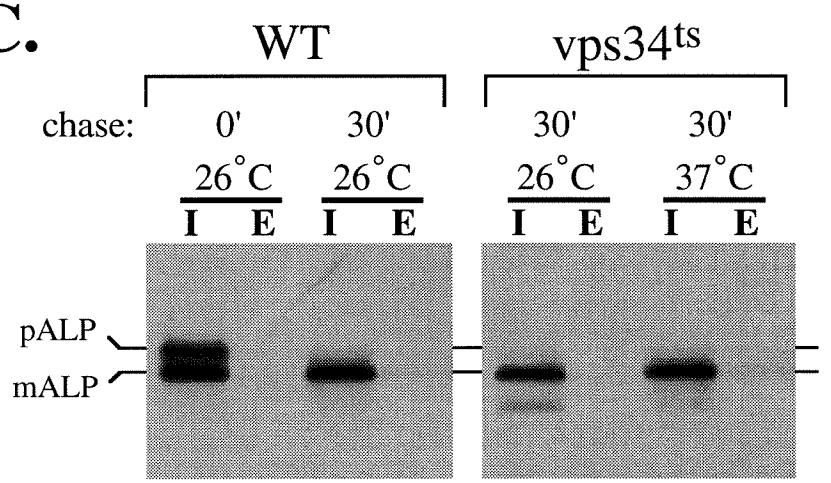

C.
WT
vps34ts
chase:
0'
30'
30'
30'
26°C
26°C
26°C
37°C
I E I E I E I E
pALP
mALP

Figure 2. The *vps34^{tsf}* allele is temperature-sensitive for PtdIns 3-kinase activity. The *vps34^{tsf}* strain was grown overnight at 26°C in the presence of ^{3}H-inositol. The cells were then resuspended in fresh media lacking labeled inositol and incubated at 37°C for the indicated times. Samples were quickly spun down and resuspended in acidified chloroform:methanol and the cells were lysed by vortexing in the presence of glass beads. The extracted lipids were deacylated using methylamine and organic extraction and the deacylated products were separated by HPLC. The positions of the deacylated products of PtdIns(3)P and PtdIns(4)P are indicated.

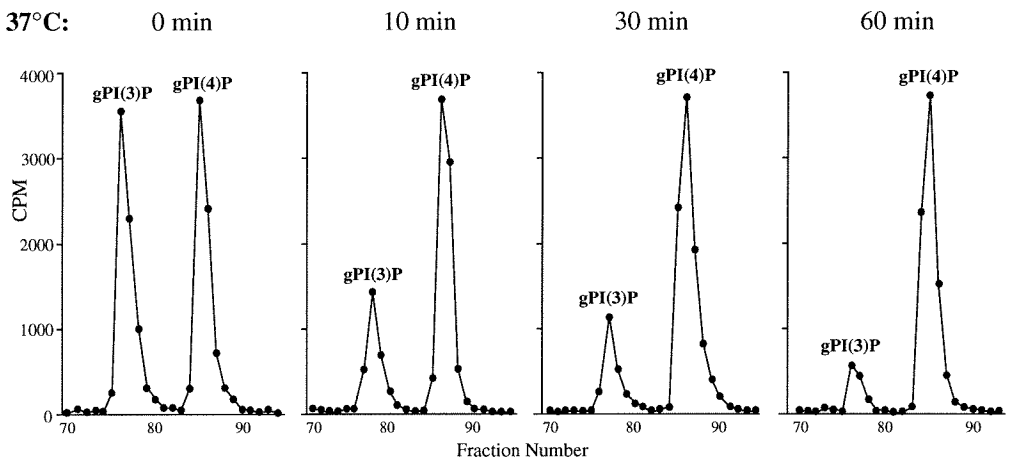

37°C:
0 min
10 min
30 min
60 min
gPI(3)P
gPI(4)P
CPM
4000
3000
2000
1000
0
70
80
90
Fraction Number

Figure 3. A temperature-conditional allele of *VPS15* is *ts* for PtdIns 3-kinase activity. A Δ*vps15* strain containing the *vps15ΔC30* allele on a low copy number plasmid was labeled and incubated as described in Fig. 2 except that the non-permissive temperature was 38°C. The labeled lipids were deacylated and analyzed by HPLC. The positions of the deacylated products of PtdIns(3)P and PtdIns(4) are indicated.

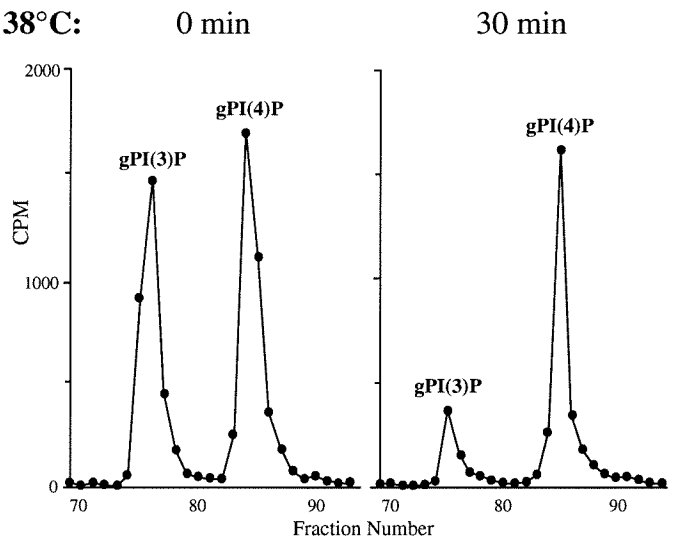

38°C:
0 min
30 min
2000
1000
0
CPM
gPI(3)P
gPI(4)P
gPI(3)P
gPI(4)P
70
80
90
70
80
90
Fraction Number

Figure 4. Catalytically inactive Vps34p mutants result in a dominant-negative phenotype. **A**. CPY sorting phenotype of strains overproducing mutant Vps34 proteins. The indicated *vps34* mutant allele on a multicopy plasmid (2μ) was introduced into *Δvps34* (Δ34) and wild-type (WT) yeast strains. The strains were labeled with Expres^{35}S-label for 10 min and chased for 30 min. The cultures were separated into pellet (I) and supernatant (E) and CPY was immunoprecipitated as described in Figure 1B. The positions of the p2 precursor and the mature forms of CPY are indicated. **B**. PtdIns 3-kinase activity in strains overproducing mutant Vps34 proteins. The indicated *vps34* mutant allele on a multicopy plasmid was introduced into *Δvps34* and wild-type strains. Extracts derived from the resulting strains were assayed for PtdIns 3-kinase activity by adding PtdIns and γ[^{32}P]ATP as described in Materials and Methods. The lipids were extracted and separated on Silica gel 60 plates developed in a borate buffer system. The positions of the products of PtdIns 3-kinase [PI(3)P] and PtdIns 4-kinase [PI(4)P] are indicated.

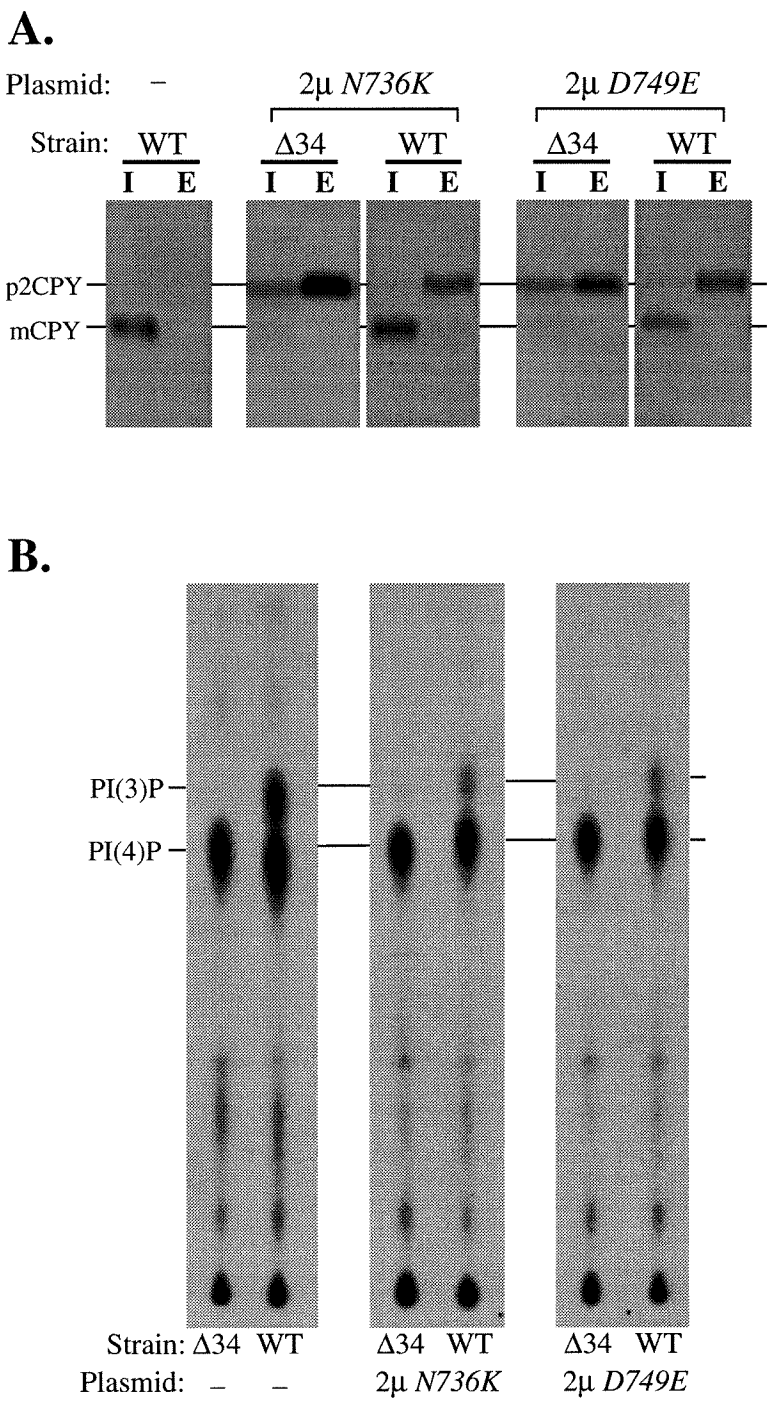

A.
Plasmid: –
2μ N736K
2μ D749E
Strain: WT
Δ34
WT
Δ34
WT
I E
I E
I E
I E
I E
p2CPY
mCPY
B.
PI(3)P
PI(4)P
Strain: Δ34 WT
Δ34 WT
Δ34 WT
Plasmid: – –
2μ N736K
2μ D749E

Figure 5. Overproduction of kinase-defective Vps15p mutants does not result in a dominant mutant phenotype. **A**. CPY sorting phenotype of strains overproducing mutant Vps15 proteins. The indicated *vps15* allele on a multicopy plasmid (2μ) was introduced into *Δvps15* (Δ15) or wild-type (WT) strains. The cells were labeled with Expres^{35}S-label, chased and CPY was immunoprecipitated from intracellular (I) and extracellular (E) fractions as described in Figure 1B. The positions of p2CPY and mCPY are indicated. **B**. PtdIns 3-kinase activity in strains overproducing Vps15p mutants. The indicated *vps15* allele on a multicopy plasmid was introduced into *Δvps15* and wild-type strains. Extracts from the resulting strains were assayed for PtdIns 3-kinase activity in the presence of PtdIns and γ[^{32}P]ATP. The labeled lipids were extracted and separated by TLC as described in Fig. 4. The positions of the products of PtdIns 3-kinase [PI(3)P] and PtdIns 4-kinase [PI(4)P] are indicated.

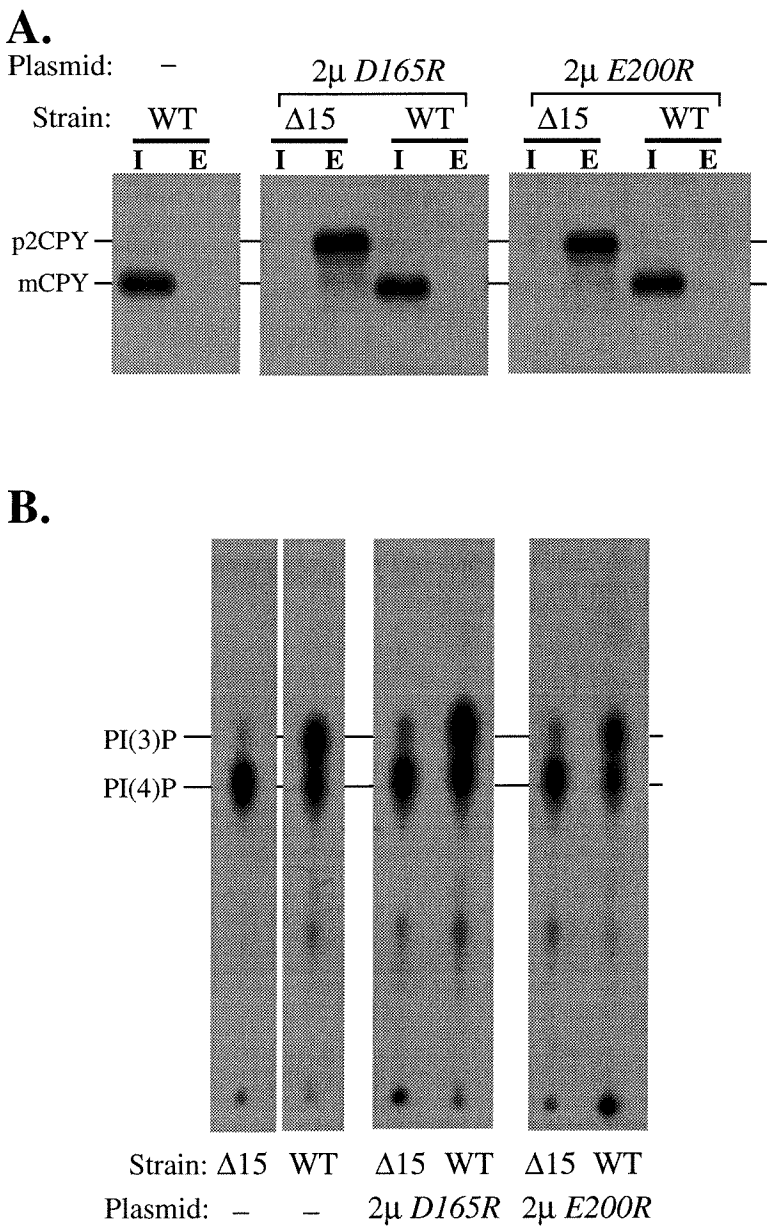

A.
Plasmid: –
2μ D165R
2μ E200R
Strain: WT
Δ15
WT
Δ15
WT
I E
I E
I E
I E
I E
p2CPY
mCPY
B.
PI(3)P
PI(4)P
Strain: Δ15 WT Δ15 WT Δ15 WT
Plasmid: – – 2μ D165R 2μ E200R

Figure 6. Cross-linking of catalytically-inactive forms of Vps15p and Vps34p. **A**. Catalytically-inactive Vps34p mutants are able to associate with Vps15p in a wild-type manner. Labeled yeast spheroplasts were osmotically lysed and treated with the thiol-cleaveable cross-linker DSP. The cross-linked extract was subjected to quantitative immunoprecipitation under denaturing but non-reducing conditions using antisera specific for Vps15p (1st Ab). The immunoprecipitates were treated with sample buffer with or without 2-mercaptoethanol (Reduction) and then re-immunoprecipitated with the indicated antisera (2nd Ab). All samples were reduced immediately prior to electrophoresis. The strains used were SEY6210 (WT) and *Δvps34* containing either the *N736K* or the *D749R vps34* allele on a low copy number plasmid. The positions of Vps15p and Vps34p are indicated. **B**. Kinase-defective Vps15p is unable to associate with Vps34p. Yeast cells were labeled, treated with cross-linker, and subjected to immunoprecipitation as described in (A). The wild-type samples are identical to those shown in (A). The strains used were SEY6210 (WT) and *Δvps15* containing either the *D165R* or the *E200R vps15* allele on a low copy number plasmid. The positions of Vps15p and Vps34p are indicated.

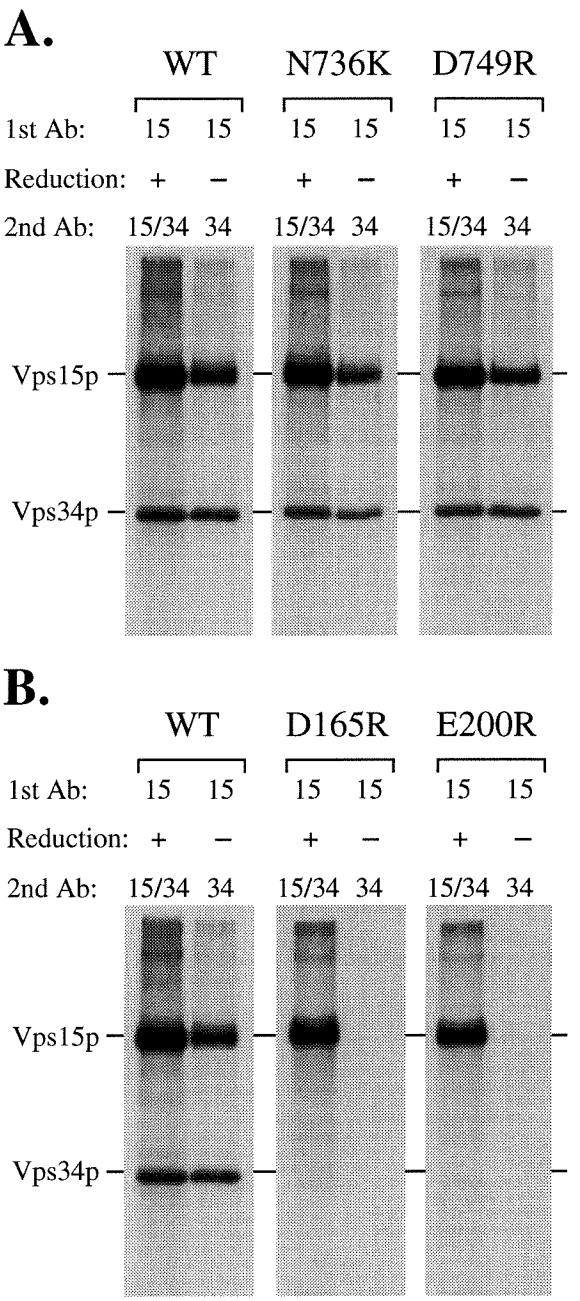

A.
WT
N736K
D749R
1st Ab:
15 15 15 15 15 15
Reduction:
+ − + − + −
2nd Ab:
15/34 34 15/34 34 15/34 34
Vps15p
Vps34p
B.
WT
D165R
E200R
1st Ab:
15 15 15 15 15 15
Reduction:
+ − + − + −
2nd Ab:
15/34 34 15/34 34 15/34 34
Vps15p
Vps34p

Figure 7. Cellular PtdIns(3)P levels are defective in *vps15* and *vps34* mutant strains. The indicated strain was labeled with [^{3}H]-inositol, the lipids were extracted and deacylated, and the deacylated products were separated by HPLC as described in Fig. 2. The strains used were SEY6210 (WT), PHY102 (Δ*vps34*), PHY112 (Δ*vps15*), PHY112 containing the *vps15E200R* allele on a low copy number plasmid (*vps15E200R*), PHY112 containing the wild-type *VPS34* gene on a multicopy plasmid (Δ*vps15*-2μ *VPS34*), and PHY112 containing the *vps15E200R* allele on a low copy number plasmid and the wild-type *VPS34* gene on a multicopy plasmid (*vps15E200R*-2μ *VPS34*). The positions of deacylated products of PtdIns(3)P and PtdIns(4)P are indicated.

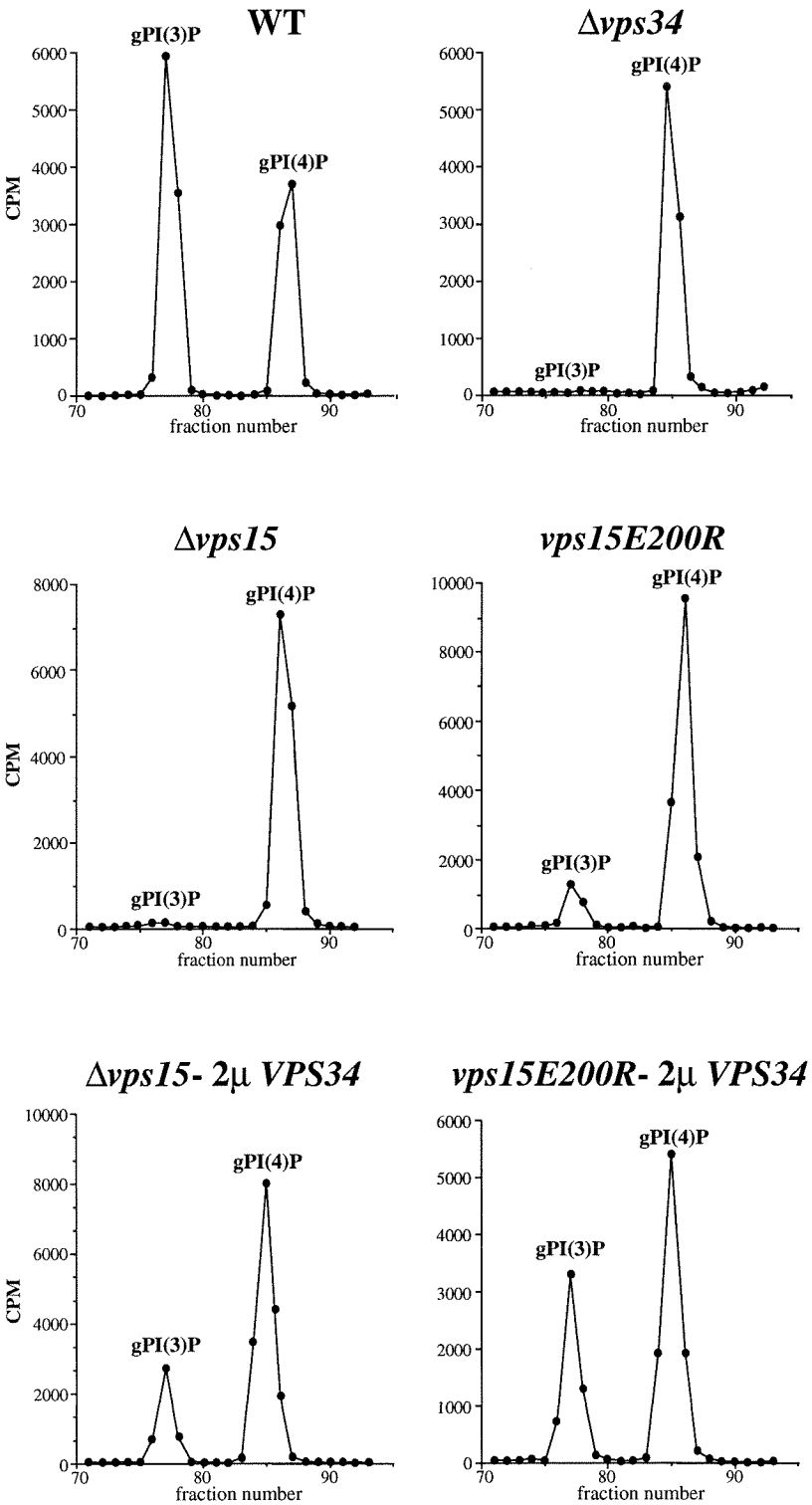

WT
gPI(3)P
gPI(4)P
CPM
fraction number
Δvps34
gPI(4)P
gPI(3)P
fraction number
Δvps15
gPI(4)P
gPI(3)P
CPM
fraction number
vps15E200R
gPI(4)P
gPI(3)P
fraction number
Δvps15- 2μ VPS34
gPI(4)P
gPI(3)P
CPM
fraction number
vps15E200R- 2μ VPS34
gPI(4)P
gPI(3)P
fraction number

Table 1. Correlation of the CPY sorting phenotype with cellular PtdIns(3)P levels. The CPY sorting defect represents the fraction of CPY that is missorted as the p2 precursor in a pulse-chase experiment. The PI(3)P/PI(4)P ratio was determined by summing all the counts above background in the peaks corresponding to PtdIns(3)P and PtdIns(4)P in the experiments depicted in Figure 7. The CPY sorting data can be found in (Herman and Emr, 1990; Herman et al., 1991a; Stack et al., 1993).

Strain	Sorting defect	$\frac{PI(3)P}{PI(4)P}$
Wild-type	<5%	1.30
Δ*vps34*	>95%	0.00
Δ*vps15*	>95%	0.01
Δ*vps15*-2μ *VPS34*	>95%	0.30
vps15E200R	>95%	0.14
vps15E200R-2μ *VPS34*	~50%	0.60

Chapter 8:

Conclusion

The genetic analysis of vacuolar protein sorting in yeast has yielded significant and unexpected insights into intracellular protein trafficking in eukaryotic cells. The work presented in this thesis has contributed to the characterization of two *VPS* gene products that function together as a membrane-associated complex. Sequence homology between Vps15p and protein kinases and between Vps34p and phosphoinositide 3-kinase (PI 3-kinase) suggested a biochemical activity for each protein. We have been able to demonstrate that Vps15p is a functional protein kinase and that Vps34p is a functional phosphatidylinositol 3-kinase (PtdIns 3-kinase), suggesting that protein and lipid phosphorylation reactions are required for the delivery of proteins to the vacuole. In addition, we have characterized the regulatory interactions between the two proteins and demonstrated that Vps15p protein kinase activity is essential for the association with and activation of Vps34p.

Vps15p represents the first characterized protein kinase demonstrated to be involved in an intracellular protein localization pathway. The involvement of protein phosphorylation in regulating protein trafficking reactions has also been suggested from work in mammalian cells. Use of protein kinase inhibitors has indicated that protein kinase activity is involved in the ER to Golgi step of the mammalian secretory pathway; however, the kinase(s) acting at these steps remain unknown (Davidson et al., 1992). Phosphorylation of the cytoplasmic tail of the 300 KD mannose-6-phosphate receptor (MPR 300) for lysosomal proteins has been correlated with the exit of the receptor from the trans Golgi network (Meresse and Hoflack, 1993). The proximity

of the phosphorylation site to the sorting signal in MPR300 for Golgi to endosome delivery suggests the possibility that phosphorylation at this site may regulate the binding of clathrin adaptin proteins (Meresse et al., 1990; Chen et al., 1993). However, the functional significance of this phosphorylation and the protein kinase responsible for it are unknown. Another possible example of phosphorylation regulating intracellular trafficking involves the polymeric immunoglobulin receptor (pIgR). The efficient transcytosis of pIgR appears to require the phosphorylation of a serine residue in its cytoplasmic tail (Casanova et al., 1990). The functional significance of this phosphorylation event is unclear due to the recent observation that the transcytosis of pIgR requires phosphorylation only in the absence of ligand binding (Hirt et al., 1993). While this does not rule out a role for phosphorylation in the intracellular trafficking of pIgR, it raises questions about the involvement of pIgR phosphorylation in the regulated sorting of the ligands of the receptor. It has been suggested that a wild-type protein kinase domain is required for the targeting of epidermal growth factor (EGF) receptors to the lysosome (Felder et al., 1990; Honegger et al., 1990). This issue remains somewhat controversial as other workers have concluded that these kinase-defective EGF receptors are actually defective in internalization from the plasma membrane rather than in sorting from an intracellular site (Lund et al., 1990; Wiley et al., 1991). A final example of a role for protein phosphorylation in intracellular protein sorting involves the fate of internalized receptor tyrosine kinases. This role is suggested by the observation that association of PI 3-kinase with cell surface tyrosine kinases may be required for their delivery to the lysosome (see below). The involvement of protein phosphorylation is due to the

fact that association of PI 3-kinase with these receptors requires the presence of phosphotyrosine residues on the receptor. Association of PI 3-kinase with the phosphorylated receptors is mediated through SH2 domains in the targeting subunit of PI 3-kinase that bind phosphotyrosine residues with high affinity (Soltoff et al., 1992). In this example, protein kinase activity of the receptors themselves appears to be required for determining their intracellular fate.

While the involvement of protein kinase activity in protein sorting for some of the examples cited above is unclear, we have established a clear requirement for Vps15p protein kinase activity in yeast vacuolar protein sorting. Mutations altering highly conserved residues in the Vps15p protein kinase domain are extremely defective for Vps15p autophosphorylation and in vacuolar protein delivery (Herman et al., 1991a; Herman et al., 1991b; Stack and Emr, 1994). In addition, the specific role of Vps15 protein kinase activity in vacuolar protein sorting appears to be in the association of Vps15p with Vps34p, which results in the recruitment of Vps34p to the membrane and activation of its PtdIns 3-kinase activity (Stack et al., 1993; Stack et al., 1994). Unresolved issues concerning Vps15p and vacuolar protein sorting include determination of the precise mechanism of association with Vps34p, possible downstream targets of Vps15p protein kinase activity other than Vps34p, and upstream regulators of Vps15p localization and protein kinase activity.

The cloning and sequencing of the catalytic subunit of mammalian PI 3-kinase revealed a high degree of homology to Vps34p and has resulted in much discussion concerning a possible role for lipid phosphorylation in regulating intracellular protein trafficking (Herman et al., 1992; Munro,

1992; Panayotou and Waterfield, 1992; Burgoyne, 1994; Exton, 1994; Kazlauskas, 1994). In work presented in this thesis, we have demonstrated that Vps34p is a functional PtdIns 3-kinase and that PtdIns 3-kinase activity is required for the sorting of soluble vacuolar proteins in yeast. Our data directly implicates PtdIns 3-kinase activity in regulating an intracellular protein trafficking pathway in yeast and has important implications on the function of PI 3-kinase in higher eukaryotic cells (see below).

There is a growing body of evidence that phospholipids play an active, dynamic role in the regulation of membrane trafficking in eukaryotic cells. Sec14p is required for Golgi function in yeast and has been shown to be a phophatidylinositol/phosphatidylcholine transfer protein (Cleves et al., 1991). This enzyme transfers a bound phophatidylinositol molecule for a phosphatidylcholine molecule in the membrane and thereby has been postulated to serve to maintain a high PI/PC ratio in the membrane. Genetic studies implicating phospholipid biosynthetic pathways in Sec14p function indicate that the relative ratios of different phospholipids in specific compartments may be important for secretory protein traffic in yeast (Cleves et al., 1991). Recent work suggests that rather than acting to physically exchange different phospholipid molecules, Sec14p may serve as a sensor of phosphatidylinositol content in the Golgi due to its relative affinities for different phospholipids (McGee et al., 1994). Lipid-bound Sec14p may then function to regulate phospholipid biosynthetic pathways in order to maintain a Golgi membrane phospholipid composition that allows secretory traffic. Another indication that the function of Sec14p involves regulation of

membrane phosphatidylinositol content comes from the observation that mutations in the yeast *SAC1* gene which will bypass Sec14p function result in inositol auxotrophy (Whitters et al., 1993). This suggests that *sac1* mutations may result in altered metabolism of phosphatidylinositol itself or of phosphoinositides derived from it. Irrespective of its exact mechanism of action, it is clear that Sec14p function involves regulation of phospholipid composition and/or metabolism and this serves to maintain secretory pathway flow through the Golgi.

Phosphoinositide metabolism has been recently implicated in the regulation of secretory vesicle transport and/or fusion with the plasma membrane. Martin and co-workers have developed an *in vitro* reconstitution of regulated secretion in neuroendocrine cells. In addition to Ca^{+2} and ATP, they have characterized a number of cytosolic factors required for this process (Walent et al., 1992). Among the necessary factors are a phosphatidylinositol transfer protein (Hay and Martin, 1993), a PtdIns 4-kinase, and a PtdIns(4)P 5-kinase. These factors suggest that the localized generation of PtdIns(4,5)P_2 is important for secretory vesicle transport/fusion. Further support for this notion comes from the seemingly unrelated findings that PtdIns(4,5)P_2 will activate phospholipase D (PLD) and that PLD is activated by the small GTP-binding protein ARF (Brown et al., 1994; Cockcroft et al., 1994; Liscovitch et al., 1994). ARF has been shown to a major constituent of coated vesicles derived from the mammalian Golgi (Rothman and Orci, 1992). A lucid synthesis of these facts was offered by Cantley and co-workers (Liscovitch et al., 1994). They suggest that PtdIns(4,5)P_2 and PLD function in a cycle that regulates vesicle targeting/fusion with the plasma membrane (Figure

1). The essential parts of this model are that PLD action produces phosphatidic acid (PA) which is known to activate PtdIns(4)P 5-kinase (Jenkins et al., 1994). This kinase phosphorylates PtdIns(4)P to form PtdIns(4,5)P_2 which then stimulates PLD to produce more PA. Termination of the cycle would appear to be regulated by the action of ARF GTPase activating protein (ARF GAP) which would convert ARF from the active GTP-bound state to the inactive GDP-bound state and cease PLD stimulation. Formation of ARF-GDP may also serve to uncoat the vesicle and possibly, together with high localized concentrations of PtdIns(4,5)P_2, catalyze the fusion of the vesicle with the membrane. PtdIns(4,5)P_2 may function in this pathway to activate or associate with proteins other than PLD or ARF GAP. Indeed, PI(4,5)P_2 has been shown to interact with numerous proteins including the actin-associated protein profilin (Lassing and Lindberg, 1985; Goldschmidt-Clermont et al., 1990), the membrane cytoskeletal protein 4.1 (Anderson and Marchesi, 1985), and protein kinase Cζ (Nakanishi et al., 1993). The existence of a functional *in vitro* assay for secretory vesicle fusion should help reveal the specific mechanisms underlying this process.

Our work with Vps34p has demonstrated that PtdIns 3-kinase activity is required for vacuolar protein sorting in yeast. We have been able to show that Vps34p possesses PtdIns 3-kinase activity *in vitro* and mutations altering conserved residues in the Vps34p lipid kinase domain abolish this activity (Schu et al., 1993). Analysis of a temperature-conditional allele that is *ts* for both CPY sorting and PtdIns 3-kinase activity has indicated that Vps34p PtdIns 3-kinase activity is directly involved in vacuolar protein sorting (Stack et al., 1994).

We have shown that Vps34p is a phosphatidylinositol-specific 3-kinase (PtdIns 3-kinase). It is able to utilize phosphatidylinositol (PtdIns), but not PtdIns(4)P or PtdIns(4,5)P_2, as a substrate in an *in vitro* PI kinase assay (Stack and Emr, 1994). We believe these data have important functional significance on the role(s) for PI 3-kinase in mammalian cells. Mammalian phosphoinositide 3-kinase (PI 3-kinase) is able to utilize PtdIns, PtdIns(4)P and PtdIns(4,5)P_2 as substrates in an *in vitro* PI kinase assay (Soltoff et al., 1992). In addition, the products of these reactions [PtdIns(3)P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3] can be detected in mammalian cells labeled with [^{3}H]-inositol (Auger et al., 1989). The kinetics of the appearance of these different phosphoinositides in mammalian cells has suggested that they may not have identical effects *in vivo*. In unstimulated mammalian cells, PtdIns(3)P is by far the most abundant 3'-phosphorylated phosphoinositide. Upon stimulation with growth factors such as platelet-derived growth factor (PDGF), the levels of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 increase dramatically while PtdIns(3)P levels remain relatively constant (Auger et al., 1989). The increase in PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 levels upon growth factor stimulation led to the suggestion that these phosphoinositides may act to signal cell proliferation (Cantley et al., 1991). In addition, the lack of any phospholipase that can hydrolyze 3'-phosphorylated phosphoinositides further suggested that these signaling effects are a function of the modified lipids themselves, i.e., that they serve as intracellular second messengers to signal cell proliferation. This notion is supported by studies where a variety of growth factor receptors have been mutated such that the binding site for PI 3-kinase has been destroyed. Cells containing these mutant

receptors are defective for cell proliferation in response to growth factor stimulation (Fantl et al., 1992; Valius and Kazlauskas, 1993). These studies must be interpreted carefully because some of the mutant receptors were expressed in cell lines that do not normally contain them and because a variety of other molecules that also have been implicated in signaling cell proliferation bind in these same regions of the receptors. Given these caveats, it appears likely that some downstream targets of PI 3-kinase are involved in growth control in mammalian cells. Indeed, it has recently been shown that PI 3-kinase serves to regulate ribosomal S6 kinase activation and thereby may exert some level of control over translational efficiency (Chung et al., 1994). In addition, mammalian PI 3-kinase has also been reported to associate with active Ras proteins and this association stimulates PI 3-kinase activity (Kodaki et al., 1994; Rodriguez-Viciana et al., 1994).

The fact that PtdIns(3)P levels do not rise significantly in response to growth factor addition suggests that it may be involved in a cellular function distinct from signaling proliferation. PI 3-kinase activity has also been implicated in processes such as histamine secretion (Yano et al., 1993), neutrophil oxidative burst (Arcaro and Wymann, 1993; Okada et al., 1994; Thelen et al., 1994), membrane ruffling and actin rearrangements (Kotani et al., 1994; Wennstrom et al., 1994; Wymann and Arcaro, 1994), and the translocation of glucose transporters to the cell surface (Hara et al., 1994; Okada et al., 1994). Our work on Vps34p has indicated a role for PtdIns 3-kinase activity in membrane trafficking in yeast. Evidence also exists of a role for PI 3-kinase in regulating protein trafficking pathways in mammalian cells. It has been shown that mutant receptors for colony

stimulating factor that lack a binding site for PI 3-kinase are internalized but fail to be degraded in the lysosome (Downing et al., 1989; Carlberg et al., 1991). Corvera and co-workers have shown that the PDGF receptor is internalized in a complex with PI 3-kinase (Kapeller et al., 1993). Mutant PDGF receptors specifically lacking the binding site for PI 3-kinase fail to accumulate inside the cells, suggesting a role for PI 3-kinase in the endocytic trafficking of cell surface receptor (Joly et al., 1994). One interpretation of these data is that PI 3-kinase may be involved at the sorting step for mammalian cell surface receptors at which a decision is made to recycle to the cell surface or to be diverted to the lysosome for degradation.

The possibility that different 3'-phosphorylated phosphoinositides may have different effects is also suggested by the fact that multiple distinct PI 3-kinase activities have been characterized in mammalian cells. These activities include two isoforms of the p110 catalytic subunit of the p85/p110 PI 3-kinase heterodimer which is known to associate with activated cell surface receptor tyrosine kinases (Hiles et al., 1992; Hu et al., 1993), a G-protein coupled PI 3-kinase activity (Stephens et al., 1994; Thomason et al., 1994) and a phosphatidylinositol-specific 3-kinase (Stephens et al., 1994). The PtdIns-specific 3-kinase has several biochemical characteristics in common with Vps34p. These include a strict substrate specificity for PtdIns and resistance to the PI 3-kinase inhibitor, wortmannin (Stack and Emr, 1994; Stephens et al., 1994). These data suggest that this mammalian PtdIns 3-kinase may represent a functional homolog of Vps34p or be a member of a family of Vps34p-like PI 3-kinases. Furthermore, the existence of multiple distinct PI 3-kinase

molecules indicated by these analyses suggest the possibility that a mammalian Vps34p homolog may function in a late Golgi compartment to regulate the sorting of lysosomal proteins in a manner directly analogous to Vps34p. These data, together with our work on Vps34p, have led us to propose that production by PI 3-kinases and PtdIns 3-kinases of a specific phosphoinositide, PtdIns(3)P, is involved in regulating intracellular protein sorting reactions in eukaryotic cells (Stack and Emr, 1994).

The demonstrated role for PtdIns 3-kinase activity in yeast vacuolar protein localization raises the question of how the phosphorylation of a specific membrane phospholipid could lead to the vesicular delivery of vacuolar proteins to their target organelle. Several models have been proposed to explain this (Stack et al., 1993). One model suggests that PtdIns(3)P serves a molecular tag whereby vesicles incorporating this modified phospholipid are designated for delivery to the vacuole or an endosomal intermediate. This model predicts the existence of recognition systems, possibly including PtdIns(3)P-specific receptors, that mediate the delivery of vesicles to the target organelle.

A second possibility is based on the idea that phospholipids may play a dynamic role in regulating the activity or membrane association of proteins involved in the vacuolar protein sorting pathway. Examples exist of such a role for phospholipids in general and phosphoinositides in particular. As mentioned earlier, PtdIns(4,5)P_2 has been shown to bind to and/or activate molecules such as profilin, protein 4.1 and protein kinase Cζ. In addition, a recent report has suggested that the pleckstrin motif found in many different proteins may be able to associate with

phosphoinositides such as PtdIns(3)P, PtdIns(4)P and PtdIns(4,5)P_2 (Harlan et al., 1994). Interestingly, many pleckstrin motif-containing proteins are known to be membrane-associated, suggesting that binding to specific modified phospholipids may contribute to the intracellular localization of these proteins. Vps34p-mediated phosphorylation of membrane PtdIns may catalyze the recruitment or activation of accessory factors involved in the budding or transport of vesicles from the sorting compartment. Candidates for such vesicle accessory proteins include coat proteins such as clathrin and clathrin-associated adaptin proteins. In addition to its role in endocytosis, clathrin has been shown to be required for the sorting of both mammalian lysosomal and yeast vacuolar proteins (Kornfeld and Mellman, 1989; Seeger and Payne, 1992). Interestingly, it has been reported that adaptins will bind to some 3'-phosphorylated forms of inositol polyphosphates, suggesting the possibility that adaptins may be able to bind to 3'-phosphorylated phosphoinositides (Voglmaier et al., 1992). Other possible vesicle transport factors include cytoskeletal elements and molecular motors which may facilitate the trafficking of vesicles between the Golgi and the endosome or vacuole (Stack and Emr, 1993).

A final model is based on the bilayer couple hypothesis (Sheetz and Singer, 1974). This hypothesis states that the asymmetric distribution of phospholipids between the two halves of a membrane bilayer would lead to membrane curvature due to the different shapes of the phospholipids. For example, phospholipids with a charged polar head group would be expected to be wedge-shaped, while zwitterionic phospholipids are cylindrical. Phosphorylation of the head group of phosphatidylinositol would lead to a large increase in the ionic character of the inositol head

group. This would result in increased charge repulsion between phospholipid head groups and could result in membrane curvature. This curvature could potentially catalyze the budding or formation of transport vesicles. Given the possible high local concentration of Vps34p substrate (PtdIns) due to its restriction within the membrane, it is conceivable that the action of Vps34p could lead to substantial production of PtdIns(3)P in a relatively restricted area and this may have implications on its mechanism of action.

In addition to suggesting roles for protein phosphorylation and PtdIns 3-kinase activity in membrane trafficking pathways, the work presented in this thesis also has yielded considerable insight into the regulatory interactions between the Vps15 protein kinase and the Vps34 PtdIns 3-kinase. We have shown that the membrane localization of Vps34p requires Vps15p as Vps34p is entirely cytosolic in a *Δvps15* strain. This, together with the fact that Vps15p and Vps34p are found as a membrane-associated complex, indicates that Vps15p acts as a targeting subunit to recruit Vps34p to the membrane site of its phospholipid substrate (Stack et al., 1993). In addition to this role, association with Vps15p also serves to stimulate Vps34 PtdIns 3-kinase activity. Indeed, the extremely low levels of PtdIns(3)P in *Δvps15* cells suggests that Vps34p is non-functional in the absence of Vps15p (Stack et al., 1994). This notion is further emphasized by the severe vacuolar protein sorting defects observed in both *Δvps34* and *Δvps15* strains (Herman and Emr, 1990; Herman et al., 1991a). Genetic analysis of catalytically inactive forms of Vps15p and Vps34p has allowed us to implicate Vps15p protein kinase activity in the association with and activation of Vps34p (Stack et al., 1994).

Collectively, these data demonstrate that the functionally active form of Vps34p is in a complex with Vps15p and indicate that formation of a stable complex between Vps15p and Vps34p is absolutely required for the efficient localization of soluble vacuolar proteins.

A number of models can be suggested which incorporate a membrane-associate complex of Vps15p and Vps34p in the vesicular delivery of proteins to the vacuole. Subcellular fractionation of Vps15p and Vps34p (Herman and Emr, 1990; Herman et al., 1991a) and the localization of precursor vacuolar proteins in yeast cells lacking Vps15p function (Vida et al., 1993) suggest that Vps15p and Vps34p act at the level of the late Golgi, most likely at the sorting compartment for vacuolar proteins. One model suggests that the Vps15p-Vps34p complex may be able to associate with the cytoplasmic tails of receptors for vacuolar protein precursors (Stack et al., 1993). Ligand binding to receptor may transduce a signal through a conformational change in the receptor cytoplasmic tail that promotes receptor association with and/or activation of Vps15p. Activation of Vps15p results in the stimulation of Vps34p PtdIns 3-kinase activity. Phosphorylation of membrane PtdIns by Vps34p would then trigger a cascade of events that ultimately results in the vesicular delivery of receptor-ligand complexes to the vacuole directly or through an endosomal intermediate. In this model, Vps15p and Vps34p effectively act as components of a signal transduction complex which couples the signal received by specific transmembrane receptor proteins into a second messenger molecule [PtdIns(3)P] that catalyzes the action of as yet unidentified effector molecules.

In conclusion, the work presented here on the function of the Vps15 protein kinase and the Vps34 PtdIns3-kinase has led to unexpected and novel mechanisms for regulating protein trafficking pathways. Our studies in yeast also have directly resulted in new concepts concerning the function of a key regulatory enzyme in mammalian cells, PI 3-kinase. While many mechanistic aspects of the specific roles for protein and phospholipid phosphorylation in membrane trafficking remain to be revealed, it is likely that work on vacuolar protein sorting in yeast will continue to yield exciting and important contributions to the regulation of intracellular protein localization in eukaryotic cells.

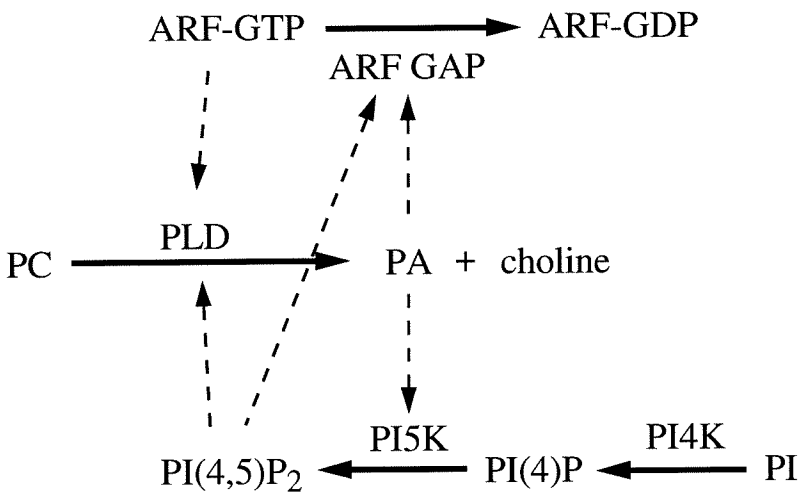


Figure 1. Phosphoinositide cycle proposed to regulate vesicle targeting/fusion. PtdIns 4-kinase (PI4K) is found associated with secretory vesicles; therefore, vesicles would contain PtdIns(4)P. The GTP-bound form of ARF (ARF-GTP) is a component of the vesicle coat and serves to activate phospholipase D (PLD). PLD hydrolyzes membrane phosphatidylcholine to form phosphatidic acid (PA) and choline. PA then activates PtdIns(4)P 5-kinase (PI5K), producing PtdIns(4,5)P_2. PIP_2 activates PLD which results in the production of additional PIP_2 by the same pathway. PIP_2 may also function at the targeting or fusion step by an unknown mechanism. PIP_2 may contribute to the down regulation of this cycle by activating a GTPase activating protein for ARF (ARF GAP). This would result in conversion to the GDP-bound form of ARF (ARF-GDP) and the uncoating of the vesicle, possibly facilitating the fusion of the vesicle with the plasma membrane. Reaction directions are indicated by the solid arrows and activations by the dashed arrows.

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