Negative Regulation of Transcription Factors by Srb10 Cyclin-Dependent Kinase

Thesis by

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In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

2001

(Defended May 22, 2001)

Acknowledgements

The success of my graduate career would not have been possible without the help from a great many people. I was fortunate to have Ray Deshaies as my advisor. His brilliance and wisdom have benefited me tremendously in my intellectual and personal growth for the past six years. I thank him sincerely for providing a vigorous, stimulating, and competitive environment for my scientific training, and for displaying great enthusiasm, patience, and support during difficult times. Members of the Deshaies lab, past and present, have made my graduate life more enjoyable. I thank Craig Correll, Renny Feldman, Wenying Shou, Jae Hong Seol, and Rati Verma for their scientific input and technical assistance, and for sharing reagents. I also thank members of my thesis committee, Pamela Bjorkman, Judy Campbell, Bill Dunphy, and Barbara Wold for their advice and support over the past years. My thesis project also benefited from a collaboration with Dr. Roland S. Annan and Dr. Steven A. Carr's group in SmithKline Beecham Pharmaceuticals, and from the stimulating scientific discussions with Dr. Richard A. Young in Whitehead Institute for Biomedical Research and Department of Biology, MIT. Finally, I owe many thanks to my parents. Their love, support, and understanding have always been a strong force that motivates me to achieve my best.

Abstract

The ubiquitin-dependent proteolytic pathway plays an important role in a broad array of cellular processes, including cell cycle control and transcription. Biochemical analysis of the ubiquitination of Sic1, the B-type cyclin-dependent kinase (CDK) inhibitor in budding yeast helped to define a ubiquitin ligase complex named SCF^{Cdc4} (for Skp1, Cdc53/cullin, F-box protein). We found that besides Sic1, the CDK inhibitor Far1 and the replication initiation protein Cdc6 are also substrates of SCF^{Cdc4} *in vitro*. A common feature in the ubiquitination of the cell cycle SCF^{Cdc4} substrates is that they must be phosphorylated by the major cell cycle CDK, Cdc28. Gcn4, a transcription activator involved in the general control of amino acid biosynthesis, is rapidly degraded in an SCF^{Cdc4}-dependent manner in vivo. We have focused on this substrate to investigate the generality of the SCF^{Cdc4} pathway. Through biochemical fractionations, we found that the Srb10 CDK phosphorylates Gcn4 and thereby marks it for recognition by SCF^{Cdc4} ubiquitin ligase. Srb10 is a physiological regulator of Gcn4 stability because both phosphorylation and turnover of Gcn4 are diminished in *srb10* mutants. Furthermore, we found that at least two different CDKs, Pho85 and Srb10, conspire to promote the rapid degradation of Gcn4 *in vivo*. The multistress response transcriptional regulator Msn2 is also a substrate for Srb10 and is hyperphosphorylated in an Srb10-dependent manner upon heat stress-induced translocation into the nucleus. Whereas Msn2 is cytoplasmic in resting wild type cells, its nuclear exclusion is partially compromised in *srb10* mutant cells. Srb10 has been shown to repress a subset of genes in vivo, and has been proposed to inhibit transcription via phosphorylation of the C-terminal domain of RNA polymerase II. Our results suggest a general theme that Srb10 represses the transcription of specific genes by directly antagonizing the transcriptional activators.

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Chapter1. Introduction

The Ubiquitin-Proteasome Pathway

Overview

Precise modulation of intracellular protein concentrations is both necessary and sometimes critical as cells switch from one state to another in response to diverse environmental stimuli or the regulated developmental programs. Cells achieve this through various regulatory mechanisms such as transcriptional, translational, and post-translational means. Among these, selective protein degradation presents advantages over the other regulatory controls. First of all, protein degradation allows rapid achievement of new steady-state level of a protein after the change in the rate of its synthesis. Second, degradation provides an irreversible means of inactivating or removing the protein. Although this mechanism of regulation of protein levels appears energy-consuming because cells have to resynthesize the destroyed proteins, only a small percentage of the cellular proteins, mostly key regulatory proteins, undergo constitutive or timing-based turnover.

Proteolysis was original regarded as a means by which a cell eliminates aberrant or damaged proteins. In the past decade, there have been ever increasing examples that establish the importance of protein degradation in cellular regulation. There exist several proteolytic pathways in the cytoplasm of eukaryotic cells. The major pathway that degrades proteins is the ubiquitin-dependent degradation pathway (Hershko and Ciechanover 1998). This pathway targets proteins to degradation by the 26S proteasome via covalent attachment of multi-ubiquitin chains (a process commonly referred to as "ubiquitination"). Ubiquitination of substrate proteins is a multistep process that generally requires three classes of enzymes: the E1 ubiquitin-activating enzyme, the E2

ubiquitin-conjugating enzyme, and the E3 ubiquitin ligase. Multiubiquitin chains are thought to serve as signals for recognition and breakdown of protein substrates by the multi-subunit and ATP-dependent protease complex called 26S proteasome. To date, ubiquitin-mediated degradation has been shown to play important roles in a broad array of cellular processes, including cell cycle transitions, signal transduction, immune response and inflammation, development and differentiation, and transcriptional regulation.

Ubiquitin

Ubiquitin is a small, highly conserved globular protein consisting of 76 amino acids. Yeast and human ubiquitin differ by only three amino acids. It was originally isolated from the thymus (Goldstein et al. 1975), but was found later to be present ubiquitously in all tissues and organisms. The first evidence of ubiquitin attachment to other proteins came in 1977, when the C-terminus of ubiquitin was found to be covalently linked via isopeptide bond to the epsilon-amino group of the lysine residue of histone 2A (Goldknopf and Busch 1977). The functional link between ubiquitin and protein degradation was not established until ubiquitin was found to be required for the ATP-dependent proteolytic activity in fractionated reticulocyte lysates and was found to be conjugated to protein substrates (Ciechanover et al. 1978; Ciechanover et al. 1980; Wilkinson et al. 1980). These and other findings led Hershko and coworkers to propose that ubiquitin attachment to proteins serves as a signal that marks them for degradation by a protease (Hershko et al. 1980). Although monoubiquitination has been observed on several proteins, its role appears to be unrelated to targeting protein for degradation. Recent developments indicate that monoubiquitination is involved in distinct cellular functions such as histone regulation, endocytosis, and the budding of retroviruses from the plasma membrane (Hicke 2001). The existence of multiubiquitin chains linked via

lysine 48 (K48) of ubiquitin was first demonstrated in 1989 (Chau et al. 1989). Inhibition of proteolysis by selective expression of K48R mutant ubiquitin led to the proposal that proteolytic targeting is primarily achieved through multiubiquitin chains assembled via K48 linkages (Finley et al. 1994).

Ubiquitin-activating enzyme (E1)

Following the discovery of ubiquitin attachment to proteins, Hershko and coworkers identified three enzymatic activities (E1, E2, and E3) required for ubiquitination (Ciechanover et al. 1981; Hershko et al. 1983). E1 is the first enzyme in this multistep process, and it catalyzes the activation of ubiquitin. This reaction proceeds in an ATP-dependent manner, resulting in the formation of a high-energy thioester bond between the C-terminal glycine residue of ubiquitin and a specific cysteine residue in the E1 enzyme. This thioester bond is labile, and ubiquitin is subsequently transferred to the ubiquitin-conjugating enzyme (E2) via transesterification. Most eukaryotes have one major functional E1 enzyme. In budding yeast *S. cerevisiae*, *UBA1* encodes the only E1 enzyme and is essential for cell viability (McGrath et al. 1991). E1 enzymes are abundant and present in both the cytoplasm and nucleus.

Ubiquitin-conjugating enzyme (E2)

Ubiquitin-conjugating enzymes (also called UBCs or ubiquitin carrier proteins) represent the second class of enzymes in the ubiquitin-conjugation pathway, An E2 accepts the ubiquitin molecule from the E1 enzyme by forming a thioester bond between an active-site cysteine residue and the ubiquitin. This allows ubiquitin to be further transferred either directly to a substrate or to the ubiquitin ligase (E3) via another transesterification. The E2 enzymes form a relative large class of enzymes in the ubiquitin-conjugation system, consisting of at least 30 enzymes, including 13 in budding yeast. They are

relatively small molecules (ranging from 16 to 34 kDa in budding yeast) and are structurally related, consisting of a highly conserved (35-40% identity) 16 kDa catalytic domain called the UBC domain (Jentsch 1992). Within this domain lies a specific cysteine residue required for E2-ubiquitin thioester formation. Although E2 enzymes in budding yeast have been implicated to play important roles in DNA repair, stress response, membrane protein turnover, cell cycle progression, and transcription (Scheffner et al. 1998), only *CDC34* is essential.

Two relatively well-characterized E2 enzymes in budding yeast are Rad6 (Ubc2) and Cdc34 (Ubc3). *RAD6* encodes a 172 amino acid, 20 kDa protein containing the conserved UBC domain (Jentsch et al. 1987). Mutation of *RAD6* leads to pleiotropic phenotypes, including slow growth, sensitivity to DNA damaging agents, deficiency in UV-induced mutagenesis, increased retrotransposition, and sporulation defects (Lawrence 1994). Some evidence suggests that the role of Rad6 in DNA repair may be mediated through ubiquitin-dependent degradation (Bailly et al. 1994). Early studies in the Varshavsky laboratory have shown that ubiquitination of certain proteins by the so-called "N-end rule" pathway (Bachmair et al. 1986; Varshavsky 1992) requires Rad6, which physically associates with the E3 enzyme called Ubr1 (Dohmen et al. 1991; Madura et al. 1993). Recent in vitro reconstitution experiments have shown that Rad6 promotes the multi-ubiquitination of a homeodomain-containing protein Cup9 (Turner et al. 2000). Interestingly, Rad6 has also been implicated in the turnover of the transcription factor Gcn4. Gcn4 is partially stabilized in a *rad6Δ* mutant, but not *ubr1Δ* (Kornitzer et al. 1994). In vitro ubiquitination experiments using fractionated yeast extracts suggest that Rad6 promotes the ubiquitination of Gcn4 (Y. Chi and R. Deshaies, unpublished data). It appears that Rad6 is involved in the ubiquitination of a variety of proteins and may associate with distinct E3 enzymes.

CDC34 is an essential gene originally identified in a screen for cell division cycle or *cdc* mutants. Temperature-sensitive mutants of *CDC34* arrest in G1 phase of the cell cycle. The biochemical function of Cdc34 remained obscure until the discovery of its sequence similarity to Rad6 (Goebl et al. 1988). The realization that Cdc34 is a ubiquitin-conjugating enzyme provided an important clue on the biological trigger at the G1 to S phase transition. Genetic analysis identified the B-type cyclin-Cdk inhibitor Sic1 as a key physiological substrate of Cdc34 and thus implicated the role of proteolysis in G1 to S phase transition (Schwob et al. 1994). *CDC34*, along with other genes (*CDC53* and *CDC4*), which are also required for Sic1 degradation, defines a ubiquitination pathway that was initially named the "CDC34 pathway" (Deshaies 1995). Besides Sic1, several other proteins are stabilized in a $cdc34^{ts}$ mutant, suggesting that they are potential CDC34 pathway substrates. These include the G1 cyclins Cln2 (Deshaies et al. 1995) and Cln3 (Yaglom et al. 1995), G1 cyclin-Cdk inhibitor Far1 (Henchoz et al. 1997), replication initiation protein Cdc6 (Drury et al. 1997), and transcription activator Gcn4 (Kornitzer et al. 1994). The evidence that Gcn4, a transcription factor involved in the up-regulation of amino acid and purine biosynthesis, is degraded in Cdc34-dependent manner suggests that CDC34 pathway is not confined to cell cycle targets.

The functions of other UBCs in budding yeast are less well characterized. Ubc4 and Ubc5 are needed for the degradation of many short-lived and abnormal proteins (Hochstrasser 1996). Ubc6 and Ubc7, along with Ubc4 and Ubc5, are required for the rapid degradation of the Matα2 repressor (Chen et al. 1993). Ubc9's role has been controversial. It was originally found to be required for cell cycle transition at the G2 or early M phase and for the degradation of B-type cyclins, suggesting that Ubc9 participates in the ubiquitination of B-type cyclins (Seufert et al. 1995). Recent evidence, however, indicates that Ubc9 promotes the conjugation of a yeast ubiquitin-like protein

Smt3 (or mammalian SUMO-1), and therefore suggests that Ubc9 may affect cyclin degradation indirectly (Schwarz et al. 1998).

Ubiquitin ligase (E3)

Ubiquitin ligases (or ubiquitin protein ligases) are the third and least understood component of the ubiquitination machinery. Although E2 enzymes can directly transfer the ubiquitin to substrates in some instances without E3 (Haas et al. 1991), they generally require E3 for efficient attachment of ubiquitin to substrate proteins. Ubiquitin ligases are particularly important because they are widely regarded as the substrate-recognition components of the ubiquitination machinery that confer specificity and regulation. They were initially defined as activities that bind to E2 and substrates, and stimulate ubiquitination reactions in the presence of E1 and E2 (Hershko and Ciechanover 1992). The discovery of E3s has been slow because, unlike E2s, they do not share easily identifiable sequence motifs. Furthermore, recent evidence suggests that E3 activities may be comprised of multi-protein complexes in addition to single subunit ligases. Early studies have identified two distinct families of ubiquitin ligases. They are the yeast Ubr1 (or mammalian homolog E3α) and the *hect* (homologous to E6-AP carboxyl-terminus) domain family defined by E6-associated protein (E6-AP). E6-AP was discovered as a 100 kDa cellular protein that is required, along with papillomavirus E6 oncoprotein, for the ubiquitination and degradation of p53 in reticulocyte lysates (Scheffner et al. 1993). Ubr1 and E6-AP appear mechanistically distinct in their actions. Whereas Ubr1 binds substrates and stimulates the reactions catalyzed by E2 by a poorly understood mechanism (Bartel et al. 1990), E6-AP catalyzes the ubiquitin transfer through an E1-E2-E3 enzyme ubiquitin thioester cascade, in which formation of the ubiquitin-E6-AP thioester intermediate is an obligatory step (Scheffner et al. 1995). The budding yeast

genome encodes five *hect*-domain proteins, and one of them, Rsp5, has been shown to ubiquitinate the largest subunit of RNA polymerase II in vitro (Huibregtse et al. 1997).

Yeast Ubr1 (mammalian counterpart is called E3α) is a non-essential, 225 kDa protein. It is the key component of the N-end rule pathway, which relates the half-life of a protein to its N-terminal residues (Bachmair et al. 1986; Varshavsky 1992; Varshavsky et al. 2000). Although the N-end rule recognition mechanism is conserved in eukaryotes, its primary physiological roles and substrates are still not fully understood. The first physiological function of Ubr1 has been shown to be the regulation of peptide uptake (Alagramam et al. 1995) by mediating the degradation of the homeodomain transcriptional repressor Cup9 (Byrd et al. 1998). Recent biochemical evidence shows that E1, Rad6, and Ubr1 ubiquitinates Cup9 in vitro, and ubiquitination of Cup9 is enhanced by dipeptides bearing destabilizing N-terminal residues (Turner et al. 2000).

Advances in cell cycle studies in the past few years have helped to enrich the repertoire of the ubiquitin ligases. Ubiquitin-mediated degradation has been shown to be important in two phases of the cell cycle: G1 to S phase transition and mitosis, which includes metaphase to anaphase transition and mitotic exit (King et al. 1996). Biochemical reconstitution of the ubiquitination of Sic1 protein defined a modular ubiquitin-protein ligase complex called SCF (for Skp1, Cdc53/cullin, F-box protein) (Feldman et al. 1997; Skowyra et al. 1997). The SCF ubiquitin ligase represents a conserved family of ubiquitin ligases that consist of at least four subunits (see details in the next section). Ubiquitination and degradation of mitotic cyclins using fractionated frog or clam egg extracts identified a 1000-1500 kDa ubiquitin ligase complex known as the cyclosome (Sudakin et al. 1995) or the anaphase-promoting complex (APC) (King et al. 1995). APC plays a key role in controlling the separation of sister chromatids and the exit from mitosis by promoting the degradation of the anaphase inhibitor Pds1 and cyclin B (King et al. 1996). The mechanism of how APC catalyzes the ubiquitination of mitotic

substrates is unclear. Although APC acts like an E3 in a reaction containing E1 and E2, none of the APC subunits identified share sequence homology to either of the two well-characterized E3 enzymes Ubr1 and E6-AP. Ubiquitin thioester intermediates were not detected with APC (King et al. 1995), suggesting that APC may facilitate the ubiquitin transfer of the E2 onto the substrate by binding to them. Interestingly, the Apc2 subunit of APC complex bears homology to the carboxyl-terminal half of Cdc53/cullin (Yu et al. 1998; Zachariae et al. 1998), raising the possibility that APC and SCF ubiquitin ligases share a common enzymatic mechanism.

The 26S proteasome

The physical destruction of multiubiquitinated proteins is carried out by a 2.5 MDa, ATP-dependent protease complex called the 26S proteasome (Voges et al. 1999). It is composed of more than 30 subunits, which assemble into two distinct subcomplexes: a central barrel-shaped proteolytic core called 20S proteasome and one or two flanking regulatory particles called the 19S caps. Bacterial and eukaryotic 20S proteasomes look very similar in structure. They are all arranged as a stack of four rings, each containing six or seven subunits. High-resolution crystal structure of the budding yeast 20S proteasome suggests slightly more complexity in organization of the eukaryotic subunits (Groll et al. 1997). The inner surface of the 20S core contains strong hydrolytic activities that processively attack the peptide bonds of the trapped proteins, releasing short oligo-peptides as end products. The functional organization of the 19S cap is still not very well characterized because few subunits have been shown to have specific functions. Structurally, the 19S cap can be further divided into two components: a base complex, which is proximal to the 20S core, and a distal lid complex (Glickman et al. 1998). The base complex contains six ATPases that appear to assemble into a six-membered ring that forms the interface between the 19S cap and the 20S core. Functions of these ATPases

are not entirely clear. Crystal structure of the yeast 20S core indicates that the ends of the cylinder are closed (Groll et al. 1997). It is been proposed recently that the ATPases in the base of the 19S cap may be involved in the gating of the proteolytic channel within the 20S core (Larsen and Finley 1997). If the multiubiquitin chains target proteins for degradation by the 26S proteasome, it is conceivable that there should be components in the proteasome that recognize and bind the multiubiquitin chains. A 50kDa subunit called S5a of the 26S proteasome from human erythrocytes was first found to bind K48-linked ubiquitin chains (Deveraux et al. 1994). The yeast homolog is Rpn10 subunit of the 19S regulatory particle. Deletion of *RPN10* in yeast does not result in a dramatic phenotype (van Nocker et al. 1996), suggesting that Rpn10 may not be the only recognition component for ubiquitin conjugates within the 19S complex. Recent evidence indicates that a tetra-ubiquitin chain serves as the minimal targeting signal for degradation (Thrower et al. 2000). A one-step affinity purification scheme of yeast 26S proteasome was developed recently (Verma et al. 2000), and in vitro degradation assays demonstrate that the purified proteasome is sufficient to rapidly degrade multi-ubiquitinated Sic1 protein (R. Verma, personal communication).

The SCF ubiquitin ligases

G1- S transition and Sic1

Cells divide to pass genetic information from one generation to the next. This is achieved through a series of ordered events that make up the so called cell division cycle. The classical eukaryotic cell cycle consists of four phases: G1, S, G2, and M. During this period, two important goals are accomplished. First, DNA is accurately duplicated once and only once in the S phase. Second, the replicated chromosomes are faithfully segregated into the daughter cells in M phase (mitosis). The gap phases that occur before (G1) and after (G2) S phase are also tightly regulated to insure the normal progression of

the cell cycle. In G1 phase, cells continue to grow in size if the nutrient or growth factors are available. Meanwhile, molecular events take place inside the cells to prepare them for the next phase, DNA replication. When cells reach a point called Start in budding yeast, or Restriction Point in animal cells, they become committed to a new round of cell division. Once a cell has passed Start or Restriction Point, it will divide regardless of the nutrient conditions.

Classical cell cycle studies identified the molecular driving force of the cell cycle: a family of protein kinases known as the cyclin-dependent kinases (CDKs) (Murray and Hunt 1993). The activities of these kinases are modulated in part by their cyclin partners, which are unstable proteins whose expressions and levels change at different stages of the cell cycle. Cell cycle progression is dependent upon the activation and inactivation of specific CDKs. In budding yeast, the cell cycle is controlled by a single CDK called Cdc28, which can associate with specific cyclins at different stages of the cell cycle to form functionally distinct CDK complexes. Three partially redundant G1 cyclins, Cln1, Cln2, and Cln3 form G1 cyclin-CDKs that control events unique to G1 phase. Six B-type cyclins (Clbs) form Clb-Cdc28 CDK complexes that drive later events. Specifically, S-phase cyclins Clb5 and Clb6 are activated in late G1 to promote S phase entry, and Clb1-4 are activated later to control the G2 and M phase events.

At Start, cells have high activities of Cln/Cdc28, and inactive Clb/Cdc28. At G1 to S phase transition, S phase-inducing Clb5/Cdc28 and Clb6/Cdc28 complexes are activated. These Clb/Cdc28 kinases play important roles in DNA replication (Epstein and Cross 1992; Schwob and Nasmyth 1993).

How are S-phase cyclin-CDKs activated? Genetic studies have identified genes that are required for G1/S phase transition, including *CDC34* (Schwob et al. 1994), *CDC4* (Hereford and Hartwell 1974), *CDC53* (Mathias et al. 1996), and *SKP1* (Bai et al. 1996). These genes are essential, and cells carrying temperature-sensitive alleles of these

genes arrest at late G1 with large multibudded morphology and unreplicated DNA. Also, G1 cyclins are required for cells to traverse Start (Nasmyth 1993). The finding that Cdc34 is a ubiquitin-conjugating enzyme (Goebl et al. 1988) hinted at a link between the G1/S transition and ubiquitin-mediated proteolysis. The break-through came when elegant work from Nasmyth's laboratory revealed that *cdc34* mutants can not enter S phase because they fail to degrade the Sic1. Sic1 was identified as a 40 kDa protein that binds tightly to Cdc28 protein and inhibits its activity (Mendenhall 1993; Nugroho and Mendenhall 1994). It was also identified as a high copy suppressor of temperature-sensitive *dbf2* mutants that exhibit a mitotic arrest phenotype (Donovan et al. 1994). Work from Schwob et al. (1994) shows that Sic1 protein appears at the end of mitosis and disappears shortly before S phase, and it is a potent inhibitor of Clb, but not Cln forms of the Cdc28 kinase. Their results also indicate that Clb5/Cdc28 activity peaks around S phase and B-type cyclins are required for DNA replication. Taken together, these observations led to the hypothesis that Cdc34 promotes the ubiquitination of Sic1 and subsequent destruction, which is required for activation of B-type cyclin/CDKs and entry into S phase. Consistent with this, deletion of *SIC1* gene alleviates the inhibition of DNA replication in *cdc34*^{ts}, *cdc4*^{ts}, *cdc53*^{ts}, and *skp1*^{ts} mutants at restrictive temperature (Schwob et al. 1994; Bai et al. 1996), and rescues the inviability of *cln1 cln2 cln3* triple deletion mutant (Schneider et al. 1996; Tyers 1996). On the other hand, overexpression of a non-degradable Sic1 would block cell cycle transition at the G1/S boundary (Verma et al. 1997a).

Identification of the prototype SCF complex

Genetic analyses have implicated a role for *CDC4*, *CDC53*, and *SKP1* gene products at the G1/S transition. However, the sequences of these proteins do not shed much light on their functions. There is no sequence homology between any of these gene

products and the well-known E3 proteins, such as Ubr1 and E6-AP. Interestingly, *CDC34*, *CDC4*, *CDC53*, and *SKP1* interact genetically and physically. Pair-wise double mutants of *cdc34*, *cdc4*, and *cdc53* exhibit a synthetic lethal phenotype, and mutations in *CDC4* and *CDC34* can be suppressed by overexpression of *CDC53* (Mathias et al. 1996). *SKP1* was identified as a suppressor of $cdc4^{ts}$ mutant, and Skp1 protein binds tightly to Cdc4 directly through a motif in Cdc4 called the"F-box" (Bai et al. 1996). Taken together with the evidence that Cdc34, Cdc53, and Cdc4 co-purify in vivo (Mathias et al. 1996), it is likely that these four proteins are part of a protein complex that act in concert to promote G1/S transition in budding yeast.

Since Cdc34 is a ubiquitin-conjugating enzyme, it appeared attractive that components of the Cdc53, Cdc4, and Skp1 may serve as the ubiquitin ligase. To test this possibility, Deshaies laboratory set up an in vitro assay system to study the ubiquitination of Sic1 (Verma et al. 1997b). This system was based on fractionated yeast extracts as first described in Deshaies et al. (1995). In vitro analysis first identified Cdc34 and Cdc4 as necessary components for Sic1 ubiquitination (Verma et al. 1997c). Finally, purified Cdc34, Cdc4, Cdc53 and Skp1 were demonstrated to be sufficient to catalyze the ubiquitination of phosphorylated Sic1 in vitro (Feldman et al. 1997; Skowyra et al. 1997). The trimeric protein complex Skp1/Cdc53/Cdc4 (F-box protein) was named as SCF. Since it qualifies as an E3 according to classical definition, SCF complex appeared to be a novel family of ubiquitin ligases. I will now refer to the ubiquitination pathway defined by Cdc34/SCF as the "SCF pathway."

Identification of a new SCF subunit

Recent work from several labs identified a fourth subunit of SCF, referred as Roc1/Rbx1 in mammals, or Hrt1 in budding yeast (Kamura et al. 1999; Ohta et al. 1999; Seol et al. 1999; Tan et al. 1999). Hrt1 was purified from Skp1 and Cdc53

immunoprecipitates and identified by mass spectrometry (Seol et al. 1999). *HRT1* is essential, and *hrt1*^{ts} cells arrest at G1/S transition. Sic1 and G1 cyclin Cln2 are stabilized in *hrt1*^{ts} cells. Biochemical studies indicates that Hrt1 is important for SCF function because recombinant Hrt1 assembles into recombinant SCF complexes and potently stimulates in vitro ubiquitination reactions (Seol et al. 1999). Moreover, using Cdc34 autoubiquitination (Banerjee et al. 1993) as an assay, in vitro evidence suggests that Cdc53 and Hrt1 comprise a ubiquitin ligase module within the tetrameric SCF complex (Seol et al. 1999). Interestingly, Roc1/Rbx1/Hrt1 is homologous to the Apc11 subunit of the APC (Zachariae et al. 1998) in that they share a highly conserved zinc-binding RING-H2 domain. Similarly, the ubiquitin ligases (Ubr1 and E3α) involved in N-end rule also contain a putative RING finger (Kwon et al. 1998). Taken together, SCF and Ubr1 may represent divergent families of RING finger-based ubiquitin ligases. Furthermore, other RING finger proteins, such as SINA (Hu and Fearon 1999), Der3/Hrd1 (Bordallo et al. 1998), Mdm2 (Honda et al. 1997), and Rad18 (Bailly et al. 1997) could also be potential ubiquitin ligases and therefore increase the repertoire of this E3 family. Two recent examples of the RING finger type ubiquitin ligases are the tyrosine kinase negative regulator c-Cbl (Joazeiro et al. 1999) and IAPs (inhibitor of apoptosis) (Yang et al. 2000).

Architecture and Conservation of SCF complexes

The Skp1 binding site within Cdc4 is a degenerate 40 amino acid motif called F-box, after human cyclin F (Bai et al. 1994). Work done in parallel to that of Sic1 has found that Cdc34, Cdc53, and Grr1 are required for the degradation of G1 cyclins (Barral et al. 1995; Deshaies et al. 1995; Willems et al. 1996). G1 cyclins are inherently unstable, and their instability is critical for the proper regulation of cell cycle progression. Grr1 is required for glucose repression in budding yeast (Flick and Johnston 1991). Sequence analysis shows that Grr1 also carries an F-box motif. Some conditional alleles

of *skp1* result in stabilization of Cln2 (Bai et al. 1996), suggesting that Skp1 is also required for G1 cyclin degradation. Sic1 and Cln2 degradation pathways appeared to share the same core components (Cdc53, Cdc34, and Skp1), but differ in the F-box protein (Cdc4 versus Grr1). Database search in budding yeast genome has identified 17 proteins that carry an obvious F-box motif (Patton et al. 1998b), and at least three (Cdc4, Grr1, and Met30) have been shown to assemble into functional complexes in vivo (Patton et al. 1998a). It is proposed that multiple SCF complexes exist in vivo, and they differ in the F-box proteins, which recruit specific substrates to Skp1 and the ubiquitination machinery (Bai et al. 1996). This idea is referred to as the "F-box hypothesis". Distinct SCF complexes are often designated by their F-box receptors (e.g. SCF^{Cdc4}, SCF^{Grr1}, and SCF^{Met30}). Besides G1 cyclins, Gic2, a protein involved in actin polarization at bud emergence (Brown et al. 1997), has been shown to be targeted for degradation by the SCF^{Grr1} complex (Jaquenoud et al. 1998). *MET30* is an essential gene involved in sulfur amino acid metabolism (Thomas et al. 1995). SCF^{Met30} complex have been implicated in promoting the degradation of the CDK-inhibitory kinase Swe1 (Kaiser et al. 1998) and transcription activator Met4 (Rouillon et al. 2000). Recent evidence, however, suggest that SCF^{Met30}-dependent ubiquitination of Met4 may not lead to its proteolysis (Kaiser et al. 2000).

F-box proteins are found in higher organisms as well, suggesting that SCF functions are conserved. Examples are Skp2 (Zhang et al. 1995; Marti et al. 1999), β-TrCP (Margottin et al. 1998), and NFB42 (Erhardt et al. 1998) in humans. SEL-10 in *C. elegans* (Hubbard et al. 1997), slimb in Drosophila (Jiang and Struhl 1998), and UFO in *A. thaliana* (Lee et al. 1997).

Skp1 is a small (163 amino acid) and highly conserved protein that serves as a linker in SCF complexes by binding to both the F-box receptor proteins and the Cdc53/Hrt1 ubiquitin ligase modules. Interesting, Skp1 has been also been found to be

part of other complexes that are distinct from SCF. These include the centromere binding protein complex CBF3 (Connelly and Hieter 1996) and RAVE, a regulator of V-ATPase assembly (Seol et al. 2001). A human Skp1-like molecule Elongin C is part of the VHL (von Hippel-Lindau) complex involved in transcription elongation (Lonergan et al. 1998).

Cdc53 is a large protein (814 amino acids) whose function has been most elusive. It was proposed that Cdc53 serves as a scaffold protein for multiple Cdc34/Skp1/F-box protein complexes (Patton et al. 1998a). However, recent evidence suggests that Cdc53, together with Hrt1, acts as a RING-H2 based ubiquitin ligase module that activates ubiquitin transfer from Cdc34 to substrates (Seol et al. 1999). The counterparts of Cdc53 in higher organisms includes Cul-1 in C. elegans (Kipreos et al. 1996) and Cul1-5 in humans (Deshaies 1999). SCF is therefore sometimes referred to as Skp1/Cullin/F-box protein.

Hrt1, despite being the smallest subunit of SCF so far, appears to bind Cdc53, the F-box proteins Grr1 and Cdc4, and also in a direct way to the E2 Cdc34 (Kamura et al. 1999; Seol et al. 1999; Skowyra et al. 1999). The mechanism behind the stimulatory effect of Hrt1 in SCF-dependent ubiquitination reactions is not clear, but may have something to do with its interaction with the E2 enzyme.

It is noteworthy that although existing evidence suggests that in budding yeast Cdc34 is the primary E2 that forms ubiquitination machinery with SCF complexes, there may be other E2-SCF combinations. The human SCF appears to collaborate with hUBC5 in ubiquitinating IκBα (Yaron et al. 1998; Spencer et al. 1999).

The role of substrate phosphorylation

The link between substrate phosphorylation and ubiquitin-dependent degradation via SCF pathway (referred to earlier as Cdc34 pathway) was first suggested by the

observations that degradation of G1 cyclins and the Cdc34-dependent ubiquitination of Cln2 in vitro requires Cdc28 activity (Deshaies et al. 1995; Yaglom et al. 1995).

More evidence came from studies of the CDK inhibitor Sic1, which is stable in early G1 cells but is abruptly degraded at the G1-S transition. What primes Sic1's destruction? Genetic studies indicate that Cln/Cdc28 kinase activity is required for Sic1 degradation because Sic1 is accumulated in cdc28 mutants and CLN-depleted cells (Schwob et al. 1994; Schneider et al. 1996). What is the exact role of Cln/Cdc28 in Sic1 turnover? In vitro ubiquitination studies of Sic1 provided biochemical evidence that phosphorylation of Sic1 by Cln/Cdc28 is required for the multi-ubiquitination of Sic1 (Verma et al. 1997c). Once Sic1 is phosphorylated, it can be ubiquitinated in a Cdc34-dependent manner in the absence of Cln2/Cdc28 activity (Verma et al. 1997c). Mutation of critical CDK phosphorylation sites in Sic1 not only dramatically reduces Sic1 ubiquitination in vitro but also stabilizes it in vivo (Verma et al. 1997a). Taken together, these results suggest that phosphorylation of Sic1 by Cln/Cdc28 is both necessary and sufficient to trigger its ubiquitination and subsequent degradation.

Why does Sic1 have to be phosphorylated in order to be ubiquitinated by Cdc34/SCF? The answer came when Sic1 ubiquitination was reconstituted in vitro using a defined set of purified protein components. Phosphorylation of Sic1 is necessary for recognition by the SCF complex, specifically by the F-box protein Cdc4 because Cdc4 binds only to phosphorylated, but not unphosphorylated, Sic1 (Feldman et al. 1997; Skowyra et al. 1997). Similarly, phosphorylation of Cln2 by Cdc28 kinase, its cognate CDK subunit, is required for its rapid turnover in vivo (Lanker et al. 1996). Grr1 binds selectively to phosphorylated Cln1 and Cln2 (Skowyra et al. 1997). Reconstitution of Cln2 using purified SCF^{Grr1} was made possible after the discovery of Hrt1 (Seol et al. 1999; Skowyra et al. 1999). It appears that substrate phosphorylation serves as a signal for the recognition and binding of the SCF complexes.

Besides Sic1 and G1 cyclins, phosphorylation by Cdc28 kinase is also required for the rapid turnover of CDK inhibitor Far1 (Henchoz et al. 1997) and replication initiation factor Cdc6 (Elsasser et al. 1999). Whereas Cln2/Cdc28 promotes the ubiquitination of Far1 in vitro, Clb5/Cdc28 is the preferential kinase that promotes the ubiquitination of Cdc6. In vitro assays using fractionated yeast extracts also show that Cdc34-dependent ubiquitination of Far1 and Cdc6 also requires Cdc4, the F-box subunit of SCF^{Cdc4} (see Chapter 2). Far1 and Cdc6 turnover have been shown to be dependent on SCF^{Cdc4} (Drury et al. 1997; Henchoz et al. 1997). Gcn4, a transcription factor involved in general control of amino acid biosynthesis, is also degraded in a SCF^{Cdc4}-dependent manner in vivo (Meimoun et al. 2000), and is ubiquitinated by SCF^{Cdc4} in vitro (see Chapter 3). Gcn4 degradation in vivo and ubiquitination in vitro also require CDK phosphorylation. Interestingly, Cdc28 is not required. Instead, Pho85 CDK, a kinase involved in phosphate metabolism, has been implicated to be required for Gcn4 turnover (Meimoun et al. 2000). Biochemical studies of Gcn4 ubiquitination in vitro also identified Srb10 CDK, a component of the RNA polymerase II holoenzyme, as a potent kinase that phosphorylates Gcn4 and promotes Gcn4 turnover by the SCF^{Cdc4} pathway. Mutation of CDK phosphorylation sites on Gcn4 greatly stabilizes Gcn4. Our work and the work from Meimoun et al. 2000 suggest that more than one kinase may be needed to insure the rapid turnover of key regulatory proteins. Similarly, Pho85 CDK has been implicated to promote Sic1 degradation in addition to Cdc28 CDK (Nishizawa et al. 1998).

So far, substrate phosphorylation (by a CDK) appears to be a common theme for SCF recognition and targeting, at least for SCF^{Cdc4}. Whether it is applicable all SCF^{Cdc4} substrates or to SCF complexes containing other F-box proteins remains to be seen. One indication that substrate phosphorylation may be a conserved feature for SCF targeting came from in vitro reconstitution of IκBα ubiquitination. The human F-box protein β-

TrCP (E3RS) binds specifically to phosphorylated IκBα to promote its ubiquitination by $SCF^{\beta\text{-}TrCP}$ (Yaron et al. 1998; Spencer et al. 1999; Winston et al. 1999).

It is still unclear how F-box receptors bind phosphorylated substrates. One possibility is that they directly bind to the phosphate epitopes. The other possibility is that they bind to other regions of the substrate molecule. The former possibility is supported by the evidence that a short IκBα phosphopeptide can compete with full length IκBα for binding to β-TrCP (Yaron et al. 1998).

Ubiquitin-dependent degradation of transcription factors

Besides the cell cycle regulators discussed above, many transcription factors are also regulated by ubiquitin-dependent degradation. The intracellular levels of proteins are often controlled at the transcriptional level, which critically depends on the rate of transcriptional initiation. It is conceivable that precise modulation of the levels of specific transcripts therefore requires tight regulation of the transcription factors. The rapid and irreversible features of the ubiquitin-dependent degradation make it an attractive means of transcriptional regulation. A few yeast transcription factors are known to have very short half-lives (3-5 min). These include Matα2 (Hochstrasser and Varshavsky 1990) and Gcn4 (Kornitzer et al. 1994). The mammalian transcription factors c-Fos (Curran et al. 1984) and c-myc (Luscher and Eisenman 1988) have half-lives of about 20 min. So far, most short-lived transcription factors are degraded through the ubiquitin-proteasome pathway. Several examples will be discussed below with the emphasis on the yeast transcription activator Gcn4. My work (see Chapter 3) and work from Meimoun et al. (2000) demonstrate that $Cdc34/SCF^{Cdc4}$ pathway ubiquitinates Gcn4 and targets it to rapid degradation in vivo.

Introduction to Gcn4

Gcn4 is a well-studied yeast transcription activator involved in the activation of amino acid and purine biosynthetic genes (Hinnebusch 1992). Yeast cells have developed a complex system to regulate the amino acid biosynthetic genes. The expression of at least 35 genes encoding enzymes in 12 different amino acid biosynthetic pathways is co-regulated such that when cells are starved for any one of a different number of amino acids, the expression of many genes from different pathways is derepressed. This cross-pathway regulation is referred to as the "general amino acid control" (Delforge et al. 1975). Genetic studies of general amino acid control have identified positive and negative regulators. *GCN* (general control non-derepressible) genes are positive regulators. Among these, GCN4 is the most proximal positive effector of gene expression. Besides amino acids, Gcn4 has also been found to regulate a gene involved in purine biosynthesis (Mosch et al. 1991).

Gcn4 has 281 amino acids which harbor two functional domains. The transcriptional activation domain was original thought to be located in a 19 acidic amino acid stretch central in the molecule (Hope and Struhl 1986). However, recent evidence suggest that multiple hydrophobic regions of Gcn4 contribute redundantly to Gcn4's transcriptional activation function (Drysdale et al. 1995; Jackson et al. 1996). The DNA binding domain is a 60 amino acid stretch that forms a basic leucine zipper. The basic region of Gcn4 makes contact with DNA, and mutations in the conserved residues abolishes Gcn4 DNA-binding in vitro and activity in vivo (Pu and Struhl 1991). The leucine zipper is required for Gcn4 dimerization, and the crystal structure of a C-terminal peptide indicates that Gcn4 leucine zipper is a classical parallel coiled-coil (O'Shea et al. 1991). Crystal structure of the protein-DNA complex shows that Gcn4 basic region binds DNA as a dimer (Ellenberger et al. 1992). Gcn4 synthesized in vitro binds to *HIS3* regulatory sequences, providing the first evidence that Gcn4 is a DNA-binding

transcription factor (Hope and Struhl 1985). The C-terminal 60 amino acids of Gcn4 are sufficient to form a homodimer that binds target DNA. Furthermore, the stable Gcn4 dimers can be formed in the absence of DNA (Hope and Struhl 1987).

Mutational analyses of a few amino acid biosynthesis genes, including *HIS3*, *HIS4*, *TRP4*, and *TRP5* have established that a short nucleotide sequence located upstream of the start sites of these genes mediates their transcriptional activation by Gcn4 in response to amino acid starvation. This sequence, about 12 base pairs long with a highly conserved hexanucleotide core TGACTC, has been shown to be the binding site for Gcn4 (Hope and Struhl 1985; Arndt and Fink 1986). The completion of the yeast genome project allows the potential characterization of a full spectrum of Gcn4 target genes. A recent computer search program using the Gcn4 binding site element has found that about 1% of the genes in the yeast genome carry three or more Gcn4-binding sites in their promoter region (Schuldiner et al. 1998). More than 50% of these genes are involved in amino acid and nucleotide metabolism. Other potential targets of Gcn4 include genes involved in transcription and cell growth.

How is Gcn4 regulated? Studies from Hinnebusch and workers in the 1980s have shown the Gcn4 expression is regulated mainly at the translational level. The 5' leader of *GCN4* mRNA contains four small uORFs (upstream open reading frames). These uORFs are essential for repression of *GCN4* to restrict the flow of scanning ribosomes to reach the GCN4 start codon when amino acids are abundant. However, the 5' proximal AUG condons play a positive role in GCN4 expression under amino acid starvation conditions (Mueller and Hinnebusch 1986). A deletion of all four uORFs results in high constitutive GCN4 expression independent of factors that normally regulate its expression (Hinnebusch 1984; Thireos et al. 1984), consistent with their role in translational control of *GCN4*. Most yeast genes do not have even a single uORF, and insertion of a uORF in the mRNA leader of a gene invariably reduces the translation of the downstream genes.

These observations suggest that the eukaryotic ribosome scanning model for translation initiation (Kozak 1978) also operates in yeast. What is the molecular mechanism governing the translational control of *GCN4* expression by uORFs? The identification of Gcn2 as a protein kinase involved in *GCN4* translation provided the first clue (Roussou et al. 1988; Wek et al. 1990). Shortly after, it was found that Gcn2 phosphorylates the α subunit of the eukaryotic translation initiation factor2 (eIF-2α) and thereby activates *GCN4* translation under amino acid starvation conditions (Dever et al. 1992).

The C-terminal sequence of Gcn4 is similar to that of mammalian AP-1 transcription factors. Gcn4 shares 44% and 28% identities with the Jun and Fos proteins respectively over the 70 C-terminal residues of Gcn4 (Vogt et al. 1987). Mammalian AP-1 transcription factors have been shown to regulate the UV response, which also involves the Ras signaling pathway (Karin 1998). Interestingly, UV irradiation of yeast leads to increased translation of Gcn4 mRNA in a Gcn2-independent manner and a post-translational event (Engelberg et al. 1994), suggesting a conservation of UV response pathway between yeast and mammals. Recently, it has been shown that *RPN11*, a gene encoding a regulatory subunit of the 26S proteasome, is required for the activation of *GCN4* target genes through an amino acid starvation-independent pathway (Stitzel et al. 2001).

Ubiquitin-dependent degradation of Gcn4

Although Gcn4 is one of the best-studied transcription factors in yeast, most of the work in the past two decades has been focused on its structure and translational control. Very little attention has been given to the post-translational control mechanisms of Gcn4. One study by Kornitzer et al. (1994) provided the first indication that Gcn4 may be regulated tightly by post-translational mechanisms such as ubiquitin-mediated degradation. In this work, they show that Gcn4 is a short-lived protein with a half-life of

5 min or less. The reason why Gcn4 is short lived is not clear. Strong overexpression of Gcn4 (e.g. under the *GAL* promoter) significantly slows cell growth (my unpublished observations), suggesting a toxic effect possibly due to wasteful biosynthesis of unneeded compounds, or a "squelching" effect (Gill and Ptashne 1988) on the transcription machinery. There may be cis-acting sequences that make Gcn4 inherently unstable. A recent study on the stability of artificially constructed transcription activators shows that the rate of degradation of activators correlates with their activation domain potency (Molinari et al. 1999). By this model, Gcn4 may be a potent activator and therefore gets degraded rapidly. In any event, it is clear that the intracellular concentration of Gcn4 is tightly regulated.

In the report by Kornitzer et al. (1994), Gcn4 was found to be ubiquitinated in vivo, and a mutant in the proteasomal regulatory subunit Cim5 inhibits Gcn4 degradation, indicating that Gcn4 is degraded by the ubiquitin-proteasome pathway. Consistent with this, they also found that Gcn4 is stabilized in a *cdc34* or *rad6Δ* mutant, suggesting that Cdc34 or Rad6 ubiquitin conjugating enzymes may be directly involved in ubiquitinating Gcn4. Although Rad6 is sufficient to ubiquitinate Gcn4 in fractionated yeast extracts (my unpublished observations), this Rad6-dependent ubiquitination pathway is not yet characterized. A novel ubiquitin ligase may associate with Rad6 in this reaction because *UBR1* is not required for rapid turnover of Gcn4 (Kornitzer et al. 1994). Moreover, the physiological role of the Rad6 in Gcn4 turnover is not clear. It appears, however, that the Rad6-dependent degradation of Gcn4 contributes less than the Cdc34 pathway in vivo (see Chapter 3). The finding of Cdc34-dependent degradation of Gcn4 is particularly interesting because the known Cdc34 substrates (G1 cyclins, Cdc6, Far1, and Sic1) are all cell cycle regulators. Study of the mechanism and regulation of Gcn4 ubiquitination provides a different angle in addressing the generality of the Cdc34-dependent (later known as SCF) ubiquitination pathway.

My thesis work (detailed in Chapter 3) started with the attempt to identify factors necessary for Gcn4 ubiquitination in vitro, and it paralleled with the effort to reconstitute Sic1 ubiquitination. Through biochemical purification and an educated guess, I identified Srb10 as a kinase that phosphorylates Gcn4 and promotes its ubiquitination in vitro. With the identification of SCF, I was able to reconstitute Cdc34/SCF^{Cdc4}-dependent Gcn4 ubiquitination using fully purified protein components. Srb10/Srb11 is a cyclin/CDK pair that associates with the RNA polymerase II holoenzyme (Liao et al. 1995). Gcn4 is phosphorylated in vivo in Srb10-dependent manner and is partially stabilized in *srb10* mutants, indicating that Srb10 CDK contribute to the rapid degradation of Gcn4 in vivo. Furthermore, mutations of CDK phosphorylation sites on *GCN4* significantly stabilize the protein, demonstrating that degradation is mediated through CDK phosphorylation.

Parallel to my work, Kornitzer and co-workers provided genetic evidence that Gcn4 is degraded in an SCF^{Cdc4}-dependent manner in vivo (Meimoun et al. 2000). Interestingly, they found that Pho85 is required for the rapid degradation of Gcn4. Pho85 is a CDK involved in phosphate assimilation and glycogen synthesis (Kaffman et al. 1994; Huang et al. 1998). However, Gcn4 is only partially stabilized in a *pho85Δ* mutant. My subsequent work shows that Gcn4 is nearly completely stabilized in a *srb10Δ pho85Δ* double mutant, suggesting Srb10 and Pho85 contribute redundantly to Gcn4 turnover in vivo. Taken together, these results indicate that the mechanisms of ubiquitination of Gcn4 and Sic1 differ in two ways. First, the kinase that targets their ubiquitination is different: Sic1 utilized G1 cyclin-CDK whereas Gcn4 utilizes Srb10 and Pho85 CDKs. Second, whereas Cdc28 is likely to be the only physiological kinase that targets Sic1 degradation, two different CDKs are required to promote rapid Gcn4 degradation in vivo. Differential targeting by substrate specific kinases therefore may not only allow a single SCF pathway to simultaneously control multiple cellular processes but also make these kinases attractive targets for regulation in the degradation pathway.

If both Srb10 and Pho85 CDKs target Gcn4 for SCF-dependent degradation, then what are the roles of each of the kinases? Gcn4 turnover appears to be constitutive when cells are grown in rich medium. However, it is stabilized upon amino acid starvation (Kornitzer et al. 1994). A recent report by Meimoun et al. (2000) shows that Gcn4 is stabilized through the down regulation of Pho85 and the loss of phosphorylation of Gcn4 at T165, which appears to be necessary for rapid Gcn4 turnover. This suggests that the Pho85-dependent degradation pathway is responsive to amino acid starvation signals. In contrast, our studies show that Srb10-dependent phosphorylation of Gcn4 is largely intact, and loss of Srb10 further stabilizes Gcn4 even under starvation conditions. This suggests that Srb10-dependent phosphorylation and degradation of Gcn4 are not regulated through amino acid starvation, and may represent a constitute pathway that promotes Gcn4 turnover. Srb10 CDK is part of the RNA polymerase II holoenzyme, which is recruited to the vicinity of the promoters of target genes during activated transcription. It has been shown that Gcn4 interacts with the RNA polymerase holoenzyme components at least in vitro (Drysdale et al. 1998; Natarajan et al. 1999). It therefore seems plausible that the role of Srb10-dependent phosphorylation and degradation of Gcn4 is to limit its duration at the promoters. Pho85 is a nuclear CDK and there has been no reports suggesting that it may be localized to DNA. Multiple cyclins form CDK complexes with Pho85 in vivo (Andrews and Measday 1998). The Pho85 CDK formed by the cyclin Pcl1 has been shown to phosphorylate Gcn4 in vitro (Meimoun et al. 2000). However, there is no evidence that phosphorylation of Gcn4 by Pcl1/Pho85 is sufficient to promote SCF-dependent ubiquitination in vitro. Furthermore, Gcn4 is not stabilized in a *pcl1Δ* mutant or several other Pho85 associated cyclin mutants (Meimoun et al. 2000), suggesting the multiple Pho85 CDK may redundantly contribute to Gcn4 turnover in vivo.

Our recent preliminary results (see Chapter 4) show that a DNA-binding mutant of Gcn4 is significantly stabilized in vivo with a half-life of about 30 min, ten-fold longer than that of the wild-type Gcn4. This raises the intriguing possibility that only promoter-bound Gcn4 molecules are subjected to the negative regulation by degradation, and phosphorylation of Gcn4 may be confined to the vicinity of the promoter DNA of target genes. Interestingly, this DNA-binding mutant of Gcn4 is not further stabilized to an appreciable extent in an *srb10Δ* or a *pho85Δ* mutant, suggesting both CDKs target promoter-bound Gcn4. The exact role of each CDK is still not clear at this moment. Conceivably, they may target different pools of Gcn4 molecules or respond to different cellular signals.

Ubiquitin-dependent degradation of other transcription factors

Met4

Met4 is a basic leucine zipper transcription factor similar to Gcn4. It is the major transcription activator of many genes involved in sulfur amino acid metabolism (Thomas and Surdin-Kerjan 1997). It was reported that the stability of Met4 depends on the concentrations of extracellular methionine. At repressive concentrations, Met4 has a short half-life (<10 min), and its degradation is dependent on SCF^{Met30} (Rouillon et al. 2000). However, a different report shows that Met4 is a stable molecule and the SCF^{Met30} –dependent ubiquitination of Met4 does not promote its turnover. Instead, this modification appears to directly inactivate Met4 (Kaiser et al. 2000). Kaiser et al. (2000) shows that Met4 is ubiquitinated in vivo, and the F-box protein Met30 binds to Met4 and is required for Met4 ubiquitination in vitro. Furthermore, Met4 ubiquitination in vivo appears to result in the attachment of short ubiquitin chains. Although this report reveals a potential proteolysis-independent function for Cdc34/SCF, it remains to be seen whether this novel function is general.

Matα2

The mating type (*MAT*) locus of budding yeast encodes transcription regulators that determine the cell type. Matα2 is a transcription repressor of **a** cell-specific genes in α cells or a/α cells. It is a short-lived protein (Hochstrasser and Varshavsky 1990), and the ubiquitin-dependence of Matα2 degradation was first suggested by the observation that Matα2 is multiubiquitinated in vivo (Hochstrasser et al. 1991). Subsequently, it was shown that four ubiquitin-conjugating enzymes, Ubc4, Ubc5, Ubc6, and Ubc7, are required for rapid degradation of Matα2 (Chen et al. 1993). These Ubc enzymes can be assigned to two separate ubiquitination pathways or complexes that work via distinct structural elements in Matα2. Ubc6 and Ubc7 can physically associate and define one of the pathways that target the *Deg1* degradation signal of Matα2 (Chen et al. 1993). A recent report demonstrates that the degradation signal of Matα2 confers nuclear-specific turnover to a reporter protein (Lenk and Sommer 2000). These observations suggest that the half-life of a short-lived protein may be determined by the utilization of both combinatorial ubiquitination machinery and the compartment-specific turnover.

c-Fos and c-Jun

The proto-oncogene products c-Fos and c-Jun heterodimerize through their leucine zippers to form the AP-1 transcription factor, which is involved in cell proliferation following mitogenic stimuli (Angel and Karin 1991). Both c-Fos and c-Jun have relatively short half-lives (about 20 and 90 min, respectively) as opposed to their oncogenic counterparts v-Fos and v-Jun (Papavassiliou et al. 1992; Treier et al. 1994). This relative stability may account at least partly for their transforming potential. Degradation of c-Fos in crude reticulocyte extracts (Papavassiliou et al. 1992) or by purified 26S proteasome (Tsurumi et al. 1995) requires the presence of c-Jun. Tsurumi et

al. (1995) also shows that multiple protein kinases, including mitogen-activated protein kinase (MAPK) and CDC2 kinase, stimulate the degradation of c-Fos. c-Fos degradation is mediated by ubiquitin pathway in vivo because it is stabilized in a E1 mutant cell line (*ts*20), and the E2 enzymes E2-F1 or UbcH5 and a novel E3 enzyme promote c-Fos ubiquitination in vitro (Stancovski et al. 1995). The in vivo ubiquitination of c-Jun was detected first by Treier, et al. (1994). They showed that c-Jun degradation is mediated by the δ domain in c-Jun. MAPK phosphorylates c-Jun on sites in the vicinity of the δ domain (Pulverer et al. 1991). Phosphorylation of c-Jun by MAPK has been shown to reduce the ubiquitination of c-Jun and increase its stability (Musti et al. 1997). In this respect, degradation of c-Jun is quite different from that of yeast Gcn4.

MyoD

MyoD is a basic helix-loop-helix (bHLH) transcription factor involved in the activation of genes encoding skeletal muscle-specific proteins. It has a half-life of 30-60 min (Thayer et al. 1989; Song et al. 1998). MyoD can be ubiquitinated by E2-F1 and a novel E3 enzyme, E3L (Gonen et al. 1996). MyoD is degraded by the ubiqutin system in vivo (Abu Hatoum et al. 1998; Breitschopf et al. 1998), and its ubiquitination and degradation in vitro is inhibited by DNA containing MyoD binding sites (Abu Hatoum et al. 1998). Breitschopf et al. (1998) shows that the N-terminal residue of MyoD is necessary and sufficient for the ubiquitin conjugation and subsequent degradation of the protein. MyoD contains several consensus CDK phosphorylation sites. Song et al. (1998) found that S200 is required for MyoD hyperphosphorylation and degradation in vivo. Interestingly, they also found that the turnover of MyoD appears to require the cognate Cdc34 ubiquitin-conjugating enzyme. Their findings suggest a similar degradation mechanism for MyoD and yeast Gcn4. It remains to be seen if MyoD is targeted by the SCF-like ubiquitin ligase in vivo.

E2F-1

E2F-1 is a cell cycle regulated transcription factor that can act as both an oncoprotein and tumor suppressor protein. It was shown that E2F-1 is degraded by the ubiquitin system (Hofmann et al. 1996; Campanero and Flemington 1997). However, the mechanism and regulation of this process was elusive until SCF complexes were identified. Marti et al. (1999) show that E2F-1 accumulates in G1 phase, but is rapidly degraded in S/G2 phase, and this degradation correlates with the accumulation of SKP2, an F-box protein. E2F-1 interacts with CUL1 and SKP2 in vitro, and mutations in E2F-1 that abolish interaction with SKP2 in vitro leads to reduced ubiquitination of E2F-1 and stabilizes the protein in vivo (Marti et al. 1999). These observations suggest that the SCF^{SKP2} ubiquitination pathway may promote E2F-1 degradation at the S/G2 phase. There is no evidence that E2F-1 phosphorylation is necessary for binding to SKP2, unlike other interactions between substrates and F-box proteins. However, there is evidence that TFIIH-mediated phosphorylation of E2F-1 plays a role in triggering E2F-1 degradation during S phase (Vandel and Kouzarides 1999). The role of this phosphorylation event is unclear. Recent evidence indicates that E2F1 can be ubiquitinated in vitro by Ubc5 and multiple ROC-cullin ligases (Ohta and Xiong 2001). Furthermore, in vitro ubiquitination of E2F-1 by CUL1-ROC1 ligase does not require E2F-1 phosphorylation, and the SKP1 subunit.

In summary, mechanism and regulation of ubiquitin-mediated degradation of many transcription factors are still not very clear at this moment, especially in the case of higher eukaryotes. It appears that distinct pathways may specify the turnover of specific type of transcription factors. Although the mechanism and regulation of higher eukaryotic transcription factors appears more complex, some features observed in yeast

studies are conserved for certain mammalian proteins. These include the substrate phosphorylation requirement for MyoD and SCF–targeting for E2F-1.

Regulation of transcription factors by localization

Overview

Although ubiquitin-mediate degradation serves as an effective way of negative regulation of short-lived transcription activators, many of them are quite stable. Recent studies from several stable transcription factors indicate that transcription activators are also regulated at the level of nuclear transport (Komeili and O'Shea 2000). The β-importin family of nuclear transport receptors is responsible for the nucleocytoplasmic transport of most proteins. The yeast nuclear export receptors include Crm1 (hCRM1 in humans) and Msn5. The import receptors include Kap95 (Importin β in humans) and Pse1 (Ohno et al. 1998). CRM1 functions in the export of proteins that carry a specific, leucine-rich type of nuclear export signal (NES), and Importin β functions in the import of proteins that carry a classical nuclear localization signal (NLS). An important feature of the nucleocytoplasmic transport is directionality. This is achieved through Ran, a small GTPase, and its regulators. Ran's regulators are distributed asymmetrically across the nuclear envelopes such that GTPase activity is favored in the cytoplasm and GDP/GTP exchange in the nucleus. As a result, nuclear Ran is in the GTP-bound state whereas cytoplasmic Ran is GDP-bound. The directionality of the nuclear transport depends on this RanGTP gradient.

Transcription factors are synthesized in the cytoplasm and transported into the nucleus to activate the transcription of their target genes. The nuclear concentration of these transcription factors are modulated by the rates of import and export such that low nuclear concentration of these transcription factors are maintained when the expression of the target genes is no longer needed. Recent studies on the regulation of several

transcription factors have shed light on the molecular mechanism governing the localization of transcription activators. It is important to note that localization is not the only means by which the activity of a transcription factor is regulated, and nuclear concentration of a transcription factor does not necessarily correlate with transcriptional activation of its target genes. Other layers of regulation may include interactions with other transcription factors, post-translational modifications, and DNA binding.

Regulation of Pho4

The best-studied example is the yeast transcription factor Pho4. Pho4 is a bHLH transcription factor involved in the phosphate starvation response (Oshima 1997). Under limiting phosphate conditions, Pho4 interacts with the homeodomain transcription factor Pho2 to activate the transcription of *PHO5* gene, which encodes a secreted acid phosphatase. Genetic studies have identified both *PHO80* and *PHO85* as negative regulators of *PHO5* gene (Ueda et al. 1975). Much of our understandings of Pho4 regulation has come from a series of elegant work carried out by O'shea and colleagues. First, Pho80 and Pho85 were found to be a cyclin/CDK complex that phosphorylates Pho4 when yeast cells are grown in phosphate rich medium, and this phosphorylation correlates with negative regulation of *PHO5* (Kaffman et al. 1994). Later, it was found that Pho4 is concentrated in the nucleus when yeast cells are starved for phosphate and is predominantly cytoplasmic when cells are grown in phosphate-rich medium. Mutation of Pho80/Pho85 phosphorylation sites on Pho4 leads to nuclear concentration of Pho4 and partially constitutive expression of *PHO5* in phosphate-rich medium (O'Neill et al. 1996), suggesting that phosphorylation of Pho4 by Pho80/Pho85 promotes its nuclear export. Subsequently, the import and export receptors for Pho4 were identified. Pho4 is imported by Pse1, which associates preferentially with the unphosphorylated form of Pho4 in vitro (Kaffman et al. 1998b). Pho4 is exported by Msn5, which associates

exclusively with the phosphorylated form of Pho4 in the presence of the GTP-bound form of yeast Ran in vitro (Kaffman et al. 1998a). Finally, analysis of the Pho4 phosphorylation sites reveals that these sites have distinct roles in regulating the activity of Pho4 (Komeili and O'Shea 1999). Komeili et al. (1999) shows that phosphorylation of Pho4 on two sites is necessary and sufficient to promote export of Pho4 in vivo and to promote its interaction with Msn5 in vitro. Phosphorylation of a third site blocks its interaction with Pse1 and prevents its nuclear import. Phosphorylation of the fourth site blocks the interaction of Pho4 with Pho2. Taken together, regulation of localization is only a part of the multi-layer regulatory system acting on Pho4.

Regulation of Msn2

Msn2 and Msn4 are two partially redundant zinc finger transcription factors that bind to promoters that carry stress response elements (STREs) and activate the expression of a large number of genes involved in stress response and diauxic shift (Martinez-Pastor et al. 1996; Schmitt and McEntee 1996; Boy-Marcotte et al. 1998). Similar to Pho4, Msn2 was also found to be regulated by localization. Msn2 is predominantly cytoplasmic under normal conditions, but is rapidly relocalized to the nucleus when exposed to one of several stress condition, such as heat, ethanol, and osmotic shocks (Gorner et al. 1998). Recent evidence suggests a role for the TOR (target of rapamycin) signalling pathway in promoting the cytoplasmic retention of Msn2/Msn4 through their binding to the cytoplasmic 12-3-3 protein BMH2 (Beck and Hall 1999). The mechanism of nuclear export of Msn2/Msn4 is not as clear as that of Pho4. Gorner et al. found that nuclear localization of Msn2/Msn4 correlated inversely to cAMP levels and protein kinase A (PKA) activity. They also show that mutation of conserved PKA phosphorylation sites abolished cytoplasmic localization of Msn2 in unstressed cells. However, there is no evidence for PKA-dependent phosphorylation of Msn2 in vitro or in vivo. It remains

possible that PKA affects Msn2 localization indirectly. Microarray analysis of genomewide gene expression in *srb10-3* cells has identified 173 genes whose expression are up two-fold more (Holstege et al. 1998), and many of theses genes are involved in stress response and diauxic shift. This raises the possibility that derepression of these genes may be due to deregulation of the Msn2/Msn4 transcription factors when Srb10 function is lost. To test this, we looked at Msn2 localization in *srb10-3* cells under unstressed conditions. The result shows that a subpopulation of the cells exhibits nuclear concentration of Msn2 in *srb10-3* cells, indicating that the tight nuclear exclusion of Msn2 normally seen in wild type cells is compromised. This effect is likely to be direct because Msn2 is phosphorylated by Srb10 in vitro and phosphorylated in vivo in an *SRB10*-dependent manner in vivo when cells are stressed. Similar to Pho4, Msn2 is also exported by the nuclear export receptor Msn5. A key question then is whether Msn4 export is mediated by Srb10-dependent phosphorylation. Mapping and mutating Srb10 phosphorylation sites on Pho4 will be necessary to address this question conclusively. It will be interesting to see how much more similarities do Msn2 and Pho4 share in terms of the molecular mechanism of their regulation. For example, is Pho80/Pho85 involved in Msn2 export? Does the cytoplasmic localization of Pho4 also involve retention through a 14-3-3 protein?

Regulation of NF-AT

Although examples of transcription factor regulation by localization have also been found in higher eukaryotes, the mechanisms appear quite different. For example, the transcription factor NF-AT (nuclear factor of activated T-cells) has been found to be regulated by NES masking. NF-AT is a cytoplasmic transcription factor involved in the induction of cytokine genes during T-cell activation. It carries both a NLS and a NES, and its localization is regulated in response to intracellular calcium levels (Hogan and

Rao 1999). Under resting conditions, calcium levels are low and NF-AT is phosphorylated. Phosphorylation masks its NLS and localizes it to the cytoplasm. A rise in intracellular calcium levels activates the phosphatase calcineurin, which then binds and dephosphorylates NF-AT. Dephosphorylation unmasks the NLS, allowing the import of NF-AT into the nucleus (Hogan and Rao 1999). Recent evidence indicates that the nuclear import of NF-AT is not sufficient to activate NF-AT target genes because NF-AT is constantly exported by the nuclear export receptor Crm1. Calcineurin suppresses this effect by competing for binding to the NES of NF-AT and thereby masking the NES, leading to transcriptionally active NF-AT in the nucleus (Zhu and McKeon 1999). A different type of NES masking has also been shown to regulate the localization of the tumor suppressor protein p53 (Stommel et al. 1999).

Theme summary

The following two chapters describe my thesis work. The early phase of my thesis work (Chapter 2) was to study the mechanism and regulation of Cdc34-dependent ubiquitination of different cellular substrates. We approached this by reconstituting the ubiquitination of different substrates in vitro using fractionated yeast extracts. The goal was to identify factors necessary and sufficient for Cdc34-dependent ubiquitination and therefore better define this ubiquitination pathway. This work was done in parallel to the Sic1 reconstitution work carried out by other members of the Deshaies laboratory. I attempted to characterize the ubiquitination of several other cell cycle substrates, including the CDK inhibitor Far1, replication initiation protein Cdc6, the G1 cyclin Cln2, and the transcription factor Gcn4. Studies using fractionated yeast extracts show that ubiquitination of all four proteins are dependent on Cdc34 and Cdc4 activities in vitro. Furthermore, phosphorylation of all of these proteins is necessary for their subsequent ubiquitination by the CDC34 pathway. In vitro results indicate that Cln2/Cdc28 kinase

promotes the ubiquitination of Cln2 and Far1; Clb5/Cdc28 kinase promotes the ubiquitination of Cdc6; and an unknown kinase promotes the ubiquitination of Gcn4. These in vitro experiments provided biochemical evidence that Cdc34/Cdc4 directly participate in the ubiquitination of these protein and therefore target them to degradation in vivo. Cdc4 was later found to be the F-box component of the SCF ubiquitin ligase complex (Feldman et al. 1997; Skowyra et al. 1997). The in vitro work on Far1, Cdc6 and Cln2 has been published (see Chapter 2).

Most of thesis work has been focused on the transcription factor Gcn4. An early observation indicated that unlike Sic1, Gcn4 ubiquitination does not require G1 cyclin CDK activity. Instead, Gcn4 ubiquitination appeared to require a different kinase. Much effort was spent on identifying the kinase that promotes Gcn4 ubiquitination. As detailed in Chapter 3, this effort identified Srb10 CDK as a physiological kinase that phosphorylates Gcn4 and promotes its SCF-dependent ubiquitination in vitro and degradation in vivo. Identification of Srb10 as a Gcn4 kinase put an interest twist on Gcn4 regulation and shifted the focus of the project on transcriptional regulation. Subsequent studies have identified transcription factor Msn2 as a physiological substrate of Srb10. Srb10 influences Msn2 localization, likely through direct phosphorylation of Msn2. Srb10 is a CDK associated with the RNA polymerase II holoenzyme. Genetic and microarray analyses suggest a negative role for Srb10 in regulating a subset of yeast genes. However, the exact role of Srb10 in transcriptional repression has been elusive, and current model suggests that the negative effect is achieved through phosphorylation of the C-terminal domain of the largest subunit of RNA polymerase II. My studies suggest that Srb10 exerts its repressive effect on specific genes by antagonizing their transcriptional activators. Srb10 negatively regulates Gcn4 target genes by promoting rapid turnover of Gcn4, and it represses Msn2/Msn4 target genes by promoting their export from the nucleus.

The physical proximity between Gcn4 and Srb10 upon transcriptional activation raises an interesting idea. Since promoter binding of Gcn4 recruits RNA polymerase II holoenzyme during transcription initiation, phosphorylation of Gcn4 and therefore the negative regulation may be confined to the vicinity of the promoter DNA of target genes. Preliminary data in Chapter 4 showed that a DNA-binding mutant of Gcn4 was significantly stabilized compared to wild-type Gcn4, suggesting that DNA binding is required for rapid Gcn4 turnover in vivo. Furthermore, preliminary data also indicated that a DNA-binding mutant of Pho4 was localized predominantly in the nucleus under phosphate-rich conditions, suggesting that DNA binding is also required for nuclear export of Pho4. Taken together, these data posit a general model that the negative regulation of transcription factors is coupled to DNA binding.

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Chapter 2. Ubiquitination of cell cycle regulatory proteins in vitro

This chapter is compiled using my data from the following published research articles in which my work constitute minor contributions, and my name is listed as a co-author in each of the articles. Specifically, Figure 1 is from Henchoz, et al. (1997); Figures 2 and 4 are from Blondel, et al. (2000); and Figure 3 is from Elsasser, et al. (1999).

Blondel, M., Galan, J., Chi, Y., Lafourcade, C., Longaretti, C., Deshaies R.J., and Peter, M. 2000. Nuclear-specific degradation of Far1 is controlled by the localization of the F-box protein Cdc4. *EMBO J.*, **19**: 6085-6097.

Elsasser, S., Chi, Y., Yang, P., and Campbell, J. L. 1999. Phosphorylation controls timing of Cdc6p destruction: A biochemical analysis. *Mol. Biol. Cell*, **10**: 3263-3277.

Henchoz, S., Chi, Y., Catarin, B., Herskowitz, I., Deshaies, R.J., and Peter, M. 1997. Phosphorylation- and ubiquitin-dependent degradation of the cyclin-dependent kinase inhibitor Far1p in budding yeast. *Genes Dev.*, **11**: 3046-3060.

Abstract

Ubiquitin-mediated proteolysis plays key roles in controlling the cell cycle progression in budding yeast. Degradation of the B-type cyclin CDK inhibitor Sic1 is important for G1/S transition, and degradation of Clb2 is essential for mitotic exit. Several other cell cycle substrates are also degraded in a cell cycle-dependent manner. These include the Cln/Cdc28 inhibitor Far1, replication initiation factor Cdc6, and the G1 cyclin Cln2. Data presented here show that ubiquitination of Far1 and Cdc6 in vitro requires phosphorylation by the Cdc28 kinase. Cdc34-dependent ubiquitination of all three proteins also requires Cdc4 activity, providing biochemical evidence that SCF^{Cdc4}/Cdc34 directly promotes the ubiquitination of these proteins in vitro.

Introduction

The eukaryotic cell cycle consists of a series of ordered events. Coordination of the cell cycle events requires tight control of the regulatory proteins. In recent years, it becomes increasingly evident that ubiquitin-dependent proteolysis plays a pervasive role in driving the cell cycle machinery (King et al. 1996). The ubiquitin-proteasome pathway is the major non-lysosomal degradation pathway in eukaryotes. Proteins are targeted to the 26S proteasome for degradation by covalent attachment of multiubiquitin chains (Ciechanover et al. 2000). The multi-ubiquitination of substrate proteins is mediated by three classes of enzymes: the E1 ubiquitin-activating enzymes, the E2 ubiquitin-conjugating enzymes, and the E3 ubiquitin ligases.

The anaphase-promoting complex (APC) and Skp1-cullin-F-box protein complex (SCF) are multi-subunit E3 ubiquitin ligases that regulate the cell cycle (Peters 1998): the APC promotes entry into anaphase and exit from mitosis, whereas SCF complexes trigger G1-S transition. In budding yeast, entry into S phase requires ubiquitin-dependent degradation of the CDK inhibitor Sic1 (Schwob et al. 1994; Verma et al. 1997a), and genetic and biochemical analyses revealed that a high molecular weight complex containing Cdc4, Cdc34, Cdc53, Hrt1, and Skp1 is required for its ubiquitination (Deshaies 1999). *CDC34* encodes an E2 ubiquitin-conjugating enzyme (Goebl et al. 1988) whereas Cdc4 contains a conserved motif called F-box, which mediates its interaction with Skp1 (Bai et al. 1996). A large family of proteins containing an F-box motif has been discovered that may function as adapters to recruit specific substrates to the core ubiquitination complex (Patton et al. 1998b), and three of these, Cdc4, Grr1, and Met30, have been shown to assemble into complexes in vivo (Patton et al. 1998a). SCF^{Grr1} has been implicated in the degradation of G1 cyclins, Cln1 and Cln2 (Barral et al. 1995; Seol et al. 1999; Skowyra et al. 1999), and the bud emergence protein Gic2

(Jaquenoud et al. 1998). Degradation of the CDK-inhibitory kinase Swe1 (Kaiser et al. 1998) and the transcription factor Met4 (Rouillon et al. 2000) are thought to be mediated by SCF^{Grr1}.

In addition to Sic1, degradation of the CDK inhibitor Far1 and the replication initiation factor Cdc6 are also important for proper cell cycle progression. Whereas Sic1 regulates entry into S phase by inhibiting the Clb/Cdc28 kinases (Mendenhall 1993; Schwob et al. 1994), Far1 is specifically required to arrest the cell cycle in response to mating pheromones by inhibiting the Cln/Cdc28 kinase (Peter and Herskowitz 1994). Far1 mRNA levels fluctuate through the cell cycle, peaking in G1 (McKinney et al. 1993). Far1 levels increase several-fold in response to mating pheromones, and the protein becomes rapidly phosphorylated by the MAP kinase Fus3 (Peter et al. 1993). To study the post-translational regulation of Far1, Peter and Herskowitz (1994) have isolated a mutant Far1 (Far1-22), which arrests cells even in the absence of mating pheromones. Whereas wild-type Far1 was only present in the G1 phase, Far1-22 was found in the nucleus throughout the cell cycle. Far1-22 carries a Ser-87 to Pro-87 mutation, and Ser-87 was located to a consensus phosphorylation site for Cdc28 kinase. Far1 is stabilized in temperature-sensitive *cdc34* and *cdc53* mutants at the non-permissive temperature, suggesting Far1 is degraded by the SCF pathway (Henchoz et al. 1997). We show here that Far1 is ubiquitinated in a Cdc34/Cdc4-dependent manner in vitro and the ubiquitination requires the phosphorylation of Far1 at Ser87 by Cln/Cdc28 kinase.

In S phase, cells must coordinate DNA replication such that every origin of replication fires only once per cell cycle. Cdc6 is part of the pre-replicative complex (prePC), which also includes the six-subunit origin recognition complex (ORC), the six Mcm proteins, and Cdc45 (Newlon 1997). Once the origin fires, the pre-RC is lost from the origin and does not reassemble until the end of the following mitosis (Diffley et al. 1994). Pre-RCs normally assemble during G1 phase and can not assemble later in the

cell cycle because the Clb/Cdc28 kinases prevents pre-RC formation (Dahmann et al. 1995). Thus protein phosphorylation by Clb/Cdc28 may be an important means to prevent spurious replication. Cdc6 has been shown both to associate with and to be phosphorylated by Clb/Cdc28 (Elsasser et al. 1996). Yeast cells arrested in S or G2 degrade bulk Cdc6 rapidly, but Cdc6 is stable as cells progress through G1 (Piatti et al. 1995; Drury et al. 1997; Sanchez et al. 1999). The cell cycle-dependent instability of Cdc6 could mean that Cdc6 is degraded in a Clb/Cdc28-dependent manner. In vivo studies indicate that Cdc6 is stabilized in *cdc34*, *cdc4*, and *cdc53* mutants (Drury et al. 1997; Sanchez et al. 1999), suggesting a role for SCF^{Cdc4} in Cdc6 turnover. A mutant alleles of Cdc6 bearing multiple mutations in the putative Cdc28 phosphorylation sites in the Cdc6 N-terminal Cdc28-binding domain was strongly stabilized compared to wild-type Cdc6, suggesting that phosphorylation of Cdc6 by Cdc28 kinase is important for its turnover (Elsasser et al. 1999). We show here that both Cdc34 and Cdc4 are required for Cdc6 ubiquitination in vitro, providing biochemical support for the in vivo findings. Furthermore, phosphorylation of Cdc6 by Clb5/Cdc28 kinase is sufficient to promote Cdc34-dependent ubiquitination.

G1 cyclins Cln1 and Cln2 are constitutively unstable proteins, and they accumulate during the G1 phase and become maximal during the late G1 phase as cells transit the Start (the point as which cells commit to completion of a cell cycle). G1 cyclin abundance is important in determining the timing of cell cycle initiation, and their instabilities are therefore critical for proper regulation of cell cycle progression. Degradation of G1 cyclins Cln2 and Cln3 are *CDC34*-dependent (Deshaies et al. 1995; Yaglom et al. 1995). Phosphorylation of these proteins on Cdc28 consensus sites is also required for their rapid degradation (Yaglom et al. 1995; Lanker et al. 1996). Several other proteins have also been implicated in Cln instability, including Skp1 (Bai et al. 1996), Cdc53 (Willems et al. 1996), and Grr1 (Barral et al. 1995). Grr1 is a F-box

proteins that binds phosphorylated Cln1 and Cln2 in vitro (Skowyra et al. 1997). Cln1 and Cln2 ubiquitination have been reconstituted in vitro using purified SCF^{Grr1} that also contains the fourth subunit Rbx1/Grr1 (Seol et al. 1999; Skowyra et al. 1999). Taken together, current evidence indicates that G1 cyclins Cln1 and Cln2 are targeted for degradation by the SCF^{Grr1} pathway. Interestingly, in vitro ubiquitination of Cln2 using fractionated yeast extracts shows that SCF^{Cdc4} can also ubiquitinate Cln2 despite the lack of physiological evidence for its role in promoting Cln2 turnover.

Results

In vitro ubiquitination of Far1 depends upon Cdc34, Cdc4, and cyclins

To determine whether Far1 is ubiquitinated by the G1-S ubiquitination system, we in vitro-translated Far1 in rabbit reticulocyte lysates (Fig. 1A) or wheat germ extracts (Fig. 1B) in the presence of [^{35}S]methionine and added DEAE-fractionated yeast extracts. These extracts are devoid of the G1 cyclins Cln1, Cln2, and Cln3 and are depleted of Cdc34 (Fig. 1A) or lack functional Cdc4 (Fig. 1B). Addition of Far1, purified GST-Cln2, ubiquitin (Ub), and Cdc34 resulted in accumulation of slower migrating forms of Far1 (Fig. 1A, lane 2). Methylated ubiquitin (meUb), which blocks formation of multiubiquitin chains by preventing Ub-Ub ligation (Hershko and Heller 1985), prevented accumulation of these high molecular weight forms of Far1 (Fig. 1A, lane 3), showing that they were ubiquitinated. Ubiquitination in vitro was dependent on Cdc34 (Fig. 1A, lane 4) and Cdc4 (Fig. 1B). DEAE-fractionated extracts prepared from *cdc4ts* mutants failed to ubiquitinate wild-type Far1 (Fig. 1B, lane 1). Addition of purified Cdc34 (Fig. 1B, lane 1) or baculovirus-infected insect cell lysate containing Cdc4 (Fig. 1B, lane 2) alone did not restore activity of the extract. However, addition of Cdc34 and

Cdc4 together restored Far1 ubiquitination (Fig. 1B, lane 3). As a control, insect lysate containing Cdc28 and Cdc34 did not lead to ubiquitination of Far1 (Fig. 1B, lane 4). Ubiquitination of Far1 was also dependent on the addition of the G1 cyclin Cln2 (Fig. 1B, lane 5), indicating that Far1 may need to be phosphorylated by Cdc28/Cln2 to be recognized by the Cdc34-dependent ubiquitination system. Alternatively, Cdc28/Cln2 may be necessary to activate a component of the ubiquitination machinery. Importantly, no ubiquitination could be detected on Far1-22 (Fig. 1A, lane 7), suggesting that the increased half-life of Far1-22 in vivo results from its failure to be ubiquitinated and degraded. These results show that wild-type Far1, but not Far1-22, is ubiquitinated in a Cdc34- and Cdc4-dependent manner in vitro and further suggest that ubiquitination of Far1 may require a phosphorylation event governed by the Cdc28/Cln2 kinase.

Cytoplasmic Far1-nls1 and Far1-nls1/nls2 are efficiently ubiquitinated by reconstituted SCF^{Cdc4} in vitro

Far1 proteins containing point mutations in its major nuclear localization signals (NLSs) (Far1-nls1, Far1-nls1/nls2) and a double mutant Far1-nls1/22 are localized to the cytoplasm. These proteins have 2.5-5-fold longer half-life than wild-type Far1 protein (Blondel et al. 2000). Bondel et al. (2000) proposed that these proteins are stabilized because Cdc4 is exclusively localized to the cell nucleus. However, to exclude the trivial possibility that the mutations introduced into Far1-nls1 and Far1-nls1/nls2 may interfere with its ubiquitination, we carried out in vitro ubiquitination experiments using reconstituted SCF^{Cdc4} and in vitro-translated ^{35}S-labeled wild-type or mutant forms of Far1 (Figure 2). Clearly, SCF^{Cdc4} was able to ubiquitinate wild type Far1 (lanes 1–5), Far1-nls1 (lanes 6–8) and Far1-nls1/2 (lanes 12–14) with similar efficiency. The smear of slower migrating forms represents ubiquitinated species of Far1, because addition of

methyl-ubiquitin (Me-Ub), which cannot be extended into multi-ubiquitin chains (Hershko and Heller 1985), competes with the formation of these high molecular weight forms. As expected, ubiquitination required the addition of purified Cdc28/Cln2 (kinase) and a phosphorylatable serine 87 (lanes 9–11), confirming that ubiquitination of Far1-nls1 or Far1-nls1/2 was dependent on phosphorylation of serine 87 by Cdc28/Cln2. We conclude that stabilization of Far1-nls1 and Far1-nls1/nls2 in vivo is not due to a defect in its ubiquitination caused by these mutations.

CDC4 and CDC34 are required for phosphorylation-dependent ubiquitination of Cdc6 in vitro

Recent demonstration that Cdc6 is stabilized in a *cdc4 sic1Δ* strain suggested that SCF^{Cdc4} targets phosphorylated Cdc6 for ubiquitination rather than acting indirectly by degrading Sic1p and thus activating Clb/Cdc28 kinase. Our results confirm the direct targeting of Cdc6 by SCF^{Cdc4} action. As shown in Figure 3, the appearance of Cdc6 conjugates depends on addition of Cdc34 (compare lanes 4 and 5). To compare Cdc6 ubiquitination in the presence and absence of Cdc4, fractionated extracts prepared from both wild type and *cdc4* cells were used. Cdc6 conjugates were not obtained when using fractionated *cdc4* extracts (Fig. 3, compare lanes 5 and 10), but if insect cell lysate containing recombinant Cdc4 was added, ubiquitination of Cdc6 was restored (Fig. 3, compare lanes 10 and 12). Naturally, Cdc6p modification still required addition of Cdc34 (Fig. 3, compare lanes 11 and 12). We conclude that the slowly migrating species are ubiquitinated Cdc6, because their appearance depends on Cdc34 and Cdc4. Although a requirement for Cdc34 and Cdc4 in this reaction could derive from a requirement to degrade Sic1, we have determined that the Clb5/Cdc28 kinase added to these reactions is in considerable excess over Sic1. We therefore propose that Cdc34 and Cdc4 are directly

required for Cdc6 destruction and that Cdc4 is required for the recognition of Cdc6 as a substrate for ubiquitination. This model is consistent with the observations that Cdc6 and Cdc4 interact in a two-hybrid system (Drury et al. 1997) and that Cdc6 is stable in G2 phase cells in a *cdc4 sic1Δ* strain (Sanchez et al. 1999).

Cdc34-dependent ubiquitination of Cln2 in yeast extracts also requires Cdc4

Ubiquitination of ^{35}S-labeled Cln2 was carried out in fractionated yeast extracts prepared from wild-type (Fig. 4, lanes 2 and 3) and *cdc4* (Fig. 4, lanes 4-8) strains. Wild-type extract was able to support Cln2 ubiquitination when purified Cdc34 was added (lane 2), and the ubiquitination was confirmed by the addition of methyl-ubiquitin (lane 3). To our surprise, Cln2 ubiquitination is defective in *cdc4* extracts (lane 5). Furthermore, addition of baculovirus-infected insect lysate containing expressed Cdc4 was able to rescue the defect (lane 7). This result suggests that SCF^{Cdc4} is able to ubiquitinate Cln2, at least in vitro.

Discussion

Ubiquitin-mediated degradation plays a key role in regulating cell cycle progression in budding yeast. Degradation of Sic1 by Cdc34/SCF^{Cdc4} pathway triggers G1-S transition (Schwob et al. 1994; Feldman et al. 1997; Skowyra et al. 1997). Work shown here expands the repertoire of the SCF^{Cdc4} substrates, and provides biochemical evidence that the SCF^{Cdc4} pathway may regulate Far1 and Cdc6 turnover. It also underscores substrate phosphorylation as a potential hallmark of SCF^{Cdc4}–dependent ubiquitination (Fig. 5).

Degradation of Far1

Far1 accumulates during G1 phase of the cell cycle. This expression pattern is achieved in part by transcriptional regulation of *FAR1*, which is restricted to G1 and late M phase of the cell cycle (McKinney et al. 1993). It is required to arrest the cell cycle in the presence of mating pheromones but is not necessary for the normal cell cycle progression (Chang and Herskowitz 1990). Far1 is degraded at the G1 to S transition and remains unstable until cells exit from mitosis (Henchoz et al. 1997). The degradation of Far1 is physiologically relevant during the yeast mating. Cells arrested with α-factor that are unable to find a mating partner adapt to the presence of mating pheromones and resume cell division. Reentry into the cell cycle requires the degradation of Far1: Cells that produce nondegradable Far1 are unable to properly activate the Cln/Cdc28 kinase after release form α factor and, as a consequence, fail to recover efficiently (Henchoz et al. 1997). Henchoz et al. (1997) also show that Far1 degradation is dependent on Cdc34, Cdc53, and specific phosphorylation at Ser87. The *CDC4*-dependence of Far1 degradation has not been clearly demonstrated in vivo. By analogy to Sic1 degradation, it is likely that Far1 degradation is dependent on Cdc4. In vitro, Far1 ubiquitination requires activities of both Cdc34 and Cdc4, and the reaction also requires phosphorylation by Cln/Cdc28 (Fig. 1). This experiment lends support to the hypothesis that Far1 is degraded by the Cdc34/Cdc4-dependent ubiquitination pathway. With the subsequent identification of SCF complexes, it is feasible to reconstitute Far1 ubiquitination using purified recombinant SCF^{Cdc4} (Fig. 2). The experiment in Fig. 2 demonstrates that purified SCF^{Cdc4} is sufficient to ubiquitinate Far1, and the reaction efficiency is higher than that observed in yeast extracts (compare Figs. 1 and 2).

Degradation of Cdc6

The rapid degradation of Cdc6 at G1-S is conserved for homologues in other eukaryotes, although not in all, suggesting periodic degradation of Cdc6 is important for regulation of cell cycle progression (Drury et al. 1997; Williams et al. 1997; Hua and Newport 1998; Saha et al. 1998). There is strong evidence arguing for a role of SCF^{Cdc4} in promoting the degradation of Cdc6 in vivo. First, Cdc6 interacts with Cdc4 in a two-hybrid assay and is stabilized in *cdc4*, *cdc34*, *cdc53* mutants (Drury et al. 1997). Second, Cdc6 ubiquitin conjugates are observed in vivo and are dependent on Cdc4 function (Sanchez et al. 1999). Phosphorylation of certain N-terminal Cdc28 consensus sites is also important for rapid Cdc6 turnover (Elsasser et al. 1999). However, there has been no biochemical evidence that SCF^{Cdc4} directly ubiquitinates Cdc6. The experiment shown in Fig. 3 shows that both Cdc34 and Cdc4 are required for the ubiquitination of Cdc6 in vitro. Furthermore, phosphorylation of Cdc6 is required for its subsequent ubiquitination. When phosphorylated by Clb5/Cdc28, Cdc6 becomes competent for ubiquitination by the Cdc34/Cdc4 ubiquitination machinery. Although Cdc6 ubiquitination has yet to be reconstituted using purified proteins, it is a reasonable assumption that the reaction will likely to proceed with higher efficiency than that shown in Fig. 3.

Degradation of Cln2

Cyclins are inherently unstable proteins, and their instability is important for the regulation of CDK activities, which drive the cell cycle engine. Cln2 is unstable in G1 phase, and its instability is required for proper regulation of Start (Schneider et al. 1998). Subunits of the SCF^{Grr1} have been implicated in Cln2 turnover (Barral et al. 1995; Bai et al. 1996; Willems et al. 1996). It is proposed that phosphorylation of Cln2 by Cdc28

couples activation of the Cln2/Cdc28 kinase to degradation of the Cln2 polypeptide and thereby renders Cln2-activated CDK activity self-limiting (Lanker et al. 1996). In vitro, Cln2 was hyperphosphorylated upon incubation with yeast extracts, which contain Cdc28 (Fig. 4). Ubiquitination of Cln1 (Skowyra et al. 1999) and Cln2 (Seol et al. 1999) have been reconstituted with purified SCF^{Grr1} after the identification of Rbx1/Hrt1. Although genetic and biochemical evidence indicates that Cln2 is predominantly ubiquitinated in vivo by SCF^{Grr1}, ubiquitination of Cln2 in yeast extracts suggests that SCF^{Cdc4} may also be able to ubiquitinate Cln2 (Fig. 4). It is surprising to see that Cln2 ubiquitination was defective in *cdc4* extracts, but could be restored with recombinant Cdc4 (Fig. 4). To correctly interpret this result, it would be necessary to see if Cln2 ubiquitination can be reconstituted using purified SCF^{Cdc4}. If SCF^{Cdc4} is sufficient to ubiquitinate Cln2, it would then suggest that the fractionated *cdc4* extracts lack both Cdc4 and Grr1 activities. Two pieces of evidence suggest that SCF^{Cdc4} may have a minor role in Cln2 turnover. First, Cln2 was weakly stabilized in *cdc4* mutant cells in vivo (t1/2 = 4 min in wild type and ~7.5 min in *cdc4-1* cells; R. Deshaies, personal communication). Second, Cdc4 binds phosphorylated Cln2 in vitro, albeit weaker than Grr1 does (Skowyra et al. 1997). It is possible that SCF^{Cdc4} is capable of ubiquitinating Cln2, but it is not the physiological choice. Work from Blondel et al. (2000) suggests that there may be compartment-specific targeting of SCF substrates depending on the localization of the F-box receptor proteins.

Materials and Methods

Yeast Strains

The yeast strains used in this study are RJD885 (*ura3 leu2 trp1 cln1::URA3 cln2::LEU2 cln3::URA3 leu2::GAL-CLN3::LEU2 pep4::TRP1 cdc28::CDC28-HA::HIS3 MAT*α) and RJD893 (*cdc4 ura3 leu2 trp1 cln1::URA3 cln2::LEU2 cln3::URA3 leu2::GAL-CLN3::LEU2 pep4::TRP1 cdc28::CDC28-HA::HIS3 MAT***a**).

Recombinant proteins

GST-Cln2 and Cdc34 were expressed and purified from E. coli as described previously (Banerjee et al. 1993; Deshaies and Kirschner 1995). ^{His6}Uba1 was purified from yeast as described previously (Feldman et al. 1997). Ubiquitin was obtained from Sigma, and methyl-ubiquitin was kindly provided by R. King. Cln2, Cdc28, Clb5/Cdc28, and Cdc4 were expressed in baculovirus-infected insect cells, and cell lysates were prepared as previously described (Verma et al. 1997c; Reynard et al. 2000). SCF^{Cdc4} complex (^{PyHA}Cdc4–Cdc53–Skp1–Hrt1) was expressed in Sf9 insect cells and bound to polyoma (Py) beads as described (Seol et al. 1999). The polyoma beads were eluted with polyoma peptide and the eluate was used to supply SCF^{Cdc4} activity in Fig. 2.

Preparation of yeast extracts

Preparation of DEAE-fractionated yeast extracts was carried out as previously described (Verma et al. 1997b; Verma et al. 1997c). The 250 mM NaCl eluate was used in the assays containing yeast extracts. This fraction was essentially devoid of Cdc34,

which eluted at 500 mM NaCl. It was also depleted of G1 cyclins by genetic trickery. Strain RJD885 was used for wild-type yeast extract, and strain RJD893 was used for *cdc4* extracts.

Preparation of substrates

Wild-type and mutant *FAR1* (Fig. 1A) and wild-type *CDC6* (Fig. 3) transcription templates were generated from plasmids containing full-length *FAR1* or *CDC6* sequence by PCR using a 5' oligonucleotide containing a T7 RNA polymerase promoter (Verma et al. 1997b). Alternatively, linearized plasmids carrying T7-directed wild-type or mutant *FAR1*(Figs. 1B, 2) and wild-type *CLN2* (Fig. 4) were used. The templates were then transcribed and translated in reticulocyte lysates (*FAR1* in Fig. 1A and *CLN2*) or wheat germ extracts (*FAR1* in Fig. 1B and *CDC6*) according to the manufacturers instructions (Promega) to generate [^{35}S]methionine-labeled substrates.

Ubiquitination reactions

In Figs. 1, 3, and 4, the 250mM DEAE eluate (see above) was used to supply ubiquitination activity, and the reactions were carried out essentially as previously described (Verma et al. 1997c). Briefly, the radiolabeled substrate (1 μl) was incubated in 10 μl of reaction mixture containing wild-type or mutant yeast extract (100 μg), reaction buffer containing protease inhibitors, ATP mix, ubiquitin (10 μg), and Cdc34p (100 ng). For reactions with *cdc4* extracts, 0.5 μl of insect lysate (~5 mg/ml) containing Cdc4p or Cdc28p was used where indicated. In Fig. 2, purified recombinant SCF^{Cdc4} (see above) was used. In a complete reaction, the yeast extracts were replaced by purified Uba1 (50 ng), Cdc34 (100 ng) and SCF^{Cdc4} complex (50–100 ng). Cdc28/Cln2 kinase

activity was supplied as insect lysate (2 μg total protein) containing expressed $Cln2^{myc}$, GST–Cdc28, Cks1, and Cak1 (Seol et al. 1999). Insect lysate containing expressed Cln2 alone was used to represent absence of Cdc28–Cln2 kinase activity. All reactions were incubated at 24°C for 60 min, terminated by the addition of SDS-PAGE sample buffer, boiled for 3 min, and evaluated by SDS-PAGE and autoradiography.

Fig. 1

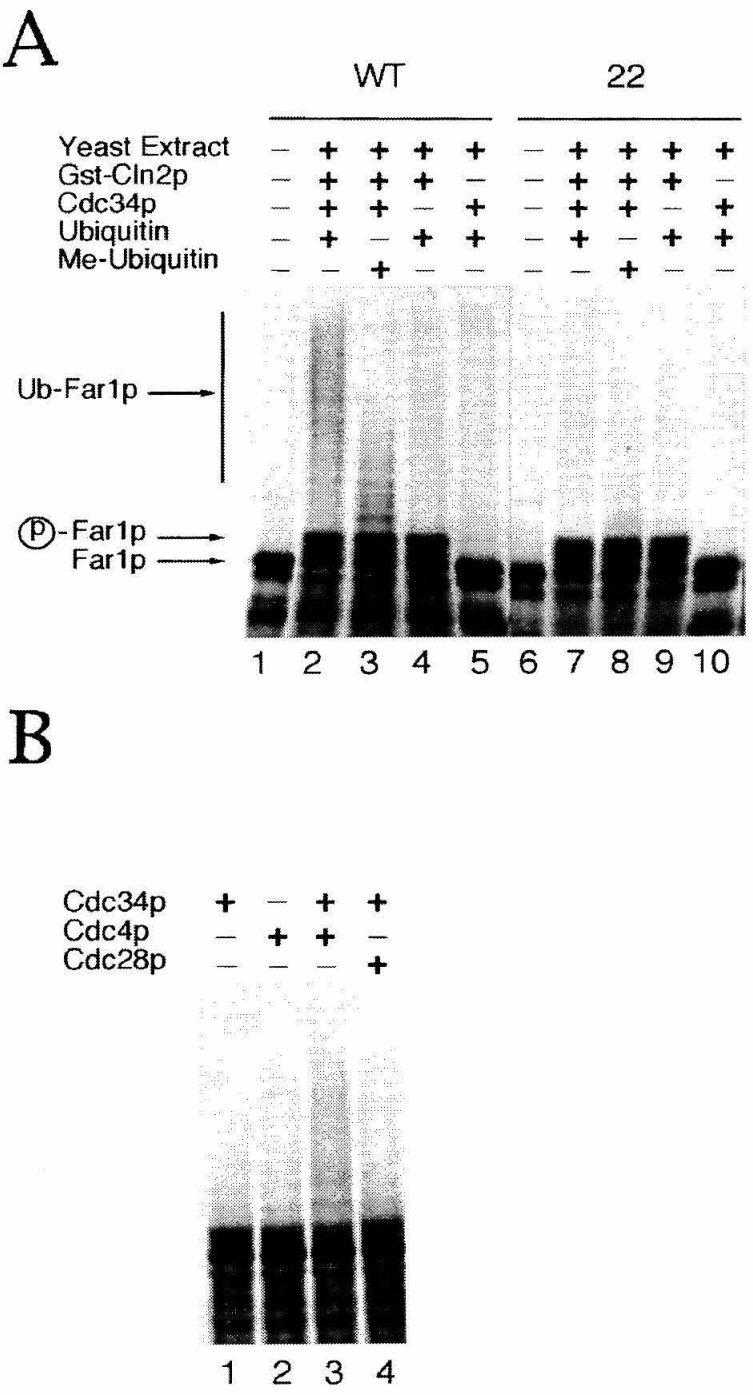

A
WT
22
Yeast Extract
Gst-Cln2p
Cdc34p
Ubiquitin
Me-Ubiquitin
Ub-Far1p
Ⓟ-Far1p
Far1p
1 2 3 4 5 6 7 8 9 10
B
Cdc34p
Cdc4p
Cdc28p
1 2 3 4

Figure 1. Wild-type Far1 but not Far1-22 is ubiquitinated in vitro.

Wild-type Far1 (A, lanes 1-5; B, lanes 1-4) or Far1-22 (A, lanes 6-10) synthesized in vitro in the presence of [^{35}S]methionine was incubated in fractionated extracts prepared from cells lacking CLN1,2, and 3 (A) or cells deleted for CLN1,2, and 3, which are also temperature-sensitive for *cdc4* (B). These extracts are devoid of Cdc34 and contain low levels of ubiquitin.

(A) Wild-type Far1 was ubiquitinated if the extract was supplemented with the addition of Cdc34, GST-Cln2, and ubiquitin (lane 2). Reduced accumulation of high molecular weight ubiquitin conjugates was observed if methyl-ubiquitin was added to block chain extension (lane 3). No ubiquitination was observed with Far1-22 (lanes 6-10).

(B) Ubiquitination of wild-type Far1p was dependent on Cdc4 (lanes 1 and 4). Ubiquitination in *cdc4* extracts was restored only by the simultaneous addition of purified Cdc34 and baculovirus-infected insect lysate containing Cdc4 (lane 3). The addition of insect lysate containing Cdc28 serves as a specificity control (lane 4).

Fig. 2

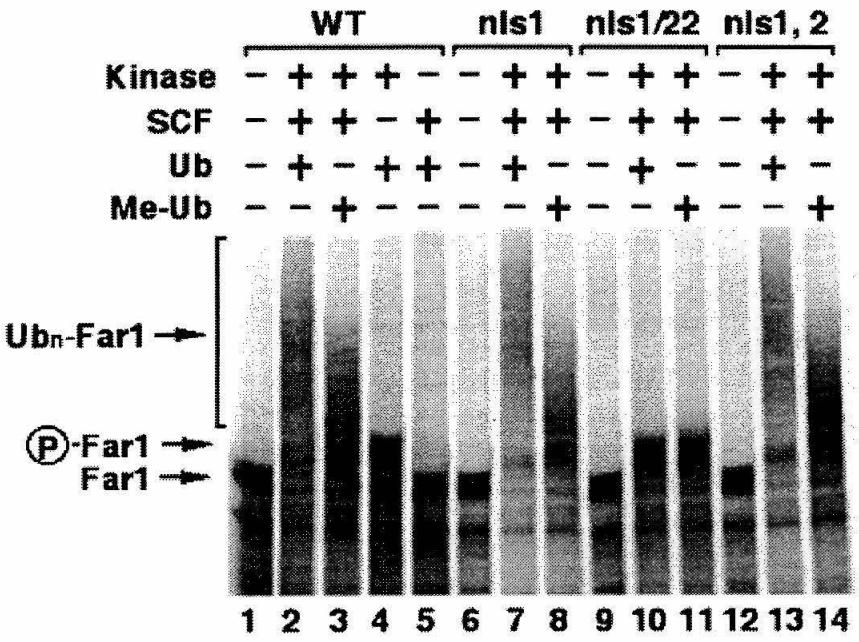

WT
nls1
nls1/22
nls1, 2
Kinase
SCF
Ub
Me-Ub
Ubn-Far1
Ⓟ-Far1
Far1
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 2. Ubiquitination of wild-type and mutant Far1 by SCF^{Cdc4} in vitro.

Wild type (lanes 1–5) or the indicated Far1 mutants (lanes 6–14) were in vitro translated in the presence of [35S]methionine and incubated as indicated with reconstituted SCF^{Cdc4} (SCF), Cdc28/Cln2 (Kinase) and ubiquitin (Ub) as described in Materials and Methods. Where indicated, Me-ubiquitin instead of ubiquitin was added as a control (Me-Ub). The arrows point to the position of unphosphorylated (Far1) and phosphorylated Far1 (P-Far1), respectively, while the brackets mark the position of multi-ubiquitinated Far1 (Ub_n-Far1).

Fig. 3

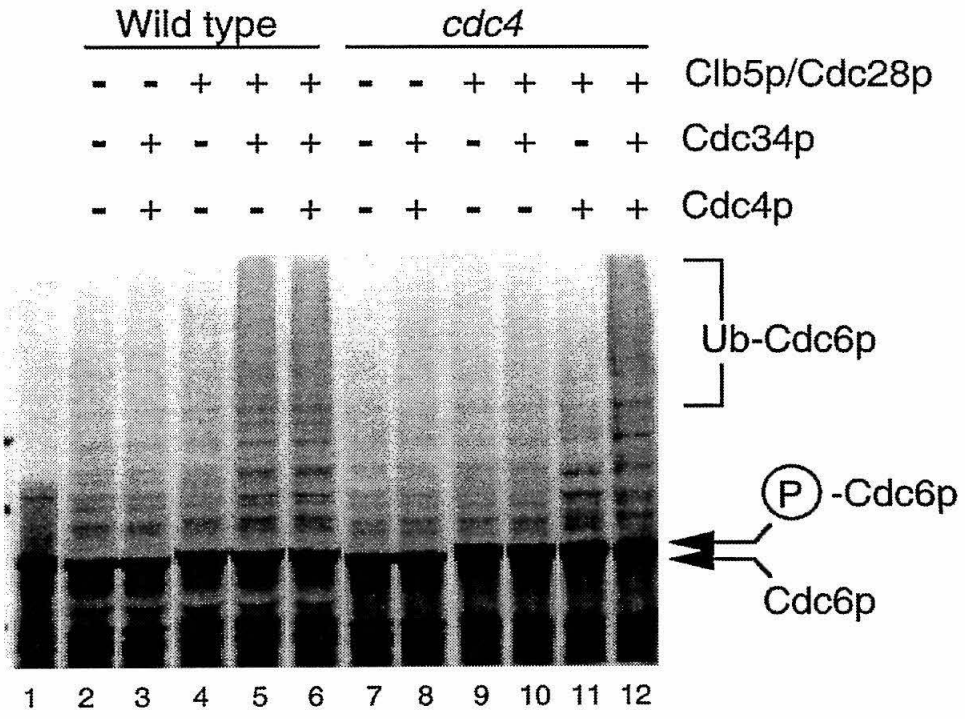
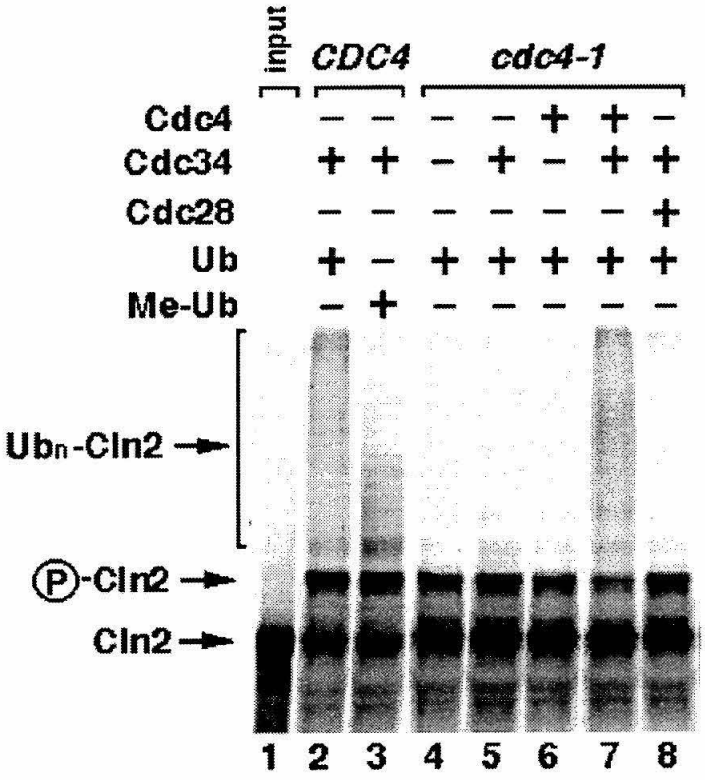

Wild type
cdc4
- - + + + - - + + + +
Clb5p/Cdc28p
- + - + + - + - + - +
Cdc34p
- + - - + - + - - + +
Cdc4p
Ub-Cdc6p
P -Cdc6p
Cdc6p
1 2 3 4 5 6 7 8 9 10 11 12

Figure 3. In vitro ubiquitination of Cdc6 is dependent on Cdc4.

^{35}S-Labeled Cdc6 was prepared by in vitro translation as described in Materials and Methods and used as a substrate for in vitro ubiquitination reactions. Lane 1 contains the input ^{35}S-labeled Cdc6 without extract. Extract prepared from RJD885 cells (wild type for ubiquitination components) was used in the ubiquitination reactions in lanes 2-6. Extract from RJD893 cells (*cdc4* and isogenic to RJD885) was used in reactions in lanes 7-12. The complete ubiquitination reaction (lanes 5 and 10) included DEAE-fractionated extract of G1-arrested cells, purified Cdc34, insect cell lysate containing Clb5/Cdc28 kinase, an ATP-regenerating system, salts, and ubiquitin. The reactions in lanes 6, 11, and 12 were supplemented with recombinant Cdc4 purified from insect cells. Cdc34 and Clb5/Cdc28 were omitted where indicated.

Fig. 4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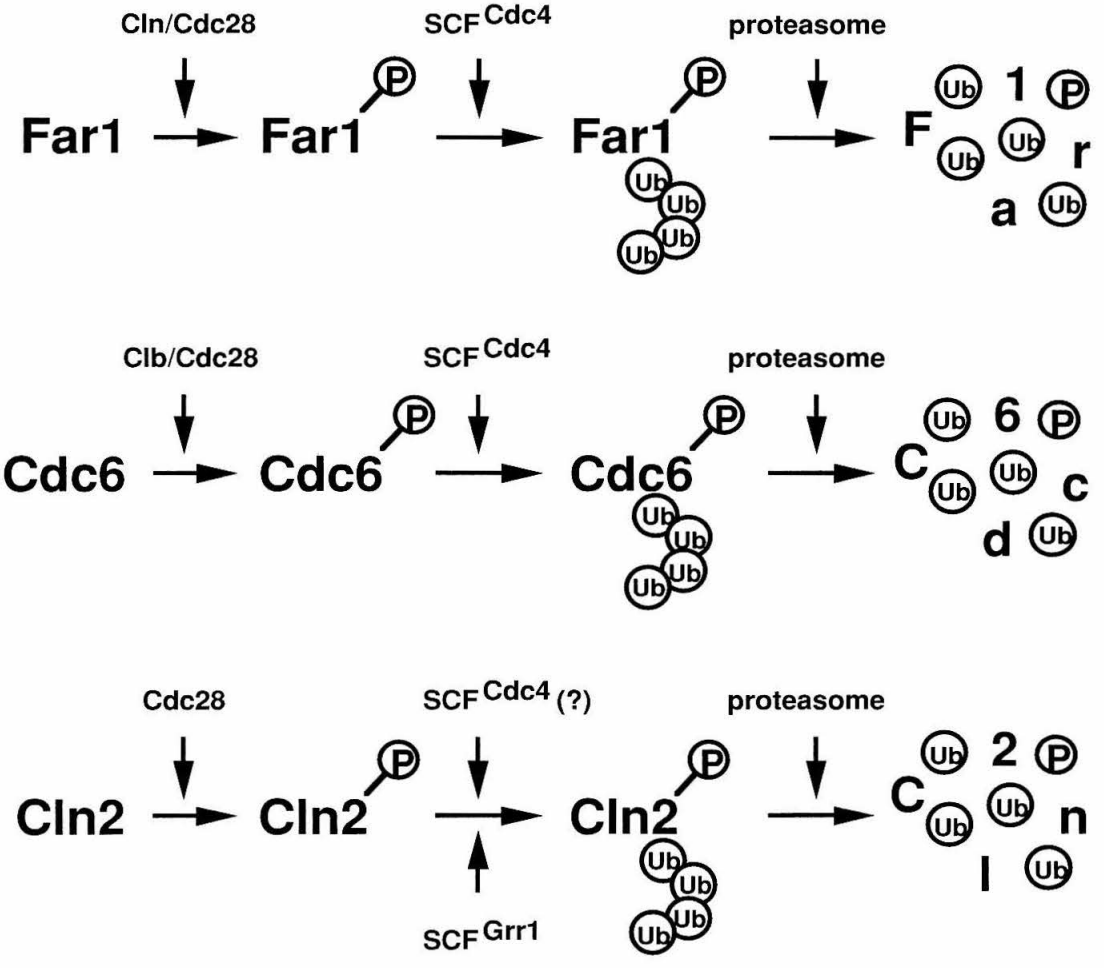
cdc4-1
Cdc4
Cdc34
Cdc28
Ub
Me-Ub
Ubn-Cln2
Ⓟ-Cln2
Cln2
1 2 3 4 5 6 7 8

Figure 4. SCF^{Cdc4} is able to ubiquitinate Cln2 in vitro.

In vitro-translated [^{35}S]methionine-labeled Cln2 (lane 1) was incubated in DEAE-extracts prepared from wild-type (lanes 2 and 3) or *cdc4-1* cells (lanes 4–8), complemented where indicated (+) with ubiquitin (Ub), and bacculo-expressed Cdc34, Cdc4 and Cdc28. In lane 3, methylated ubiquitin (Me-Ub) was added instead of ubiquitin to compete for poly-ubiquitination. The arrow points to unphosphorylated (Cln2) or phosphorylated Cln2 (P-Cln2), while the bracket marks the position of poly-ubiquitinated Cln2 (Ubn-Cln2).

Fig. 5


Cln/Cdc28
SCF Cdc4
proteasome
Far1
Far1
Far1
Ub
Ub
Ub
Ub
Ub
F
1
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r
a
Clb/Cdc28
SCF Cdc4
proteasome
Cdc6
Cdc6
Cdc6
Ub
Ub
Ub
Ub
Ub
C
6
P
c
d
Cdc28
SCF Cdc4 (?)
proteasome
Cln2
Cln2
Cln2
SCF Grr1
Ub
Ub
Ub
Ub
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C
2
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n
l

Figure 5. A model for the degradation of Far1, Cdc6, and Cln2. Phosphorylation of these proteins by Cdc28 CDK, either in the form of Cln/Cdc28 for Far1, Clb/Cdc28 for Cdc6, or autophosphorylation by cognate Cdc28 for Cln2, targets them for SCF-dependent ubiquitination. Phosphorylated Far1 and Cdc6 are recognized by SCF^{Cdc4} complex whereas phosphorylated Cln2 is recognized by SCF^{Grr1}. However, SCF^{Cdc4} can ubiquitinate Cln2 in yeast extracts and might contribute to Cln2 turnover in a minor way.

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Chapter 3. Negative regulation of Gcn4 and Msn2 transcription factors by Srb10 cyclin-dependent kinase

Work presented in this chapter has been published. I acknowledge M. J. Huddleston, X. Zhang, R. S. Annan, and S. A. Carr for mass spectrometric analysis of Gcn4 in vitro phosphorylation sites, and R. Young for intellectual input on Msn2 work.

Chi, Y., Huddleston, M.J.[1], Zhang, X.[1], Young, R.A.[2], Annan, R.S.[1], Carr, S.A.[1], and Deshaies, R.J. 2001. Negative regulation of Gcn4 and Msn2 transcription factors by Srb10 cyclin-dependent kinase, *Genes Dev.* **15**:1078-1092.

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Abstract

The budding yeast transcriptional activator Gcn4 is rapidly degraded in an SCF^{Cdc4}-dependent manner in vivo. Upon fractionation of yeast extracts to identify factors that mediate Gcn4 ubiquitination, we found that Srb10 phosphorylates Gcn4 and thereby marks it for recognition by SCF^{Cdc4} ubiquitin ligase. Srb10 is a physiological regulator of Gcn4 stability because both phosphorylation and turnover of Gcn4 are diminished in *srb10* mutants. Gcn4 is almost completely stabilized in *srb10Δ pho85Δ* cells, or upon mutation of all Srb10 phosphorylation sites within Gcn4, suggesting that the Pho85 and Srb10 CDKs conspire to limit the accumulation of Gcn4. The multistress response transcriptional regulator Msn2 is also a substrate for Srb10 and is hyperphosphorylated in an Srb10-dependent manner upon heat stress-induced translocation into the nucleus. Whereas Msn2 is cytoplasmic in resting wild type cells, its nuclear exclusion is partially compromised in *srb10* mutant cells. Srb10 has been shown to repress a subset of genes in vivo, and has been proposed to inhibit transcription via phosphorylation of the C-terminal domain of RNA polymerase II. We propose that Srb10 also inhibits gene expression by promoting the rapid degradation or nuclear export of specific transcription factors. Simultaneous down-regulation of both transcriptional regulatory proteins and RNA polymerase may enhance the potency and specificity of transcriptional inhibition by Srb10.

Introduction

Precise modulation of intracellular protein concentration is an important means by which diverse cellular processes are regulated. One way cells effectively achieve this is through proteolysis of key regulatory proteins. The ubiquitin system is the major cytoplasmic pathway by which proteins are degraded. Ubiquitin-mediated degradation of cellular proteins involves attachment of ubiquitin chains to substrate proteins, which are subsequently targeted for degradation by the 26S proteasome (Ciechanover et al. 2000). Assembly of a multiubiquitin chain upon a substrate typically requires three classes of enzymes: the ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3). The E1 enzyme activates ubiquitin in an ATP-dependent manner by linking to its C terminus via a thioester bond. The E2 enzyme accepts the thioesterified ubiquitin and transfers it to a lysine residue of the target protein. This step usually requires assistance from an E3 enzyme. There are potentially 13 E2-like proteins in budding yeast. In contrast, there are two distinct classes of E3s: the HECT domain family and the RING domain family (Deshaies 1999; Seol et al. 1999; Jackson et al. 2000). The RING domain family of E3s includes multimeric complexes such as SCF and anaphase-promoting complex/cyclosome (APC/C), as well as single subunit ligases like Ubr1. Whereas HECT E3s act as a direct intermediary by accepting ubiquitin from E2 and transferring it to substrate, RING-based E3s are thought to catalyze ubiquitination by enabling the direct transfer of ubiquitin from E2 to substrate (Seol et al. 1999). Regardless of their mechanism of action, E3s typically bind directly to E2 and to substrate, suggesting that they provide substrate specificity in cellular ubiquitination reactions.

One salient example of how proteolysis can provide a regulatory switch stemmed from genetic analysis of the G_1/S transition in budding yeast (Schwob et al. 1994). Cells harboring temperature-sensitive mutations in *SKP1*, *CDC53*, and *CDC4* arrest in G1 phase at the nonpermissive temperature because they fail to degrade the S phase cyclin/cyclin-dependent kinase (CDK) inhibitor Sic1 (Schwob et al. 1994; Bai et al. 1996). Subsequent in vitro reconstitution of Sic1 ubiquitination led to the identification of SCF^{Cdc4}, the prototype of the SCF (for Skp, Cdc53/Cullin, F-box receptor) family of ubiquitin ligases (Feldman et al. 1997; Skowyra et al. 1997; Verma et al. 1997a). Recently, Hrt1 (also known as Roc1 and Rbx1), an essential fourth subunit of the SCF complex, was identified (reviewed in Deshaies 1999). The SCF family of ubiquitin ligases is potentially large given that the yeast genome encodes at least 17 potential F-box receptor subunits (Patton et al. 1998b), and at least two other SCF complexes-SCF^{Grr1} and SCF^{Met30}-have been identified in budding yeast (Patton et al. 1998a). Cdc34 appears to be the primary E2 enzyme that interacts with SCF complexes and catalyzes ubiquitination of their substrates in budding yeast.

Besides Sic1, the CDK inhibitor Far1 (Henchoz et al. 1997) and the replication initiation protein Cdc6 (Drury et al. 1997; Elsasser et al. 1999) have been shown to be substrates of SCF^{Cdc4}. A common feature in the ubiquitination of SCF^{Cdc4} substrates is that they must be phosphorylated by the major cell cycle CDK, Cdc28 (Henchoz et al. 1997; Verma et al. 1997a; Elsasser et al. 1999). Phosphorylation appears to serve as a general signal that promotes binding of the F-box receptor Cdc4 to the substrates (Feldman et al. 1997; Skowyra et al. 1997). To investigate the generality of the Cdc34/SCF^{Cdc4} pathway, we initiated biochemical analysis of the roles of these proteins in Gcn4 ubiquitination. Gcn4, a transcription activator involved in the regulation of amino acid and purine biosynthetic genes (Hinnebusch 1992) is very unstable, and its degradation is dependent on Cdc34 and proteasome function (Kornitzer et al. 1994). Very

recently, it was shown that Gcn4 is stabilized in *cdc4*, *cdc53* and *skp1* temperature-sensitive mutants and in *pho85Δ* cells (Meimoun et al. 2000). This suggests that SCF^{Cdc4} contributes to the rapid degradation of Gcn4 in vivo and that a CDK other than Cdc28 is involved in Gcn4 degradation. However, there is no biochemical evidence to date that either SCF^{Cdc4} or Pho85 directly promotes ubiquitination of Gcn4.

Here we provide evidence that the Srb10 CDK of the SRB/mediator complex phosphorylates both Gcn4 and the multistress response transcription factor Msn2. Whereas Srb10 targets Gcn4 for SCF^{Cdc4}-dependent degradation, it helps enforce the nuclear exclusion of Msn2. It has been proposed that Srb10 negatively regulates transcription of certain genes by binding and phosphorylation of the C-terminal domain (CTD) of the largest subunit of RNA polymerase II (Hengartner et al. 1998). Our results suggest that Srb10 can also repress the transcription of specific genes by directly antagonizing transcriptional activators.

Results

Ubiquitination of Gcn4 in yeast extracts

Our in vitro studies on Gcn4 ubiquitination were prompted by the observation that Gcn4 turnover in vivo depends on Cdc34 (Kornitzer et al. 1994). As a first step toward understanding the mechanism and regulation of Gcn4 turnover, we set out to reconstitute Gcn4 ubiquitination in vitro. Ubiquitination of [^{35}S]methionine-labeled Gcn4 was evaluated in G1-cyclin-depeleted whole cell yeast extracts as described for Sic1 (Verma et al. 1997a). Although ubiquitination of Sic1 required the addition of GST-Cln2 to supply Cdc28 kinase activity (Fig. 1A, lanes 6,7), ubiquitination of Gcn4 did not (lanes 2,3). Multiubiquitination of both substrates was confirmed by the addition of methyl-

ubiquitin (lanes 4 and 8), a chain-terminating derivative of ubiquitin. Concomitantly with ubiquitination, Gcn4 exhibited a characteristic molecular weight (MW) upshift upon incubation in yeast extract (compare lanes 1,2). To test if this modification was due to phosphorylation, we first incubated $Gcn4^{HA}$ with a yeast extract fraction enriched for this activity, and then immunoprecipitated $Gcn4^{HA}$ and treated it with calf intestinal alkaline phosphatase (CIAP). As shown in Fig. 1B, CIAP treatment reversed the MW shift of Gcn4, indicating that it arises from phosphorylation.

The results above suggested that Gcn4 might be targeted for ubiquitination by a novel pathway. Alternatively, a protein kinase besides Cdc28 may be able to direct Gcn4 to the SCF pathway. To distinguish between these possibilities, we fractionated whole cell extract from mutant *cdc4*^{ts} cells into a protein kinase fraction and an SCF-containing fraction (the SCF fraction lacks the E2 Cdc34; Verma et al. 1997a). As shown in Fig. 1C, multiubiquitination of Gcn4 occurred only when both yeast fractions and exogenous Cdc34 and Cdc4 were added. Since ubiquitination of Sic1 did not require the 'kinase fraction' (data not shown), we concluded that Gcn4 must be phosphorylated by an unknown protein kinase before it can be ubiquitinated by the SCF^{Cdc4}/Cdc34 pathway

Identification of the Srb10 subunit of RNA Polymerase II holoenzyme as a Gcn4 kinase

To identify the activity required for Gcn4 ubiquitination in vitro, we first attempted to purify this activity by conventional column chromatography. We used both Gcn4 kinase assays and ubiquitination assays to monitor this activity during purification. Unfortunately, we did not purify enough activator to obtain protein sequence. However, upon partial purification (Fig. 2A), we identified a high molecular weight (MW) Gcn4 kinase activity that co-fractionated with the ubiquitination-promoting activity (Fig. 2B). Note that although there are two contaminating low-MW Gcn4 kinases that peak in

fractions 11 and 15, neither of these fractions can sustain ubiquitination of Gcn4 by SCF^{Cdc4}. We suspected the kinase activity was a CDK since the Cdc28 CDK is required for SCF^{Cdc4}-dependent ubiquitination of Sic1, Far1, and Cdc6. Given that Kin28 and Srb10, both of which are components of the RNA polymerase II holoenzyme (Myer and Young 1998), are the only CDKs known to reside in a high MW complex, we tested if either of these kinases was responsible for the purified activity. Extract fractions enriched for the high MW Gcn4 kinase activity were prepared from *SRB10* and *srb10Δ* cells and tested for their ability to support Gcn4 ubiquitination by Cdc34 and purified recombinant SCF^{Cdc4} complex. As shown in Fig. 2C, the kinase fraction from *SRB10* but not *srb10Δ* cells supported Gcn4 ubiquitination. In contrast, we did not observe any defect when the kinase fraction was prepared from a $kin28^{ts}$ strain (data not shown). Taken together, these observations suggest that Gcn4 is directly ubiquitinated by $Cdc34/SCF^{Cdc4}$, and that the Srb10 subunit of RNA Polymerase II holoenzyme is the predominant protein kinase in yeast extract that is able to specify ubiquitination of Gcn4 by this pathway.

Srb10, but not Kin28, phosphorylates Gcn4 in vitro and targets it for SCF-dependent ubiquitination

Since both Srb10 and Kin28 associate with the RNA polymerase II holoenzyme complex (Myer and Young 1998), we next tested if either Srb10 or Kin28 could directly phosphorylate Gcn4 by immunoprecipitating each of the kinases from yeast whole cell extracts and performing kinase assays using purified Gcn4 expressed in *E. coli*. Anti-myc immunoprecipitates from $SRB10^{myc9}$ cells exhibited potent Gcn4 kinase activity, whereas those from $KIN28^{myc9}$ cells had little or no activity (Fig. 3A, lanes 2-4). As expected (Hengartner et al. 1998), GST-CTD (C-terminal domain) was phosphorylated

by both Srb10^{myc9} and Kin28^{myc9} immunoprecipitates (lanes 6-8). GST-CTD kinase activity observed in the Kin28^{myc9} immunoprecipitate was not due to contaminating Srb10 because similar activity was recovered from an *srb10Δ* strain (lane 8). These data demonstrate that the high MW Gcn4 kinase activity that we enriched for is due to Srb10, not Kin28. To further confirm the specificity of the kinase activity present in Srb10^{myc9} immunoprecipitates, we evaluated the Gcn4 kinase activity of immunoprecipitates prepared from an *srb10-3*^{myc9} strain, which harbors an active-site mutant form of the Srb10 kinase that is incorporated into the RNA polymerase II holoenzyme (Liao et al. 1995). Although similar amounts of Srb protein were present in Srb10^{myc9} and Srb10-3^{myc9} immunoprecipitates (Fig. 3B, lower panel), only the former phosphorylated Gcn4 (Fig. 3B, upper panel).

In our initial experiments to test the ability of Srb10 to phosphorylate Gcn4, we used a strain (Z689) that expressed HA-tagged Srb11, the cyclin partner of Srb10. Anti-HA immunoprecipitates from Z689 cells contained Gcn4 kinase activity similar to that shown in Fig. 3A (data not shown). [^{35}S]methionine-labeled Gcn4, after pre-incubation with an Srb11^{HA} immunoprecipitate, was ubiquitinated in the presence of purified Uba1, Cdc34 and SCF^{Cdc4}(Fig. 3C, lane 4). This reaction was specific because it was dependent on all of the above components and ubiquitin (lanes 2, 5-8). Ubiquitination of Gcn4 by the Cdc34/SCF^{Cdc4} pathway was also reconstituted with Gcn4 produced in *E. coli* (Fig. 3D). The greater reaction efficiency observed in this experiment was most likely due to the use of SCF preparations containing Hrt1. Taken together, these results indicate that phosphorylation of Gcn4 by Srb10 promoted its recognition and ubiquitination by SCF^{Cdc4}.

Srb10 phosphorylates Gcn4 and regulates its stability in vivo

To investigate the physiological relevance of our biochemical data, we first tested if Srb10 influences the phosphorylation state of Gcn4 in vivo by evaluating the electrophoretic mobility of $Gcn4^{myc9}$ immunoprecipitated from pulse-radiolabeled wild-type and *srb10* mutant cells. Gcn4 was phosphorylated in vivo in an Srb10-dependent manner as evidenced by a phosphatase-sensitive (Fig. 4A, right panel) MW upshift that was diminished in *srb10* mutant cells (Fig. 4A, left panel). This, combined with our in vitro observations, strongly suggests that the Srb10 CDK is a physiological Gcn4 kinase.

If Srb10 targets Gcn4 for turnover by the SCF^{Cdc4} pathway, we predicted that Gcn4 should be stabilized in *srb10* mutants. To test this possibility, we carried out pulse-chase experiments to measure the turnover rate of $Gcn4^{myc9}$ in wild-type and *srb10* mutants. It is important to note that the tagged Gcn4 was expressed from the endogenous *GCN4* locus, under the control of the native promoter. Consistent with previously published results (Kornitzer et al. 1994; Meimoun et al. 2000), $Gcn4^{myc9}$ was rapidly degraded in wild-type cells, with a half-life of 2.5-5 min (Figs. 4B and 5B). In contrast, $Gcn4^{myc9}$ was moderately stabilized in both *srb10-3* and *srb10Δ* mutants with a half-life of 10-12 min (Figs. 4B and 5B). Consistent with this difference in half-life, the steady-state level of $Gcn4^{myc9}$ is about two-fold higher in *srb10* mutant cells compared to wild type (Fig. 4C). Careful examination of autoradiograms revealed that the phosphorylation-dependent upshifted form of $Gcn4^{myc9}$ was strongly reduced, but not eliminated, in *srb10* cells (Figs. 4B, 5A, and 5B). Furthermore, $Gcn4^{myc9}$ was more effectively stabilized in $cdc34^{ts}$ cells at the restrictive temperature ($t_{1/2} > 20$ min; Fig. 4D). We thus inferred that there might be additional protein kinases that can target Gcn4 for degradation via the $Cdc34/SCF^{Cdc4}$ pathway.

Both Srb10 and Pho85 contribute to Gcn4 turnover in vivo

Meimoun et al. (2000) noted in their report that Gcn4 is not stabilized in an *srb10-3* mutant. However, we reproducibly observed partial stabilization of Gcn4 in *srb10* mutants. Since Meimoun et al. performed their pulse-chase analyses with overexpressed derivatives of Gcn4, we evaluated whether overproduction influenced the apparent rate of Gcn4 degradation. To test this, we directly compared the rates of $Gcn4^{myc9}$ degradation in strains that expressed $GCN4^{myc9}$ from a *GAL* promoter with the strains that expressed $GCN4^{myc9}$ from its own promoter. Indeed, when $Gcn4^{myc9}$ was overproduced, its rate of degradation appeared very similar in wild-type and *srb10Δ* cells (Fig. 5A, top panels). At normal levels of expression, however, Gcn4 was clearly stabilized in an *srb10Δ* mutant (Fig. 5A, bottom panels). Immunoblotting confirmed that the steady-state levels of $Gcn4^{myc9}$ were at least five-fold higher in *GAL*-driven $GCN4^{myc9}$ strains compared with native-promoter-driven $GCN4^{myc9}$ strains (data not shown). Interestingly, we also noticed a progressive, *SRB10*-independent post-translational modification of $Gcn4^{myc9}$ when it was overproduced (Fig. 5A, arrowhead; note that Gcn4 expressed from its own promoter does not show the same extent of progressive modification in *srb10Δ* cells), suggesting that the overexpressed protein is more susceptible to a protein kinase that normally does not act (or acts poorly) upon endogenously expressed Gcn4.

We evaluated $Gcn4^{myc9}$ stability in several other CDK mutants in an attempt to address whether multiple CDKs play a redundant role in Gcn4 turnover. $Gcn4^{myc9}$ degradation was unaffected in *ctk1Δ* and α-factor arrested cells, suggesting neither Ctk1, a CDK homolog that phosphorylates CTD, nor Cdc28 contributed to turnover (data not shown). However, we found that $Gcn4^{myc9}$ was moderately stabilized in a *pho85Δ* mutant with a half-life of about 20 min (Fig. 5B), similar to what was reported by Meimoun, et al. (2000). To test if Srb10 and Pho85 contribute independently to Gcn4 turnover, we

tested Gcn4^{myc9} stability in an *srb10Δ pho85Δ* double mutant strain. As shown in Fig 5B, Gcn4^{myc9} was further stabilized in *srb10Δ pho85Δ* cells with a half-life of over 40 min, similar to what was observed in *cdc34^{ts}* cells at 37°C (Fig. 4D). Since *srb10* and *pho85* mutations had an additive effect, we conclude that at least two CDKs (Srb10 and Pho85) contribute to rapid Gcn4 turnover in vivo.

Srb10-dependent degradation of Gcn4 is not regulated by amino acid starvation

Since stabilization of Gcn4 under amino acid starvation conditions is achieved at least in part by the down regulation of Pho85 activity (Meimoun et al. 2000), we wanted to test if Srb10-dependent degradation of Gcn4 was regulated in a similar fashion. To test this, we imposed amino acid starvation conditions by culturing cells in minimal medium lacking a specific amino acid that the cells were auxotrophic for and then measured Gcn4 stability under starved and unstarved conditions in wild-type, *srb10Δ*, and *pho85Δ* cells. Although Gcn4^{myc9} was significantly stabilized upon amino acid starvation with a half-life of about 20 min (Fig. 6A), it is further stabilized in *srb10Δ* cells with a half-life of about 40 min (Fig. 6B). Furthermore, Srb10-dependent phosphorylation of Gcn4^{myc9} (as indicated by the MW shift) was not diminished upon starvation (Fig 6A, B), suggesting that it is not regulated by amino acid starvation. Whereas amino acid starvation significantly stabilized Gcn4^{myc9} further in *srb10Δ* cells, it only increased Gcn4^{myc9} half-life slightly in *pho85Δ* cells (Fig. 6C). Taken together, these results suggest that Srb10 is not involved in the starvation-regulated aspect of Gcn4 turnover, and down-regulation of Pho85 is the primary means of stabilizing Gcn4 upon amino acid starvation.

CDK phosphorylation is required for rapid Gcn4 turnover in vivo

The protein sequence of Gcn4 reveals five putative CDK phosphorylation sites (S/TP): S17, T61, T105, T165 and S218. Our analyses of Gcn4 ubiquitination in vitro and stability in vivo suggest that these sites may be important for rapid Gcn4 turnover. To test this possibility, we phosphorylated purified Gcn4 using $Srb10^{myc9}$ immunoprecipitates and subjected the in vitro phosphorylated Gcn4 to mass spectrometry analysis. All phosphopeptides that were recovered contained one of the five consensus S/TP sites. Moreover, direct sequencing confirmed that four of the consensus sites (S17, T61, T105, and S218) were phosphorylated. The exact site of phosphorylation on T165-containing peptides could not be determined, but was presumed to be T165. To analyze if any of these sites is important for Gcn4 turnover, we first mutated each of the five sites by site-directed mutagenesis, and produced mutant forms of Gcn4 protein by in vitro translation. We then tested the ability of the five single and a quintuple (3T2S) phosphorylation-site mutants of Gcn4 to serve as substrates for SCF^{Cdc4}-dependent ubiquitination in vitro. Although we did not observe a marked defect for any of the single-site mutants, ubiquitination of the 3T2S quintuple mutant was greatly diminished (Y. Chi and R. Deshaies, unpublished data). Based on our in vitro data, we constructed a strain in which the wild-type *GCN4* locus was replaced with *gcn4-3T2S*^{myc9}, and the stability of the encoded mutant protein was tested by pulse-chase analysis. As shown in Fig. 7B, Gcn4-$3T2S^{myc9}$ was very stable in vivo, and there was no appreciable degradation during the 40-min chase period. Noticeably, the mutant protein did not exhibit the characteristic MW shift caused by Srb10-dependent phosphorylation.

The *gcn4-3T2S*^{myc9} strain did not show any obvious morphology or growth defect compared with wild-type. Since we utilized a C-terminally *myc9*-tagged version of *GCN4* for all of our in vivo studies, we were concerned that the myc9 tag might render

Gcn4 inactive. To test the activities of various Gcn4 derivatives in our strains, we employed a starvation plate assay similar to that described by (Natarajan et al. 1999). Cells were streaked on a control plate with normal leucine supplement and a plate with excess leucine, which causes isoleucine (Ile) and valine (Val) starvation (Niederberger et al. 1981). This assay monitors Gcn4-dependent activation of Ile and Val biosynthetic genes. As shown in Fig. 7A, all strains (tagged or untagged *GCN4*), except *gcn4* deletion mutants, grew on -Ile, -Val plates equally well as on control plates, suggesting that the myc9 tag did not diminish the function of Gcn4. Furthermore, normal growth of *gcn4-3T2S*^{myc9} cells on the starvation plate suggests that CDK phosphorylation is not essential for Gcn4 function.

Srb10 phosphorylates Msn2 and influences its localization

Microarray analysis of genome-wide gene expression in the *srb10-3* mutant reveals 173 genes whose expressions are up two-fold or more than wild-type, and a significant fraction of these genes are involved in stress response and diauxic shift (Holstege et al. 1998). Msn2 and Msn4 are two partially redundant zinc-finger transcription factors that activate expression of a suite of stress-response genes (Martinez-Pastor et al. 1996; Schmitt and McEntee 1996; Boy-Marcotte et al. 1998), including those induced in *srb10-3* cells. Based on our analysis of Gcn4 regulation, we hypothesized that Srb10 might also directly repress the activities of transcription factors, including Msn2 and Msn4, by targeting them for degradation by the SCF pathway. To this end, we tested the ability of Msn2 to serve as a substrate for Srb10 in vitro. Using an assay similar to that shown in Fig. 3A, we found Srb10 to be a potent kinase for recombinant GST-Msn2 (Fig. 8A, lane 6). Furthermore, phosphorylated GST-Msn2 was efficiently ubiquitinated by SCF^{Cdc4} (data not shown). However, pulse-chase analysis revealed that Msn2^{myc9} was a stable

protein in wild-type cells with a half-life of more than 1 hr (data not shown). Interestingly, when pulsed-labeled at 27°C and chased under heat stress conditions (37°C), Msn2 was rapidly (within 5 min) phosphorylated in an *SRB10*-dependent manner (Fig. 8B). Taken together, these data suggest that Srb10 phosphorylates Msn2 in vivo in response to stress. However, Srb10-dependent phosphorylation did not appear to target Msn2 for degradation via the Cdc34/SCF^{Cdc4} pathway.

Recent evidence suggests that Msn2 and Msn4 are regulated by shuttling between the cytoplasm and the nucleus in response to environmental conditions. Under normal conditions, Msn2 and Msn4 are retained in the cytoplasm, presumably by a combination of active nuclear export and cytoplasmic anchorage (Gorner et al. 1998; Beck and Hall 1999). To test if Srb10 influences the localization of Msn2/Msn4, we monitored Msn2 localization in *MSN2*^{myc9} strains by indirect immunofluorescence. As expected, Msn2^{myc9} was mostly cytoplasmic in wild-type cells under unstressed conditions (Fig.8C, panel *a*; Gorner et al. 1998). Interestingly, Msn2^{myc9} was localized to the nucleus in a sub-population (15-30%) of unstressed *srb10-3* cells (Fig. 8C, panel *c*) and *srb10Δ* cells (data not shown). Although we do not understand why Msn2 was mislocalized in only a fraction of *srb10Δ* cells, our results are consistent with the elevated expression of *MSN2/MSN4*-dependent genes in *srb10-3* cells as determined by microarray analysis of a population of cells (Holstege et al. 1998).

Because Msn5 has been shown to be a nuclear exporter for Pho4 that has been phosphorylated by Pho85 (Kaffman et al. 1998), we next tested if Msn5 was also required for the export of Msn2. Strikingly, Msn2^{myc9} was concentrated in the nucleus in >90% of *msn5Δ* cells under normal conditions (Fig. 8C, panel *b*), suggesting that Msn5 is the primary export receptor for Msn2.

Discussion

SCF ubiquitin ligases have been implicated in the regulation of multiple transcription factors, including β-catenin, Gcn4, and Met4 (Deshaies 1999). Here, we provide four major lines of evidence that the Srb10 CDK complex of the SRB/mediator module of the RNA polymerase II holoenzyme contributes to Gcn4 instability by phosphorylating Gcn4 and thereby targeting it to SCF^{Cdc4}. First, a high MW Srb10-containing complex was purified as the most prominent activity in yeast extract that sustains SCF^{Cdc4}-dependent ubiquitination of Gcn4. Second, immunopurified Srb10 phosphorylated recombinant Gcn4, thereby rendering it a substrate for recombinant SCF^{Cdc4}. Third, Gcn4 was phosphorylated in an *SRB10*-dependent manner in vivo. Fourth, Gcn4 was partially stabilized in *srb10* mutants. Taken together, our observations indicate that Gcn4 is a physiological target of Srb10, and raise the intriguing possibility that recruitment of SRB/mediator may limit how long a transcriptional regulatory protein can occupy a promoter.

Regulation of Gcn4 by Srb10 and Pho85

During the course of our work, Meimoun et al (2000) reported that Pho85 is required for rapid turnover of Gcn4. Although they show that Gcn4 is phosphorylated in vitro by recombinant Pcl1/Pho85, there is no direct evidence that this modification triggers its ubiquitination by SCF^{Cdc4}. Thus, it remains possible that Pho85 influences both Gcn4 and Sic1 (Nishizawa et al. 1998; Meimoun et al. 2000) degradation through an indirect effect on SCF^{Cdc4} activity. Nevertheless, we favor the hypothesis that both Srb10 and Pho85 directly promote Gcn4 turnover, and that the kinases act independently to target Gcn4 degradation, based on the observation that Gcn4 was modestly stabilized in

pho85Δ and *srb10Δ* cells, but strongly stabilized in *pho85Δ srb10Δ* double mutants. Our observations coupled with those of Meimoun et al. suggest that it may be possible to generate more sophisticated regulatory controls by making the SCF-dependent turnover of a substrate require its phosphorylation by two or more kinases. Because distinct protein kinases can target different substrates to a single type of SCF complex (e.g. G1-CDK targets Sic1, whereas either Srb10 or Pho85 targets Gcn4, to the SCF^{Cdc4} complex), and because there are potentially 17 different types of SCF complexes and 120 protein kinases in yeast, combinatorial interactions between these elements can potentially generate a staggering array of regulatory controls.

Recent evidence suggests that amino acid starvation stabilizes Gcn4 through the negative regulation of Pho85-dependent phosphorylation (Meimoun et al. 2000). Interestingly, we found that Gcn4 was only moderately stabilized upon starvation and was further stabilized in *srb10Δ* cells. Moreover, Srb10-dependent phosphorylation of Gcn4 remained intact in amino acid-starved cells (Fig. 6A, B). This indicates that whereas Pho85 is down-regulated upon starvation, Srb10-dependent degradation of Gcn4 persists. It appears that Srb10 and Pho85 contribute to SCF^{Cdc4}-dependent degradation of Gcn4 in different ways, either by targeting different pools of Gcn4 or by responding to different cellular signals.

If Srb10 negatively regulates Gcn4 by promoting its turnover, one would expect to see increased expression of Gcn4 target genes in *srb10* mutants. Although microarray analysis of genome-wide gene expression in the *srb10-3* mutant reveals 173 genes whose expressions are up two-fold or more compared to wild-type (Holstege et al. 1998), only two of these are potential Gcn4 targets. This suggests that *srb10* mutation does not lead to global derepression of Gcn4 target genes under normal growth conditions. We provide the following possible reasons to explain the microarray data. First, stabilization of Gcn4 in *srb10* mutants only leads to ~1.5-2 fold increase in the steady state level of Gcn4 (Fig.

4C). Compensatory regulatory mechanisms, such as redundant kinases that promote Gcn4 turnover and the well-characterized Gcn4 translational controls, may serve to limit the accumulation of Gcn4 in *srb10* cells. The former possibility is supported by the observation that the 3T2S mutations have a greater effect on Gcn4 stability than the *pho85 srb10* double disruption (compare Fig. 5B, Fig. 7B). Second, Srb10 may have self-canceling positive and negative effects on Gcn4. It is possible that phosphorylation of Gcn4 by Srb10 stimulates its activity while also promoting its turnover. If so, the loss of Srb10-dependent phosphorylation of Gcn4 may not only stabilize the protein but also decrease its potency as a transcriptional activator. Third, pleiotropic effects of *srb10* mutation, such as derepression of the stress response genes (Holstege et al. 1998) and slow growth, may indirectly alter the expression profile of Gcn4 target genes. Despite the absence of major change in the transcriptional output of Gcn4 in *srb10* mutants, it is evident that Srb10 negatively regulates Gcn4 by promoting its rapid turnover.

Regulation of Msn2 and Msn4 by Srb10

The transcriptional activities of the partially redundant Msn2 and Msn4 proteins are tightly regulated. In resting cells these factors are inactive, whereas in stressed cells they promote transcription of a large suite of genes (Martinez-Pastor et al. 1996; Schmitt and McEntee 1996; Boy-Marcotte et al. 1998). Srb10 is implicated in the regulation of Msn2/Msn4, in that multiple transcriptional targets of these proteins are induced in *srb10-3* cells (Holstege et al. 1998). Msn2 was phosphorylated by Srb10 in vitro, and the phosphorylation state of Msn2 was modulated by Srb10 in vivo (Fig. 8A, B). In contrast to Gcn4, however, Msn2 was stable in resting or stressed cells, regardless of whether it was localized to the cytoplasm or nucleus (data not shown).

Activation of Msn2 in stressed cells is linked to its translocation from the cytoplasm to the nucleus (Gorner et al. 1998). Whereas Msn2 is exclusively cytoplasmic in resting wild type cells, Msn2 was primarily nuclear in 15-30% of *srb10* cells (Fig. 8C). Although our data show that *SRB10* can influence Msn2 localization, other factors, including the TOR and cAMP-dependent protein kinases, govern the nucleocytoplasmic shuttling of Msn2 in stressed cells (Gorner et al. 1998; Beck and Hall 1999), and regulated trafficking of Msn2 still occurred properly in the majority of *srb10* cells (data not shown). The underlying basis for the heterogeneity of *srb10* cell populations is unclear. Perhaps the majority of *srb10Δ* cells adapt to chronic Msn activity in the nucleus by inducing factors that partially bypass Srb10's role in Msn2 export. Regardless of the exact relationship between stress, Msn2/Msn4, and Srb10, the most parsimonious hypothesis is that the global induction of Msn target genes in *srb10-3* cells (Holstege et al. 1998) results, at least in part, from failure to properly phosphorylate and thereby promote Msn5-dependent export of Msn2/Msn4 from the nucleus. Mapping and mutation of Srb10 phosphorylation sites in Msn2 will be needed to vigorously establish a direct role for Srb10 in the nuclear export of Msn2.

Implications for negative regulation of transcription by SRB/mediator: beyond CTD phosphorylation

The Srb10/Srb11 CDK/cyclin pair is part of a heterotetrameric module, which associates with the multiprotein complex termed "SRB/mediator" (Myer and Young 1998). Current models suggest that the Srb10 module functions through CTD of RNA polymerase II, in part by controlling its phosphorylation (Hengartner et al. 1998). Both genetic and microarray analyses indicate that Srb10 serves as a negative effector of specific transcriptional programs (Carlson 1997; Holstege et al. 1998). These

observations pose a key question: how does Srb10 activity selectively repress the expression of a small subset of the genome? Biochemical studies revealed that phosphorylation of CTD by Srb10 blocked the recruitment of RNA polymerase holoenzyme to promoter DNA (Hengartner et al. 1998). It was postulated that gene-specific control by Srb10 might be mediated by promoter-bound factors that influence whether or not Srb10/Srb11 phosphorylates CTD before a promoter-bound preinitiation complex is formed.

An expanded view for how Srb10 negatively regulates transcription of selected genes is supported by our work. Specifically, we propose that Srb10 directly phosphorylates transcription factors, thereby altering their activity. This provides a simple and logical explanation for how Srb10 mediates gene-specific transcriptional regulation. Interestingly, the exact mechanism of regulation appears to differ for different transcription factors. Whereas Srb10 targets Gcn4 for SCF^{Cdc4}-dependent degradation, it appears to down-regulate Msn2 activity by promoting nuclear exclusion (although the exact manner in which this occurs remains to be determined). In support of a direct role for Srb10 in the regulation of transcription factors, phosphorylation of the S699 residue of Gal4 by Srb10 was recently shown to be essential for Gal4-dependent induction of *GAL* genes (Hirst et al. 1999). Coupled with phosphorylation of the CTD, direct phosphorylation of specific transcription factors by Srb10 might potentiate its inhibitory effect on transcription and help focus its repressive activity upon specific promoters.

An interesting idea that emerges from ours and other studies is that direct negative regulation of transcriptional activators by Srb10 may be confined to the vicinity of promoters to limit the lifetime of transcription factors that are actively engaged in promoting transcription. We propose that Srb10 comprises a "timer" that constitutively targets promoter-bound Gcn4 for degradation to limit the number of transcripts that a

DNA-bound molecule of Gcn4 can promote. Intriguingly, analysis of a set of synthetic transcriptional activators revealed an inverse relationship between their potency and their stability, suggesting that the degradation of transcription factors may be mechanistically coupled to their ability to promote assembly of active transcriptional complexes on promoter DNA (Molinari et al. 1999). Thus, spatially restricted activation of transcription factor proteolysis may be a general regulatory theme. Note that a related argument can also be advanced for Msn2 and Msn4, whereby Srb10-dependent phosphorylation initiates the nuclear export of Msn molecules located at promoter elements. Although our studies highlight roles for Srb10 in the control of Gcn4 stability and Msn2 export, it is possible that Srb10 modulates other aspects of transcription factor activity in a spatially restricted manner. It will be interesting to see if the mammalian homolog of Srb10 likewise mobilizes diverse regulatory strategies to enable tight regulation of gene expression.

Materials and methods

Yeast strains

The yeast strains used in this study are listed in Table 1. All YC strains were derived from Z719, Z690, and Z687 (Liao et al. 1995). Yeast cells were cultured in standard rich or synthetic medium supplemented with 2% dextrose, raffinose, or galactose as described (Sherman 1991). The *GCN4* locus was modified to encode Gcn4 tagged with three copies of the HA epitope (HA3) at its C-terminus as described (Schneider et al. 1995), and a *gcn4* deletion strain (*gcn4::HA3*) was generated using a similar scheme. The *GCN4*, *SRB10*, *srb10-3*, *KIN28* and *MSN2* loci were modified to encode proteins tagged with nine copies of the myc epitope (myc9) at their C-termini as

described (Seol et al. 1999). *GAL-GCN4* alleles were generated by replacement of the entire *GCN4* promoter region with a PCR fragment containing *URA3* and *GAL1, 10* promoter sequences. *cdc34*^{ts} strains were constructed by homologous recombination using a plasmid carrying *cdc34-2* (Kornitzer et al. 1994). The *gcn4*, *pho85* and *msn5* deletion strains were generated by PCR-based one-step replacement. Deletion alleles were confirmed by scoring mutant phenotypes and by PCR. To generate the *gcn4-3T2S*^{myc9} strain, sequences spanning the five SP/TP sites within *GCN4*^{myc9} were first replaced by *URA3*. The resulting *GCN4*^{myc9}*::URA3* strain was subsequently transformed with a *gcn4-3T2S* fragment that also contained adjacent 5' untranslated sequences. Transformants were cultured in YPD for 6-10 hours prior to plating on 5-fluoroorotic acid (5-FOA) medium. 5-FOA resistant transformants were screened by PCR and confirmed by sequencing. All strains derived from Z719, Z690 or Z687 were constructed either by direct transformation using lithium acetate method or by genetic cross. Details of the strain constructions or oligonucleotide sequences are available upon request.

Fractionation of yeast extracts

Whole cell or DEAE Sepharose FF (Amersham Pharmacia) fractions of yeast extracts from G1 cyclin-depleted RJD885 or RJD893 cells were prepared as described (Verma et al. 1997b) with the following modifications. After the frozen cell powder was thawed in Buffer93 (B93), saturated (100%) ammonium sulfate solution was added slowly to a final concentration of 10% to allow further extraction of proteins. To concentrate fractionated proteins prior to assay, the flow-through and 0.15-0.25 M NaCl eluate of the DEAE Sepharose column were concentrated (to ~30 mg/ml) using Ultrafree-15 centrifugal filter (Biomax-10K, Millipore) and dialyzed against B93.

To resolve Gcn4 kinase and ubiquitination activities, RJD893 whole cell extract (WCE) was first fractionated by ammonium sulfate precipitation. The 0-35% and the 45-60% cuts were resuspended in CWB (25 mM HEPES [pH7.6], 25 mM NaCl and 1 mM DTT) and applied to a DEAE Sepharose column. The 0.25 M eluate from each DEAE fractionation was concentrated to 10-15 mg/ml and dialyzed against B93. The 0.25 M DEAE eluate from the 0-35% ammonium sulfate cut contained Gcn4 kinase activity, whereas that from the 45-60% cut contained Gcn4 ubiquitination activity (when supplemented with the kinase fraction plus Cdc34). For assays shown in Fig. 2C, the dialyzed 0-35% ammonium sulfate cut was used directly without further purification.

To partially purify the Gcn4 kinase, we grew RJD481 cells in YPD to late log phase [optical density at 600 nm (OD_{600})=2.5-3] and harvested and lysed them as described (Verma et al. 1997b). WCE (~1000 mg protein) was first fractionated by ammonium sulfate precipitation, and the 0-40% cut (~150 mg protein) was resuspended in CWB and loaded onto a 10-ml SP Sepharose FF (Amersham Pharmacia) column. The column was washed with 30 ml CWB and eluted with a 100-500 mM NaCl gradient. Fractions containing the peak activity (as measured by kinase and ubiquitination assays, see below) were pooled (~7 mg protein), diluted in CWB and applied to 2 ml of glutathione agarose (Sigma) previously loaded with ~4 mg GST-Gcn4. The affinity column was washed with 5 ml CWB +100 mM NaCl and eluted with 4 ml CWB +400 mM NaCl. The eluate was concentrated to 200 μl (~300 μg protein) and 50 μl was loaded onto a 2.4 ml Superdex200 column (Amersham Pharmacia) equilibrated with CWB +500 mM NaCl. 50 μl fractions were collected and 2 μl of each fraction was used in the activity assays.

Preparation of assay substrates

GCN4 and *SIC1* were transcribed from EcoRI-linearized YEp88-GCN4 and RDB445 by SP6 and T7 polymerase, respectively. *GCN4-HA* transcription template was generated by PCR using a 5' oligonucleotide containing a T7 RNA polymerase promoter (Verma et al. 1997b) and a 3' oligonucleotide containing sequences that encode the HA epitope. All mRNAs were translated in rabbit reticulocyte lysates according to manufacturer's instructions (Promega). For ubiquitination assays, in vitro-translated Gcn4 was partially purified by batch chromatography on DEAE resin as described for Sic1 (Verma et al. 1997a). The 100-400 mM NaCl DEAE eluate containing Gcn4 was heated at 65°C for 5 min to destroy a non-specific Gcn4 kinase activity present in reticulocyte lysates (brief heating at up to 90°C did not significantly compromise the ability of Gcn4 to serve as a ubiquitination substrate). The eluate was then centrifuged at 10,000 g for 5 min and the supernatant was exchanged into 30 mM Tris-HCl (pH7.5), 50 mM potassium acetate and 1 mM DTT by three cycles of dilution and concentration to the original volume in a Centricon-10 (Amicon). Recombinant Gcn4 was also expressed in *E. coli* and purified as described (Kim et al. 1994). GST, GST-Gcn4, and GST-Msn2 were expressed in BL21(DE3) containing pLyS, and were purified by glutathione affinity chromatography. GST-CTD was expressed and purified as described (Thompson et al. 1993).

Immunoprecipitations, kinase assays and immunoblots

Active protein kinases were isolated from yeast strains in which the relevant genes were modified to encode proteins with a myc9 epitope at the C-terminus. Cells were grown to late log phase (OD_{600} 2.5-3) and whole cell extracts were prepared as described above. All purification procedures were performed on ice or at 4°C. Typically, cell extract (200-300 µl of 30-40 mg/ml) was diluted three fold with Buffer B

(30 mM Tris-HCl [pH7.5], 6% ammonium sulfate, 1 mM EDTA, 1 mM DTT, 0.02% Triton X-100 and 0.5 mM PMSF), and pre-incubated with 15 μl protein A beads for 1 hr. After brief centrifugation, the supernatant was incubated with 1 μl of anti-myc monoclonal antibody 9E10 ascites fluid for 2 hrs and then 10-15 μl of protein A beads for additional 1-2 hrs. The protein A beads were washed three times with Buffer B and once with kinase assay buffer (KAB: 30 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 10 mM NaCl, 1 mM DTT and 0.02% Triton X-100).

^{32}P kinase assays were performed by incubating immobilized kinase with 1 μg substrate (Gcn4, GST, GST-CTD or GST-Msn2) in a 10 μl reaction containing KAB, 0.5 μl γ-^{32}P-ATP (4500 Ci/mmol), and 50 μM ATP at 22°C for 1 hr with occasional mixing. The reaction was briefly centrifuged and the supernatant was either used immediately as substrate for the ubiquitination reactions (Figs. 3D) or mixed with Laemmeli sample buffer, boiled for 3 min, resolved by SDS-PAGE and visualized by autoradiography. For [^{35}S]methionine-labeled Gcn4 substrates, kinase assays (Figs. 1B and 3C) were carried out for 30 min at 25°C in KAB supplemented with ATP regenerating system.

Immunoblotting was performed with either monoclonal 9E10 ascites fluid (1:2000 dilution) or affinity-purified anti-Cdc28 polyclonal antibody. Blots were visualized using horseradish peroxidase-conjugated secondary antibodies and a chemiluminescence (ECL+) kit (Amersham). For experiment in Fig. 4C, blots were scanned and quantitated using the STORM system (Molecular Dynamics).

Ubiquitination assays

Ubiquitination reactions in yeast extracts (Verma et al. 1997a) or with purified components (Feldman et al. 1997; Seol et al. 1999) were carried out essentially as described. Two forms or SCF^{Cdc4} were used during the course of this study: a triple

infection complex (Skp1/Cdc53^{PyHA}/Cdc4^{PyHA}) and a quadruple infection complex (Hrt1/Skp1/Cdc53/Cdc4^{PyHA}), both of which were expressed in baculovirus-infected Sf9 insect cells and purified by binding to anti-polyoma (α-Py)-conjugated protein A beads and eluted using Py peptide as described (Seol et al. 1999). Approximately 100-150 ng of SCF^{Cdc4} as estimated by Coommassie-stained gels was used in each reaction. Reactions were incubated at 25°C for 1 hr, terminated by addition of Laemmeli sample buffer, and evaluated by SDS-PAGE and autoradiography.

Pulse-chase experiments

GCN4^{myc9}, *MSN2^{myc9}*, and untagged control cells were grown overnight in SD +leucine +histidine + uracil at 30°C to mid-log phase (OD_{600} ~0.5). Approximately 3 x 10^8 cells were harvested and concentrated by centrifugation into 3 ml of the same medium. Cells were pulse-labeled for 5 min with 500 μCi of Tran^{35}Slabel (1175 Ci/mmol, ICN), then chased in the same medium containing 1mM ammonium sulfate, 5mM methionine and 1mM cysteine. For *cdc34-2* mutants, cells were shifted to 37°C for 1 hr before labeling. At various times of the chase, 0.5 ml of the culture was removed and diluted immediately into 1.5 ml cold stop buffer (50 mM Tris-HCl [pH7.5], 50 mM NaF and 0.1% NaN_3). Cells were centrifuged briefly and cell pellets were frozen in liquid nitrogen (and stored at –80°C if necessary). The cell pellets were then each supplemented with 100 μl of 0.5 mm glass beads and 100 μl lysis buffer (30 mM Tris-HCl [pH7.5], 0.5% SDS, 6% ammonium sulfate, 1 mM EDTA, 1 mM DTT and 0.5 mM PMSF), vortexed for 2 s, and boiled for 3 min. The samples were cooled briefly, vortexed for 2 min, boiled again for 2 min and vortexed again for 1 min. Samples were then centrifuged at 10,000 g for 10 min, and the supernatant was diluted five-fold with Triton buffer (30 mM Tris-HCl [pH7.5], 1% Triton X-100, 1 mM DTT and 1 mM PMSF)

and immunoprecipitated with 9E10 antibody and protein A beads. The protein A beads were washed three times with Buffer B, aspirated to dryness, and mixed with 2X Laemmeli buffer. Samples were boiled for 3 min prior to being evaluated by SDS-PAGE followed by autoradiography and PhosphorImager (Molecular Dynamics) analysis.

ES–MS Analysis of phosphorylated Gcn4

Phospho–Gcn4 (70 pmol) was digested with modified trypsin (Promega) for 6 hours at 37°C and then subjected to multi-dimensional phosphopeptide analysis by ESMS (Verma et al. 1997c). Briefly, phosphopeptides were detected by online LC-ESMS. The flow from the column was split with 95% going to a fraction collector and 5% to the mass spectrometer. The mass spectrometer is optimized to produce and detect CID generated m/z 79 (PO_3^-) product ions, which are highly specific for phosphorylated peptides (Huddleston et al. 1993). The MS is operated in a single ion-monitoring mode for enhanced sensitivity. Phosphopeptide-containing HPLC fractions (containing m/z 79 ions) were then analyzed by negative-ion nanoelectrospray using precursor ion scans to distinguish phosphopeptides from unmodified peptides, and determine their mass (Carr et al. 1996). Candidate phosphopeptides were sequenced by tandem mass spectrometry using with nanoES (Carr et al. 1996) or on LC-ES MS/MS (Zhang et al. 1998). Phosphorylation site stoichiometry was determined by measuring the ratio of phosphorylated to nonphosphorylated peptide in the positive ion ES spectrum. Positive ion spectra for each phosphorylated and nonphosphorylated peptide were extracted and summed from a full scan LC-MS analysis of 18 pmol of Gcn4 tryptic digest.

Immunofluorescence

Cells were growing overnight in 30 ml YPD at 27°C to mid-log phase (OD_{600} ~0.5). 4 ml of cells were then removed and fixed with 4.5% formaldehyde at room temperature for 1 hr. Indirect immunofluorescence was carried out essentially as described (Pringle et al. 1991) using 9E10 (1:3000) as primary antibodies and fluorescein-conjugated-goat-anti-mouse antibodies (1:3000) as secondary antibodies. Fujichrome Provia 400 slide film was used to record data images on a Zeiss Axioskop microscope.

Acknowledgements

We thank K. Struhl, R. Kornberg, D. Kornitzer, R. Feldman, G. Reynard, C. Correll and J. H. Seol for supplying plasmids, strains and baculovirus-expressed proteins. We thank R. Conaway and M. Petroski for comments on the manuscript. This work was financed in part by a grant from the NIH (GM52466), and support from the Lucille P. Markey Charitable Trust and the Howard Hughes Medical Institute (all to R.J.D.) Y.C. was supported by a National Research Service Award (T32GM07616) from the National Institute of General Medical Sciences.

Table 1. Yeast strains

Strain	Genotype
RJD885	*MATα leu2 trp1 ura3 cln1::URA3 cln2::LEU2 cln3::URA3 pep4::TRP1 leu2::GAL-CLN3::LEU2 cdc28::CDC28^{HA}::HIS3*
RJD893	*MATa leu2 trp1 ura3 cln1::URA3 cln2::LEU2 cln3::URA3 pep4::TRP1 leu2::GAL-CLN3::LEU2 cdc28::CDC28^{HA}::HIS3 cdc4*
RJD481	*MATα leu2 trp1 ura3 pep4::LEU2*
Z719	*MATa his3Δ200 leu2-3,112 ura3-52*
Z687	*MATa his3Δ200 leu2-3,112 ura3-52 srb10Δ1::hisG*
Z690	*MATa his3Δ200 leu2-3,112 ura3-52 srb10-3::hisG*
Z689	*MATa his3Δ200 leu2-3,112 ura3-52 srb11Δ::hisG*
	RY7038[*SRB11^{HA} CEN URA3*]
YC1	*MATa his3Δ200 leu2-3,112 ura3-52 GCN4^{HA3}*
YC2	*MATa his3Δ200 leu2-3,112 ura3-52 GCN4^{HA3} srb10Δ1::hisG*
YC6	*MATa his3Δ200 leu2-3,112 ura3-52 GCN4^{HA3} SRB10^{myc9}*
YC15	*MATa his3Δ200 leu2-3,112 ura3-52 GCN4^{HA3} KIN28^{myc9}*
YC16	*MATa his3Δ200 leu2-3,112 ura3-52 GCN4^{HA3} KIN28^{myc9} srb10Δ1::hisG*
YC17	*MATa his3Δ200 leu2-3,112 ura3-52 SRB10^{myc9}*
YC7	*MATa his3Δ200 leu2-3,112 ura3-52 srb10-3^{myc9}::hisG*
YC19	*MATa his3Δ200 leu2-3,112 ura3-52 GCN4^{myc9}*
YC21	*MATa his3Δ200 leu2-3,112 ura3-52 GCN4^{myc9} srb10Δ1::hisG*
YC23	*MATa his3Δ200 leu2-3,112 ura3-52 GAL-GCN4^{myc9}*
YC24	*MATa his3Δ200 leu2-3,112 ura3-52 GAL-GCN4^{myc9} srb10Δ1::hisG*
YC45	*MATa his3Δ200 leu2-3,112 ura3-52 GCN4^{myc9} srb10–3::hisG*
YC40	*MATa his3Δ200 leu2-3,112 ura3-52 cdc34-2*
YC35	*MATa his3Δ200 leu2-3,112 ura3-52 GCN4^{myc9} cdc34-2*
YC67	*MATa his3Δ200 leu2-3,112 ura3-52 pho85::URA3*
YC59	*MATa his3Δ200 leu2-3,112 ura3-52 GCN4^{myc9} pho85::URA3*
YC69	*MATa his3Δ200 leu2-3,112 ura3-52 pho85::URA3 srb10Δ1::hisG*
YC61	*MATa his3Δ200 leu2-3,112 ura3-52 GCN4^{myc9} pho85::URA3 srb10Δ1::hisG*
YC25	*MATa his3Δ200 leu2-3,112 ura3-52 gcn4::HA3*
YC57	*MATa his3Δ200 leu2-3,112 ura3-52 gcn4^{myc9}::URA3*
YC94	*MATa his3Δ200 leu2-3,112 ura3-52 gcn4-3T2S^{myc9}*
YC84	*MATa his3Δ200 leu2-3,112 ura3-52 MSN2^{myc9}*
YC124	*MATa his3Δ200 leu2-3,112 ura3-52 MSN2^{myc9} srb10–3::hisG*
YC111	*MATa his3Δ200 leu2-3,112 ura3-52 msn5::URA3*
YC107	*MATa his3Δ200 leu2-3,112 ura3-52 MSN2^{myc9} msn5::URA3*

Fig. 1

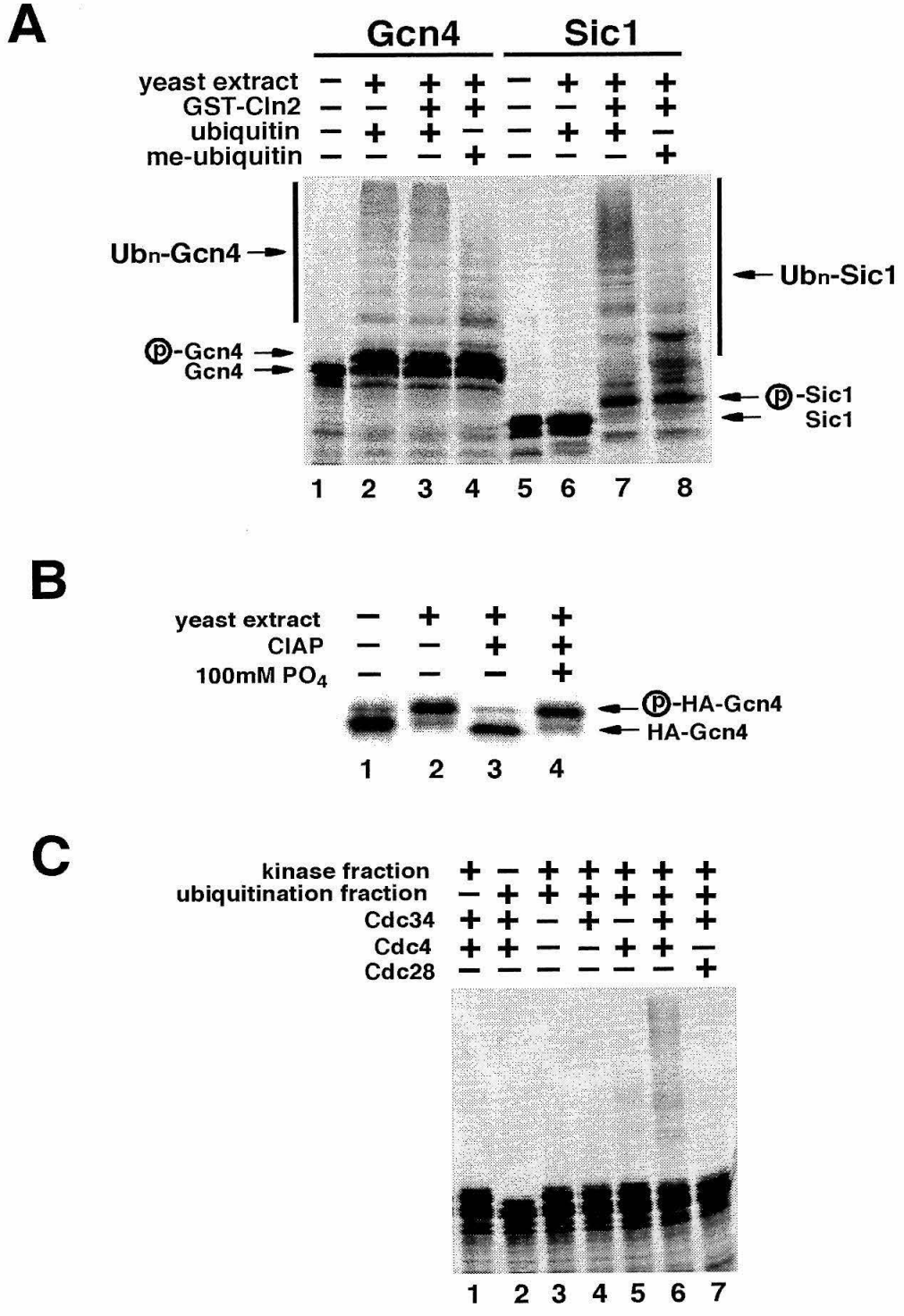
A
Gcn4
Sic1
yeast extract
GST-Cln2
ubiquitin
me-ubiquitin
Ubn-Gcn4
Ubn-Sic1
Ⓟ-Gcn4
Gcn4
Ⓟ-Sic1
Sic1
1 2 3 4 5 6 7 8
B
yeast extract
CIAP
100mM PO4
Ⓟ-HA-Gcn4
HA-Gcn4
1 2 3 4
C
kinase fraction
ubiquitination fraction
Cdc34
Cdc4
Cdc28
1 2 3 4 5 6 7

Figure 1. Gcn4 ubiquitination in yeast extracts requires SCF components and a protein kinase activity other than Cdc28.

(*A*) [^{35}S]methionine-labeled Gcn4 (lanes *1-4*) or Sic1 (lanes *5-8*) synthesized by in vitro translation were incubated with G1-cyclin depleted yeast whole cell extracts from RJD885 strain in the absence (lane *2,6*) or presence (lane *3,7*) of purified GST-Cln2. Uba1, Cdc34 and SCF activities were supplied by the yeast extracts. Lanes *1* and *5* contained only input substrates. Methylated ubiquitin (me-ubiquitin) was used instead of wild-type ubiquitin in lanes *4* and *8*. Encircled P refers to phosphorylated forms of Sic1 and Gcn4. All samples in panels A-C were evaluated by SDS-PAGE followed by autoradiography.

(*B*) [^{35}S]methionine-labeled Gcn4^{HA} translation product was phosphorylated by DEAE flow-through fraction of RJD885 whole cell extract (see Materials and Methods). An aliquot of the reaction was immunoprecipitated using 12CA5 antibody and mock treated (lane *2*) or treated with calf intestinal alkaline phosphatase (CIAP) in the absence (lane *3*) or presence (lane *4*) of phosphate inhibitor as described (Verma et al. 1997a). Immunoprecipitated input Gcn4^{HA} is shown in lane *1*.

(*C*) Ubiquitination of Gcn4 in fractionated yeast extracts from a *cdc4^{ts}* strain (RJD 893). Extract fractions lacking Cdc34 but supplying either SCF components or a kinase activity were used (see Materials and Methods). A complete reaction (lane *6*) included a kinase fraction (~10 μg), a ubiquitination fraction (~15 μg), Cdc34 (100 ng) and insect cell lysate containing baculovirus-expressed Cdc4 (~4 μg total protein, Verma et al. 1997a). In lanes *1-5*,one or more of the above components was omitted, as indicated. Insect cell lysate containing baculovirus-expressed Cdc28 was used as specificity control (lane *7*) for the Cdc4 lysate.

Fig. 2

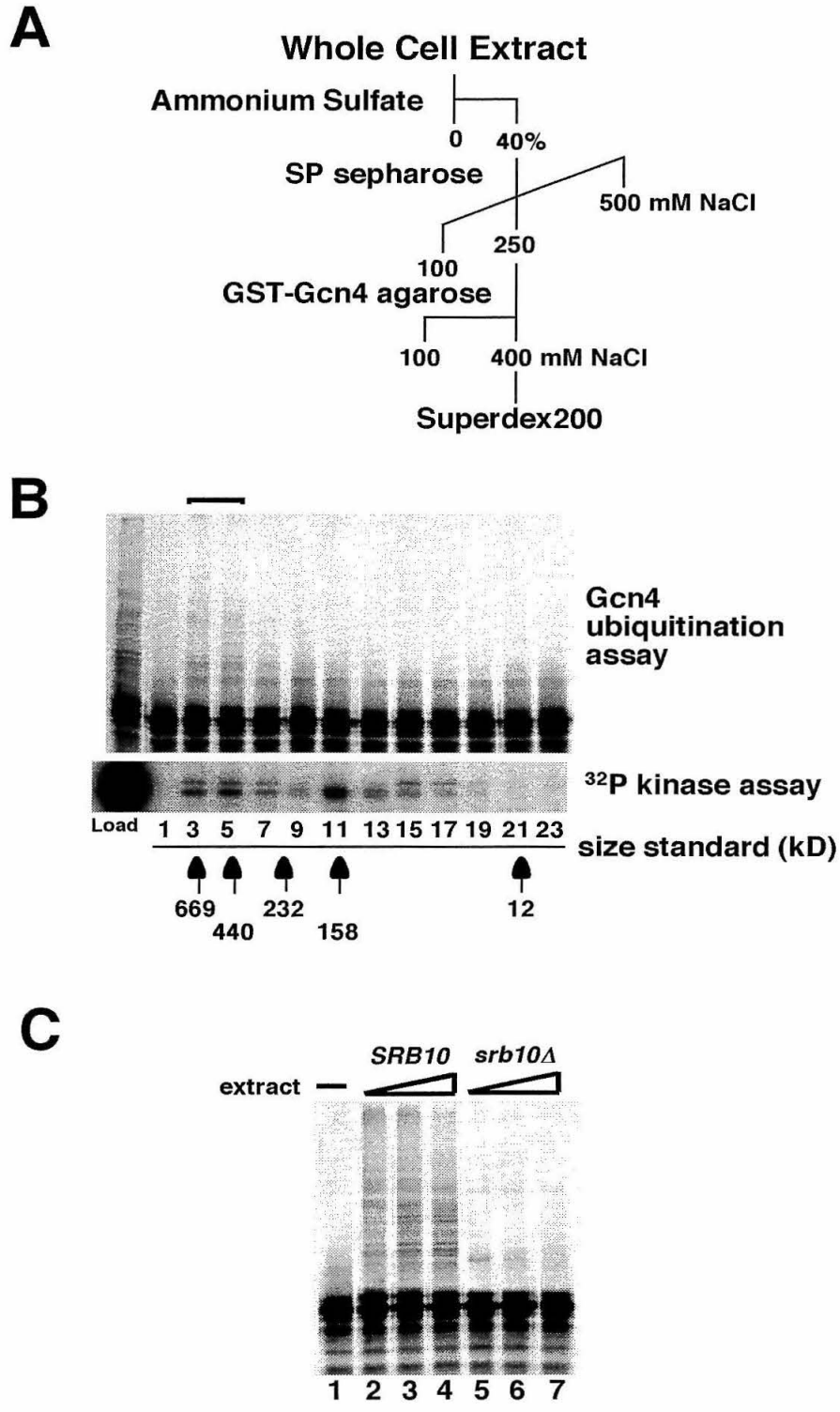
A
Whole Cell Extract
Ammonium Sulfate
0
40%
SP sepharose
500 mM NaCl
250
100
GST-Gcn4 agarose
100
400 mM NaCl
Superdex200
B
Gcn4
ubiquitination
assay
32P kinase assay
Load
1 3 5 7 9 11 13 15 17 19 21 23
size standard (kD)
669
440
232
158
12
C
SRB10
srb10Δ
extract
1 2 3 4 5 6 7

Figure 2. Identification of Srb10 as the protein kinase that promotes Gcn4 ubiquitination in yeast extracts.

(*A*) Purification scheme (see Materials and Methods for details).

(*B*) The indicated fractions from a Superdex 200 column were evaluated for both protein kinase activity using Gcn4 expressed in *E. coli* (*bottom* panel) and ubiquitination-promoting activity using [^{35}S]methionine-labeled Gcn4 (*top* panel). Each ubiquitination reaction included ~100ng of "triple infection" SCF^{cdc4} (see Materials and Methods). The bracket highlights the peak ubiquitination-promoting activities (fractions *3,5*).

(*C*) Ubiquitination reactions were carried out with [^{35}S]methionine-labeled Gcn4 (lane *1*) in the presence of "triple infection" SCF^{cdc4} and a fraction of yeast extract that was enriched for Gcn4 kinase activity (see Materials and Methods). Varying amounts (5, 10 and 20 μg) of "kinase fraction" prepared from an *SRB10* (Z719, lanes *2-4*) or an *srb10Δ* (Z687, lanes *5-7*) strain were tested.

Fig. 3

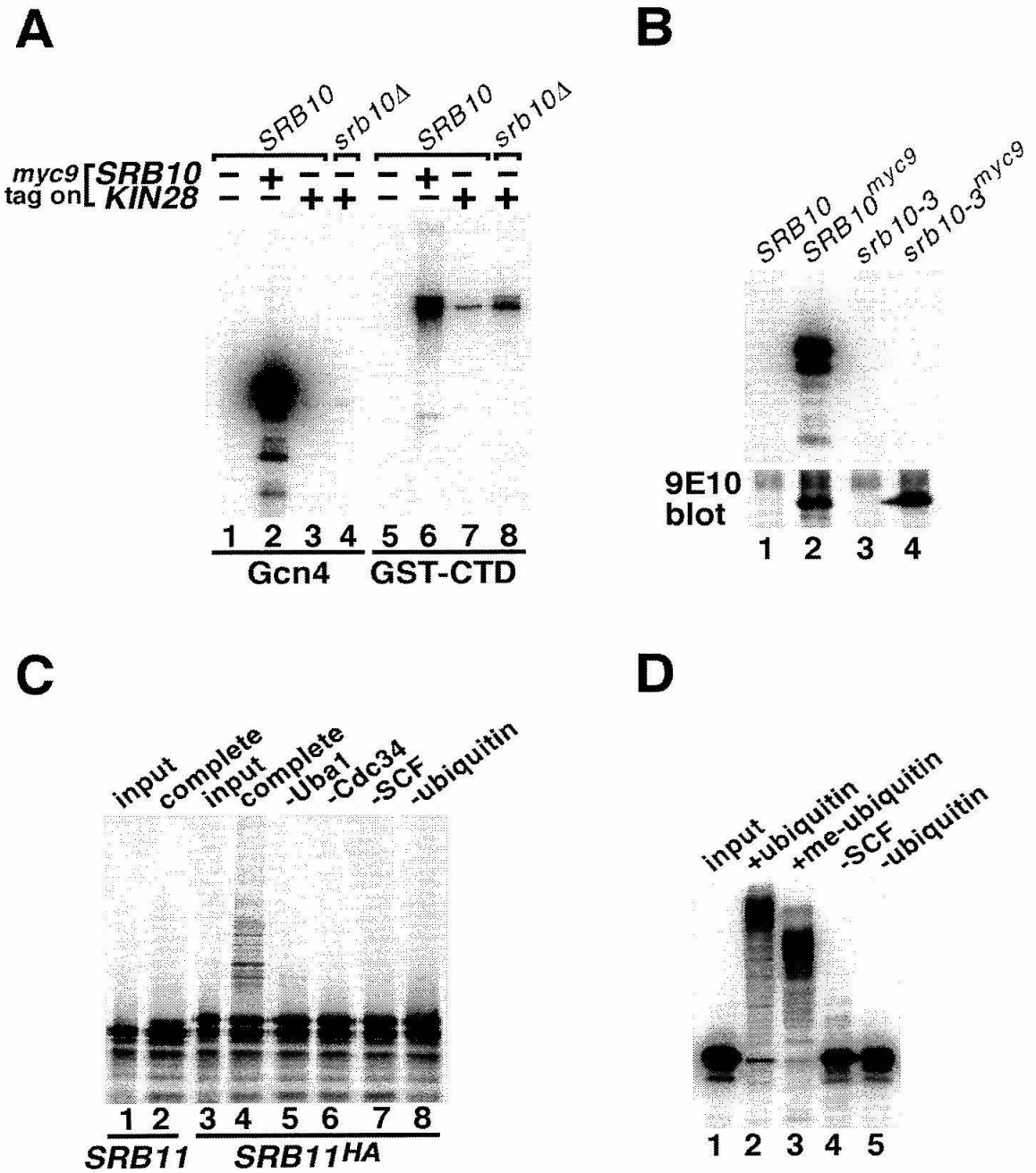
A
SRB10
srb10Δ
SRB10
srb10Δ
myc9 tag on
SRB10
KIN28
1 2 3 4 5 6 7 8
Gcn4
GST-CTD
B
SRB10
SRB10myc9
srb10-3
srb10-3myc9
9E10 blot
1 2 3 4
C
input
complete
input
complete
-Uba1
-Cdc34
-SCF
-ubiquitin
1 2 3 4 5 6 7 8
SRB11
SRB11HA
D
input
+ubiquitin
+me-ubiquitin
-SCF
-ubiquitin
1 2 3 4 5

Figure 3. Srb10 directly phosphorylates Gcn4, and targets it for SCF^{Cdc4}-dependent ubiquitination.

(*A*) Immunoprecipitates from *SRB10*^{myc9} strain YC6 (lanes *2, 6*), *KIN28*^{myc9} strains YC15 (lanes *3,7*) and YC16 (lanes *4,8*) and untagged strain YC1 (lanes *1,5*) were assayed for kinase activity using, as substrate, Gcn4 (lanes *1-4*) or GST-CTD (lanes *5-8*) purified from *E. coli*. Reactions were evaluated by SDS-PAGE and autoradiography.

(*B*) Same as (*A*), except immunoprecipitates from *SRB10*^{myc9} strain YC17 (lane *2*) and *srb10-3*^{myc9} strain YC7 (lane *4*) were compared (*top* panel). Untagged strains Z719 (lane *1*) and Z690 (lane *3*) were used as controls. Antigen levels were monitored by immunoblotting duplicate immunoprecipitates with 9E10 antibody (*bottom* panel).

(*C*) Srb10 protein kinase activity was immunoprecipitated from Z689 (*SRB11*^{HA}) with 12CA5 antibody. This immunoprecipitate had strong Gcn4 kinase activity similar to *SRB10*^{myc9} strains shown in (*A*) and (*B*) as judged by ^{32}P kinase assays (data not shown). The ubiquitination assay was carried out by first incubating [^{35}S]methionine-labeled Gcn4 with $Srb11^{HA}$ bound to protein A beads. An aliquot of the supernatant was used in the subsequent ubiquitination reactions (lanes *3-8*). Immunoprecipitates from untagged strain Z719 were used as controls (lanes *1,2*). Ubiquitination reactions were either supplemented with a complete set of components (lanes *2,4*) including SCF^{Cdc4} (triple infection) or a subset of components in which the factor indicated above each lane was omitted (lanes *5-8*).

(*D*) Gcn4 purified from *E. coli* was first phosphorylated in a kinase assay as shown in panel B, lane 2. ^{32}P-labeled Gcn4 was then added to ubiquitination reactions that were carried out as described in (*C*), except SCF^{Cdc4} (quadruple infection) was used.

Fig. 4

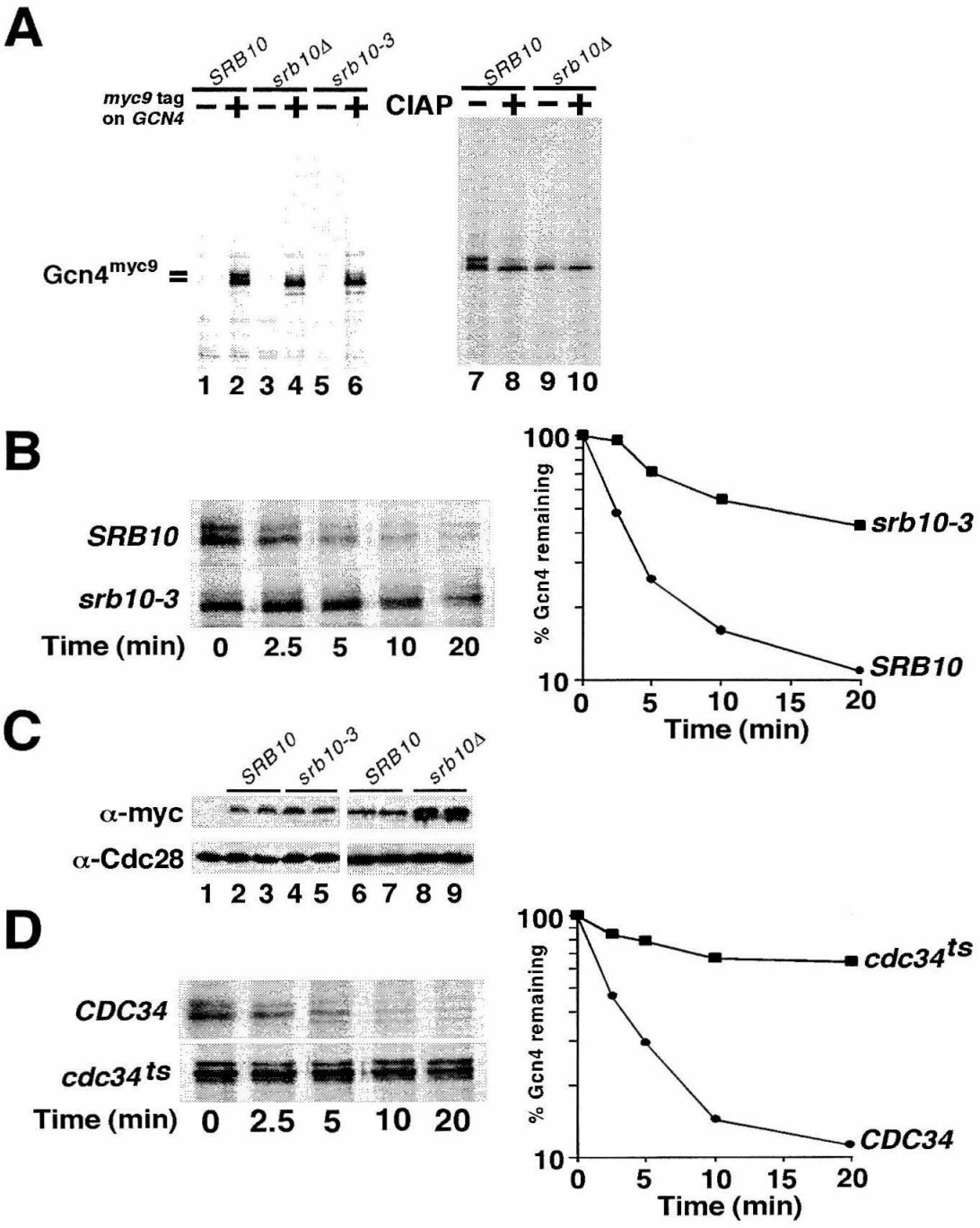
A
SRB10
srb10Δ
srb10-3
myc9 tag on GCN4
− + − + − +
CIAP
SRB10
srb10Δ
− + − +
Gcn4myc9 =
1 2 3 4 5 6
7 8 9 10
B
SRB10
srb10-3
Time (min) 0 2.5 5 10 20
100
% Gcn4 remaining
10
srb10-3
SRB10
0 5 10 15 20
Time (min)
C
SRB10
srb10-3
SRB10
srb10Δ
α-myc
α-Cdc28
1 2 3 4 5 6 7 8 9
D
CDC34
cdc34ts
Time (min) 0 2.5 5 10 20
100
% Gcn4 remaining
10
cdc34ts
CDC34
0 5 10 15 20
Time (min)

Figure 4. Srb10 phosphorylates Gcn4 and regulates its stability in vivo.

(*A*) (*Left* panel) Cells from strains carrying untagged *GCN4* (Z719, lane *1*; Z687, lane *3*; Z690, lane *5*) or *GCN4*^{myc9} (YC19, lane *2*; YC21, lane *4*; YC45, lane *6*) were pulse-labeled for 5 min (without chase) in SD medium containing Tran^{35}Slabel. Cell lysates were immunoprecipitated using 9E10 antibody and evaluated by SDS-PAGE and autoradiography as described in Materials and Methods. (*Right* panel) Same as *left* panel, except the immunoprecipitated samples were either mock-treated (lanes *7,9*) or treated (lanes *8,10*) with 2 U CIAP at 37°C for 30 min.

(*B* and *D*) Pulse-chase analysis of Gcn4^{myc9} stability in *SRB10* (YC19), *srb10-3* (YC45), *CDC34* (YC19) and *cdc34*^{ts} (YC35) cells. Quantitations of the experiments are shown on the right. The experiment in *D* was performed at 37°C.

(*C*) *SRB10* (Z719, lane *1*; YC19, lanes *2,3,6,7*), *srb10-3* (YC45, lanes *4,5*), or *srb10Δ* (YC21, lanes *8,9*) cells were cultured as described for the pulse-chase experiments, harvested and lysed by boiling in SDS-containing buffer. Equal amounts (~50 μg) of cell lysates were analyzed by immunoblotting using 9E10 (*top* panels) and anti-Cdc28 (*bottom* panels) antibodies. Each sample (except the untagged control) was run in duplicate lanes, and lanes *1-5* and lanes *6-9* are from separate experiments. Bands were quantitated, and Gcn4 signals were normalized against Cdc28. Compared to wild-type, Gcn4 level is 1.7 times higher in *srb10-3* and 2.0 times higher in *srb10Δ*.

Fig. 5

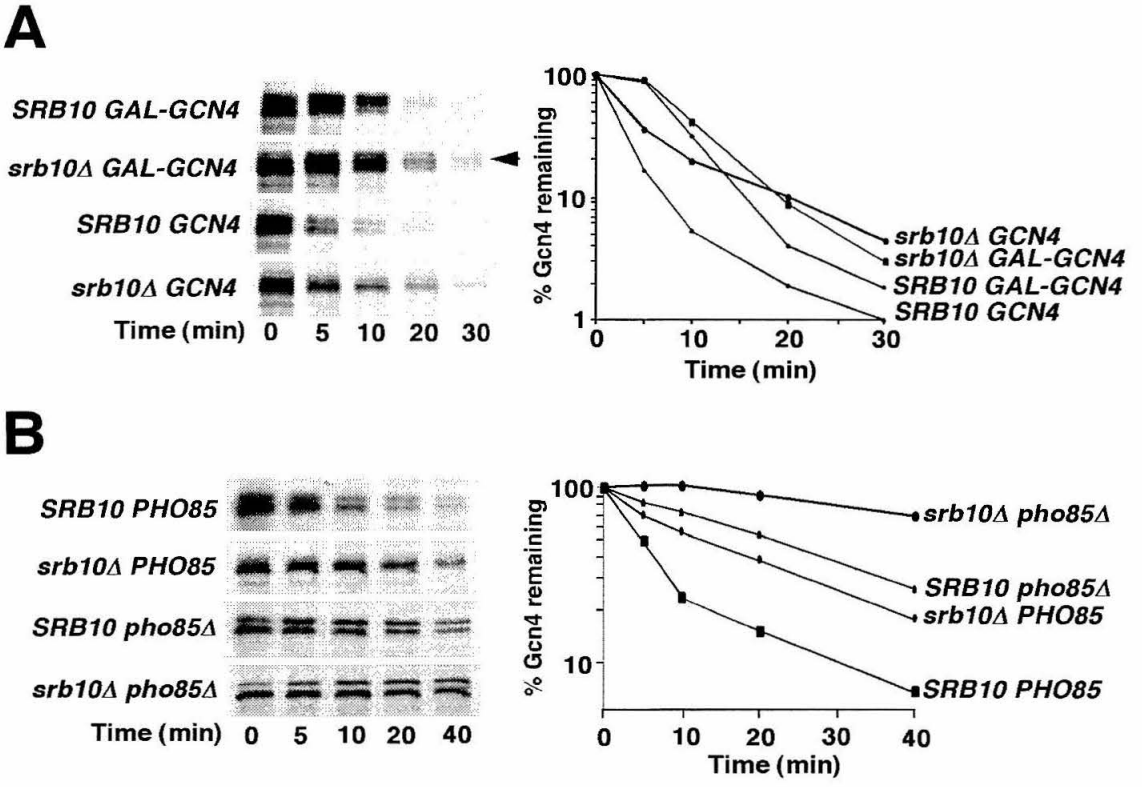
A
SRB10 GAL-GCN4
srb10Δ GAL-GCN4
SRB10 GCN4
srb10Δ GCN4
Time (min) 0 5 10 20 30
100
10
1
% Gcn4 remaining
0 10 20 30
Time (min)
srb10Δ GCN4
srb10Δ GAL-GCN4
SRB10 GAL-GCN4
SRB10 GCN4
B
SRB10 PHO85
srb10Δ PHO85
SRB10 pho85Δ
srb10Δ pho85Δ
Time (min) 0 5 10 20 40
100
10
% Gcn4 remaining
0 10 20 30 40
Time (min)
srb10Δ pho85Δ
SRB10 pho85Δ
srb10Δ PHO85
SRB10 PHO85

Figure 5. Both Srb10 and Pho85 contribute to Gcn4 turnover in vivo.

(*A*) Gcn4^{myc9} stability under normal and overproduced conditions. Wild-type (YC19, YC23) and *srb10Δ* (YC21, YC24) cells grown in YP-raffinose medium were either transferred to synthetic galactose medium (*top* two panels) or synthetic dextrose medium (*bottom* two panels) for 1 hr prior to being subjected to pulse-chase analysis as described for Figure *4A*. Quantitation of the results is shown on the right. The arrowhead denotes a post-translationally modified form of Gcn4^{myc9} that appeared rapidly in *SRB10* cells, but was only slowly generated in *srb10Δ* strains. Note that the biphasic degradation observed for *GAL-GCN4^{myc9}* strains was likely due to the carry-over synthesis of Gcn4^{myc9} during the first 5 min of chase.

(*B*) Gcn4 stability in wild-type (YC19), *srb10Δ* (YC21), *pho85Δ* (YC59) and *srb10Δ pho85Δ* (YC61) cells was evaluated by pulse-chase analysis (see Fig. *4A* legend). Quantitation of experiment is shown on the right. In two independent experiments, the half-life of Gcn4 in the various strains varied by less than 2 min.

Fig. 6

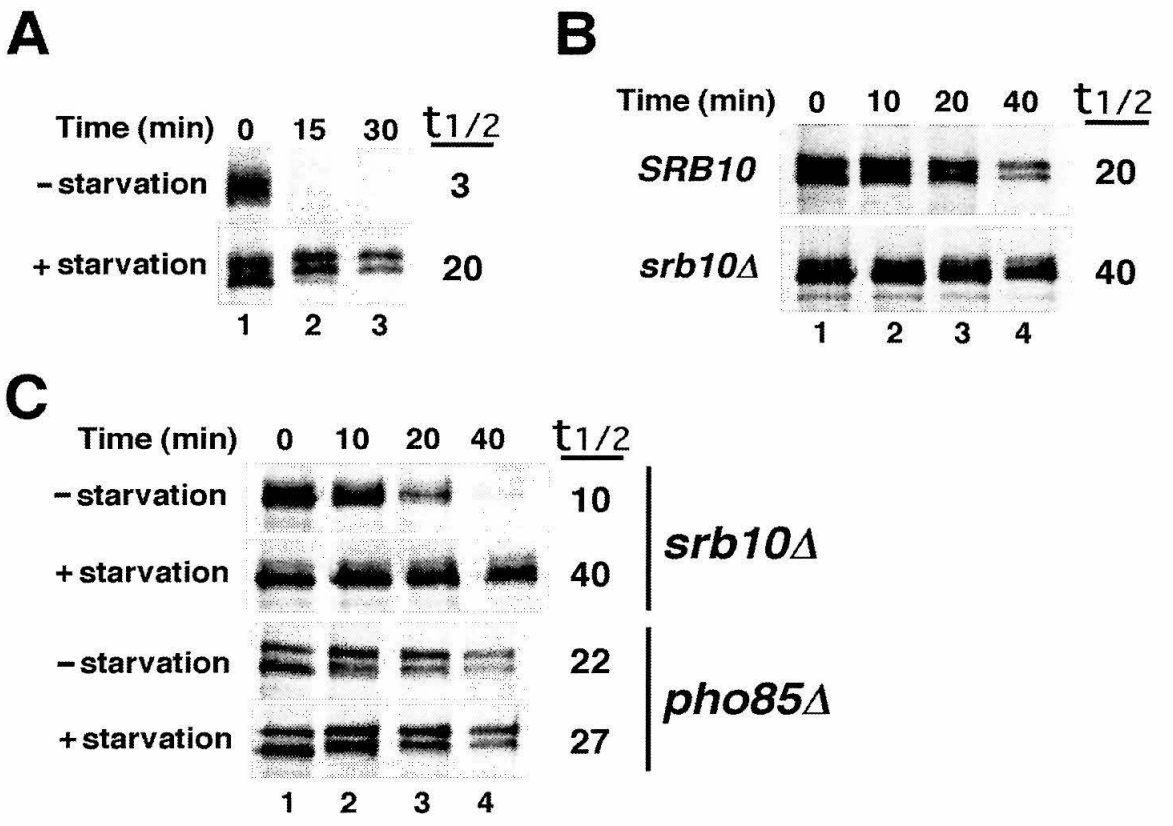
A
Time (min) 0 15 30 t1/2
- starvation 3
+ starvation 20
1 2 3
B
Time (min) 0 10 20 40 t1/2
SRB10 20
srb10Δ 40
1 2 3 4
C
Time (min) 0 10 20 40 t1/2
- starvation 10
+ starvation 40
srb10Δ
- starvation 22
+ starvation 27
pho85Δ
1 2 3 4

Figure 6. Srb10-dependent degradation of Gcn4 is not regulated by amino acid starvation.

(*A*) *SRB10* (YC19) cells were grown to mid-log phase and transferred to either SD +leucine +histidine + uracil (-starvation) or SD -leucine +histidine + uracil (+starvation) medium. Cells were cultured for 15 min and pulse chase analysis was carried out as described in Materials and Methods. Starving cells for 30 or 60 min resulted in similar magnitude of stabilization of Gcn4 (data not shown). Half-life of Gcn4 (t1/2) was determined from quantitation of the data using phosphorimager.

(*B*) Same as (*A*), except *SRB10* (YC19, *top* panel) and *srb10Δ* (YC21, *bottom* panel) cells were assayed for Gcn4 stability in SD -leucine +histidine + uracil (+starvation) only.

(*C*) Same as (*A*), except *srb10Δ* (YC21, *top* panels) cells and *pho85Δ* (YC59, *bottom* panels) cells were assayed for Gcn4 stability.

Fig. 7

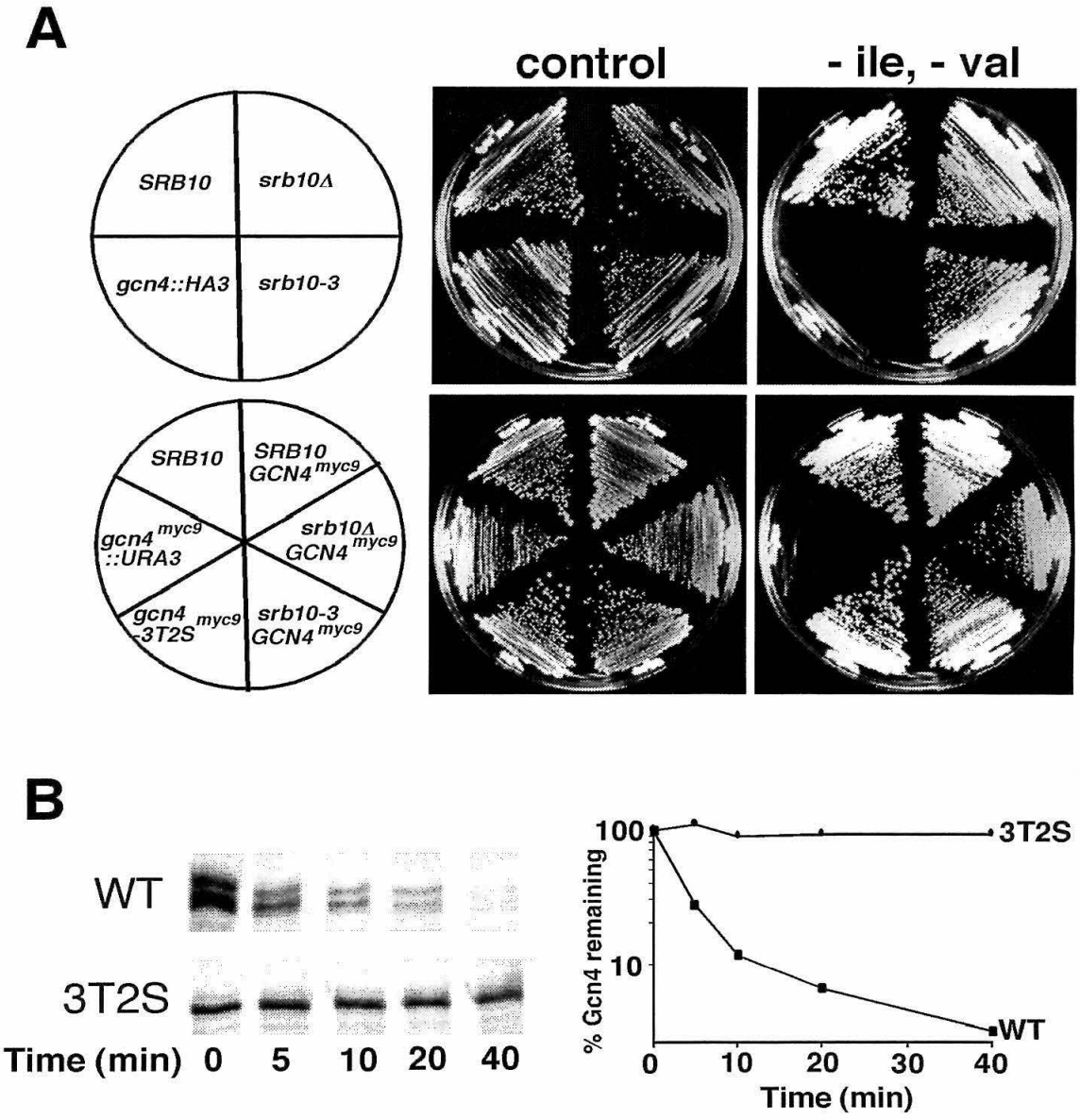
A
control
- ile, - val
SRB10
srb10Δ
gcn4::HA3
srb10-3
SRB10
SRB10 GCN4myc9
gcn4myc9 ::URA3
srb10Δ GCN4myc9
gcn4myc9 -3T2S
srb10-3 GCN4myc9
B
WT
3T2S
Time (min) 0 5 10 20 40
100
10
% Gcn4 remaining
3T2S
WT
0 10 20 30 40
Time (min)

Figure 7. CDK phosphorylation is not essential for Gcn4 activity, but is required for its rapid degradation in vivo.

(*A*) Strains with the indicated genotypes were grown on SD +leu (~0.23 mM) +his + ura medium (control) and the same medium supplemented with 40 mM leucine (-Ile, -Val) to induce starvation for isoleucine and valine. Plates were photographed after incubation at 30°C for 3 days. *Top row* of panels show wild-type strain (Z719) and mutant strains *srb10Δ* (Z687), *srb10-3* (Z690) and *gcn4::HA3* (YC25). *Bottom row* of panels show strains containing *myc9*-tagged *GCN4* alleles (YC19, YC21, YC45, YC57 and YC94). (*B*) Pulse-chase analysis (see Fig. *4A* legend) of $Gcn4^{myc9}$ stability in wild-type (YC19) and *gcn4-3T2S* (YC94) cells. Quantitation of the experiment is shown on the right.

Fig. 8

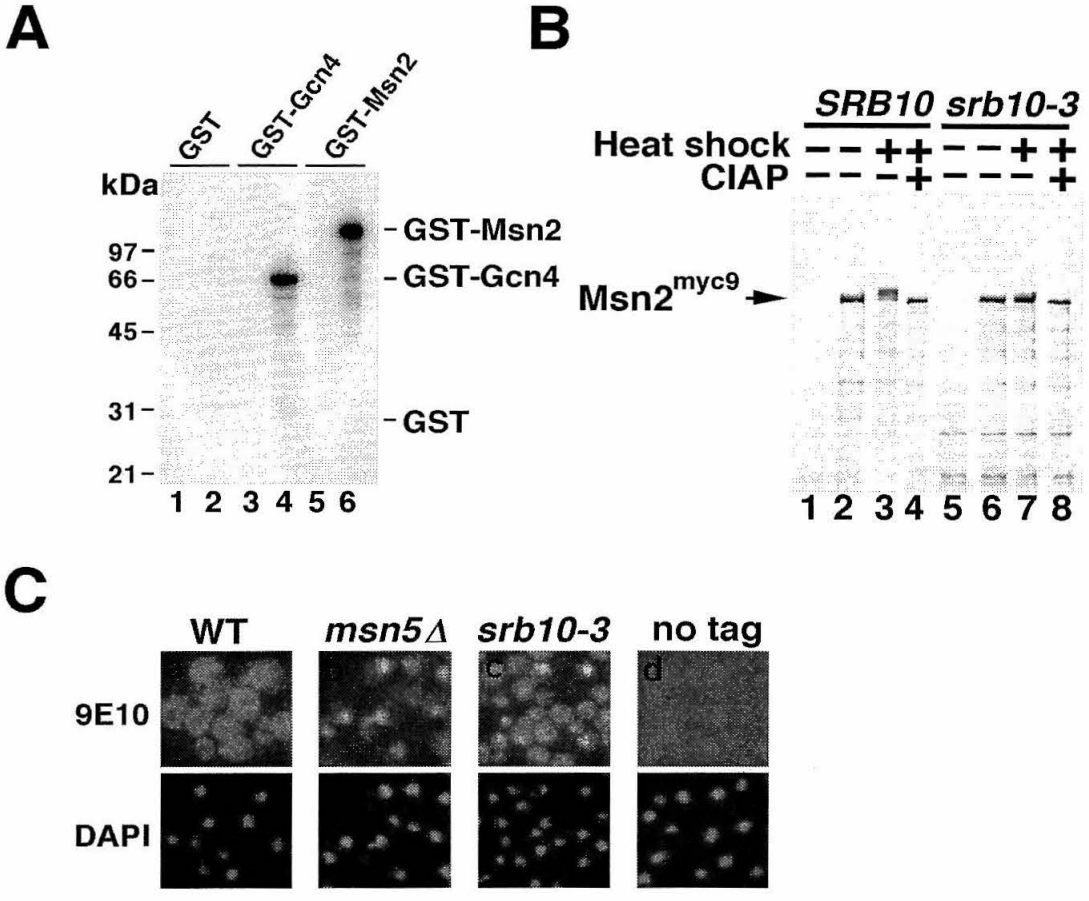
A
GST
GST-Gcn4
GST-Msn2
kDa
97
66
45
31
21
GST-Msn2
GST-Gcn4
GST
1 2 3 4 5 6
B
SRB10
srb10-3
Heat shock
CIAP
Msn2myc9
1 2 3 4 5 6 7 8
C
WT
msn5Δ
srb10-3
no tag
9E10
DAPI

Figure 8. Msn2 is phosphorylated in an Srb10-dependent manner in vitro and in vivo, and its localization is influenced by Srb10.

(*A*) Kinase assays were carried out with purified GST (lanes *1,2*), GST-Gcn4 (lanes *3,4*), and GST-Msn2 (lanes *5,6*) using immunoprecipitates from *SRB10*^{myc9} strain (YC17, lanes *2,4,6*) and *srb10-3*^{myc9} strain (YC7, lanes *1,3,5*) as described in Fig. *3A*.

(*B*) Cells from *MSN2*^{myc9} (YC84, lanes *2-4*) and *MSN2*^{myc9} *srb10-3* (YC124, lanes *6-8*) strains grown at 27°C were pulse-labeled with Tran^{35}Slabel at 27°C for 5 min, and chased at 37°C for 5 min. Aliquots (1/3 before the heat shock and 2/3 after the heat shock) of the cultures were subjected to immunoprecipitation with 9E10 antibody. Immunoprecipitates from the heat shock samples were divided and either mock-treated (lane *3,7*) or treated (lanes *4,8*) with 2 U CIAP for 30 min at 37°C. Untagged strains Z719 (lane *1*) and Z690 (lane *5*) were used as controls.

(*C*) Localization of Msn2^{myc9} in wild-type (YC84,*a*), *msn5Δ* (YC107, *b*), and *srb10-3* (YC124, *c*) cells was evaluated under resting conditions by indirect immunofluorescence with 9E10 antibody. DAPI was used to stain cell nuclei. An *srb10-3* strain containing untagged *MSN2* (Z690) was used as control *(d)*.

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Chapter 4. Future directions: Negative regulation of transcription factors through DNA binding

Introduction

The primary task of a newly synthesized transcriptional activator is to activate the transcription of its target genes in the nucleus. Once it travels into the nucleus, the transcriptional activator effects gene-specific transcriptional activation in a series of steps as suggested by current models (Lee and Young 2000). The activator first binds to the regulatory elements of target genes. DNA binding of the activator then recruits chromatin remodeling and modifying complexes such as Swi/Snf and SAGA to promoters. Chromosomal remodeling may increase the stability of the activator-DNA complex or affect access of promoter sequences for binding of the transcription apparatus. Once the repressive effect of the nucleosomal structure is resolved, the activator then recruits the transcription initiation apparatus to promoters. This is probably achieved through interactions between the activation domain of the activator and the transcription apparatus, such as components of the RNA polymerase II holoenzyme. Finally, the activator stimulates the activity of the assembled transcription apparatus, resulting in robust transcriptional initiation of its target genes.

Although activators may also influence the events subsequent to assembly of the initiation apparatus, the eventual fate of these activators is not clear. How long do they bind to the promoters, and how are they negatively regulated after they have performed their tasks? Recent evidence and studies in Chapter 3 suggest at least two modes of negative regulation of transcription factors: ubiquitin-dependent degradation (e.g. Gcn4) and nuclear exclusion (e.g. Msn2 and Pho4). The former appears to be mainly applicable

to short-lived transcription factors and the latter for relatively long-lived ones. Since there may be two different pools of transcription activators present in the nucleus, promoter-bound and free ones, an obvious question is: how are the different pools of activators regulated? The work described in Chapter 3 indicates that two different kinases (Srb10 and Pho85) redundantly contribute to Gcn4 turnover. Given that Srb10 CDK is part of the RNA Polymerase II holoenzyme, we initially hypothesized that the two kinases account for the degradation of different pools of Gcn4: whereas Srb10 promotes the degradation of promoter-bound Gcn4, Pho85 targets primarily Gcn4 molecules that are not bound to DNA. Here we show preliminary studies on the DNA-binding mutants of Gcn4 and Pho4. Our results indicated that a DNA-binding mutant of Gcn4 was significantly stabilized compared to wild type Gcn4, suggesting that DNA binding is required for rapid Gcn4 turnover in vivo. This DNA-binding mutant of Gcn4 was not further stabilized to appreciable extent in an *srb10Δ* or a *pho85Δ* mutant, suggesting that both kinases target promoter-bound Gcn4 for degradation. Finally, cells carrying a DNA-binding mutant of Pho4 showed predominantly nuclear localization of this protein under phosphate-rich conditions, suggesting that DNA binding is required for the nuclear export of Pho4. Taken together, we propose a general model that negative regulation of transcription factors is coupled to DNA binding.

Preliminary Results

Rapid degradation of Gcn4 in vivo requires DNA binding

The finding that the Srb10 CDK phosphorylates Gcn4 and promotes its turnover raises an interesting possibility that this regulation may be confined to the vicinity of promoter DNA. To test this possibility, we constructed DNA-binding mutants of Gcn4

and tested their stability in vivo by pulse-chase analysis. A single point mutation (A238G) in the basic region of Gcn4 DNA-binding domain nearly eliminates the DNA-binding of Gcn4 in vitro and its activity in vivo (Fig.1; Pu and Struhl 1991). Pulse-chase analysis of this DNA-binding mutant indicated that it had a half-life about 30 minutes compared to the wild type Gcn4, which had a half-life of about 2 minutes (Fig. 2). A similar result was obtained for another Gcn4 DNA-binding mutant (Gcn4-N235K, data not shown). This suggests that DNA binding is required for rapid Gcn4 turnover in vivo. Since CDK phosphorylation is also required for Gcn4 turnover (Chapter 3), we infer that the specific phosphorylation of Gcn4 that leads to its degradation requires DNA binding. Consistent with this, Srb10-dependent phosphorylation of Gcn4 was reduced in a DNA-binding mutant of Gcn4 as indicated by the change in the molecular weight (MW) up-shifted form of Gcn4 (Fig. 2, compare lanes 1 and 9). The residual phospho-shift in Gcn4-A238G is *SRB10*-dependent (data not shown) and may be due to the residual DNA binding of Gcn4-A238G because the activity of Gcn4-A238G was not completely eliminated in vivo (Fig.1, compare *gcn4Δ* with *A238G*). Alternatively, it may be due to phosphorylation of DNA-unbound form of Gcn4 by Srb10.

Both Srb10 and Pho85 CDKs target DNA-bound form of Gcn4 for degradation

We originally hypothesized that Srb10 CDK and Pho85 CDK target different pools of Gcn4 for degradation. Specifically, we suspected that Pho85 may target a large pool of Gcn4 proteins that are not bound to DNA. To test this possibility, we measured the stability of Gcn4-A238G in *srb10-3* or *pho85Δ* strains. Interestingly, neither deletion of *SRB10* nor deletion of *PHO85* further stabilized Gcn4 significantly (Fig. 3), suggesting that both kinases target primarily the DNA-bound form of Gcn4 for degradation.

DNA binding is required for nuclear exclusion of Pho4

To test if DNA binding is a general requirement for the negative regulation of transcription factors, we investigated the regulation of the transcription factor Pho4, which is involved in phosphate metabolism (Oshima 1997). Unlike Gcn4, Pho4 is relatively stable and is regulated by localization (Komeili and O'Shea 2000). Under phosphate-rich conditions, Pho85/Pho80 CDK phosphorylates Pho4 and promotes its export from the nucleus (Kaffman et al. 1994; O'Neill et al. 1996). As a result, Pho4 appears predominantly cytoplasmic when cells are grown in phosphate-rich medium. Based on the crystal structure of Pho4 bHLH domain-DNA complex (Shimizu et al. 1997), we constructed a DNA-binding mutant of Pho4 (Pho4-db), which had three of its conserved base-contact residues mutated to alanine (H255A, E259A, R263A). We then looked at the localization of wild type Pho4 and Pho4-db by indirect immunofluorescence. If DNA binding is required for Pho4 export, we would predict a defect in the nuclear exclusion of Pho4 when cells are grown in phosphate-rich medium. As shown in Fig. 4, this was indeed the case: whereas wild type Pho4 was predominantly cytoplasmic in most cells, Pho4 was predominantly nuclear.

Discussion

Regulation of Gcn4 and Pho4 through DNA binding

The preliminary results shown above suggest that the negative regulation of transcription activators may be coupled to DNA binding because transcription factors (e.g. Gcn4 and Pho4) are not subject to the tight regulation if they fail to bind DNA (Figs. 2 and 4). Since phosphorylation of Gcn4 and Pho4 is required for their regulation but not

for their activity (Chapter 3; O'Neill et al. 1996), it is likely that DNA binding is required for the phosphorylation of Gcn4 and Pho4. Consistent with this, Srb10-dependent phosphorylation of a DNA-binding mutant of Gcn4 is reduced compared to wild type (Fig. 2). Pulse-labeling or immunoblot experiments are needed to monitor the phosphorylation state of wild type Pho4 versus Pho4-db since Pho85-dependent phosphorylation of Pho4 in vivo causes electrophoretic mobility shift of Pho4 (Kaffman et al. 1998). The prediction is that Pho85-dependent phosphorylation seen for wild type Pho4 would be diminished for Pho4-db. Although more control experiments need to be done to solidify these preliminary findings, the combined results of Gcn4 and Pho4 suggest a model that phosphorylation of transcription factors at the promoter DNA may serve as timers that restrict the amount of transcripts they can activate, and the substrate-specific phosphorylation events lead to their inactivation by distinct means, including degradation and nuclear export (Fig. 5).

Future directions

Similar to Pho4, Msn2/Msn4, two partially redundant multistress transcription factors, are also regulated by localization (Chapter 3; Gorner et al. 1998). Phosphorylation of Msn2 by Srb10 (Chapter3) and protein kinase A (Gorner et al. 1998) have been implicated in this regulation. We are currently constructing a DNA-binding mutant of Msn2 to test if DNA binding is also required for Msn2 regulation.

Since Pho85 CDKs have been shown to negatively regulation of both Gcn4 and Pho4, an interesting possibility is that Pho85 CDKs may be localized to the vicinity of the DNA, or they may be recruited to DNA by transcription factors upon DNA binding. One can envision two possible mechanisms of how DNA binding mediates the regulation of Gcn4 and Pho4. One possibility is that DNA binding causes a conformational change in

Gcn4 and Pho4 that renders them better substrates for Pho85. The second possibility is that Pho85 is concentrated in the vicinity of DNA, and DNA-bound forms of Gcn4 and Pho4 have a better chance of being phosphorylated by Pho85. We are designing experiments to distinguish these possibilities.

Immunopurified Srb10 and Pho85 kinase activities are sufficient to phosphorylate Gcn4 in vitro (Chapter 3; Meimoun et al. 2000). If DNA-bound Gcn4 is the physiological substrate, one would expect that the rate of phosphorylation by these kinases may be greatly enhanced if the kinase assays are performed in the presence of DNA fragments containing Gcn4 binding sites. If so, phosphorylation pattern of wild type Gcn4 can be compared with that of DNA-binding mutant of Gcn4 to reveal critical site(s) whose phosphorylation is strongly DNA-dependent.

Phosphorylation of Gcn4 by Pho85 CDK promotes its degradation whereas phosphorylation of Pho4 by Pho85 CDK promotes its nuclear export. An interesting question is why Pho85 CDK complexes regulate transcription factors by distinct means? How is the specificity achieved? The specificity may derive from the sequences surrounding the critical phosphorylation sites on the substrates, or it may have to do with the exact region in the nucleus where the phosphorylation occurs. Genetic results indicate that the Pho80/Pho85 is the only Pho85 CDK complex involved in Pho4 regulation (Oshima 1997) whereas there may be multiple Pho85 CDK complexes involved in Gcn4 turnover, including Pcl1/Pho85 (Meimoun et al. 2000). Studies using a Gcn4-Pho4 hybrid protein may shed some light on this question.

Materials and Methods

Yeast strains

The yeast strains used in this study were derived from Z719 (*MAT**a** his3Δ200 leu2-3,112 ura3-52*) and Z690 (*MAT**a** his3Δ200 leu2-3,112 ura3-52 srb10-3::hisG*) (Liao et al. 1995). Yeast cells were cultured in standard rich or synthetic medium supplemented with 2% dextrose as described (Sherman 1991). The *GCN4* and *PHO4* loci were modified to encode proteins tagged with nine copies of the myc epitope (myc9) at their C-termini as described (Seol et al. 1999). The *gcn4* and *pho85* deletion strains were generated by PCR-based one-step replacement (Baudin et al. 1993). Deletion alleles were confirmed by scoring mutant phenotypes and by PCR. The *gcn4-A238G*^{myc9} and *pho4-db*^{myc9}strains were generated first by PCR-based site-directed mutagenesis using mutagenic oligos as previously described (Verma et al. 1997). DNA fragments containing the mutated coding sequences of *GCN4* or *PHO4* were used to transform Z719 as described for wild type to generate myc9-tagged chromosomal alleles of these genes. All mutant alleles were confirmed by direct sequencing. Other derivative strains were constructed by genetic crosses.

Pulse-chase experiments

Cells were grown overnight in SD +leucine +histidine + uracil at 30°C to mid-log phase (OD_{600} ~0.5). Approximately 3 x 10^8 cells were harvested and concentrated by centrifugation into 3 ml of the same medium. Cells were pulse-labeled for 5 min with 500 μCi of Tran^{35}Slabel (1175 Ci/mmol, ICN), then chased in the same medium containing 1mM ammonium sulfate, 5mM methionine and 1mM cysteine. At various

times of the chase, 0.5 ml of the culture was removed and diluted immediately into 1.5 ml cold stop buffer (50 mM Tris-HCl [pH7.5], 50 mM NaF and 0.1% NaN_3). Cells were centrifuged briefly and cell pellets were frozen in liquid nitrogen (and stored at –80°C if necessary). The cell pellets were then each supplemented with 100 µl of 0.5 mm glass beads and 100 µl lysis buffer (30 mM Tris-HCl [pH7.5], 0.5% SDS, 6% ammonium sulfate, 1 mM EDTA, 1 mM DTT and 0.5 mM PMSF), vortexed for 2 s, and boiled for 3 min. The samples were cooled briefly, vortexed for 2 min, boiled again for 2 min and vortexed again for 1 min. Samples were then centrifuged at 10,000 g for 10 min, and the supernatant was diluted five-fold with Triton buffer (30 mM Tris-HCl [pH7.5], 1% Triton X-100, 1 mM DTT and 1 mM PMSF) and immunoprecipitated with 9E10 antibody and protein A beads. The protein A beads were washed three times with Buffer B, aspirated to dryness, and mixed with 2X Laemmeli buffer. Samples were boiled for 3 min prior to being evaluated by SDS-PAGE followed by autoradiography and PhosphorImager (Molecular Dynamics) analysis.

Immunofluorescence

Cells were diluted from stationery culture (1:500) and grown for 8-10 hr in 10 ml YPD at 30°C to mid-log phase (OD_{600}=~0.5). 1 ml of cells were then removed and fixed with 4.5% formaldehyde at room temperature for 30-45 min. Indirect immunofluorescence was carried out essentially as described (Pringle et al. 1991) using 9E10 (1:3000) as primary antibodies and fluorescein-conjugated-goat-anti-mouse antibodies (1:3000) as secondary antibodies. Zeiss Axiovision software was used to record digital images on a Zeiss Axioskop microscope.

Fig. 1

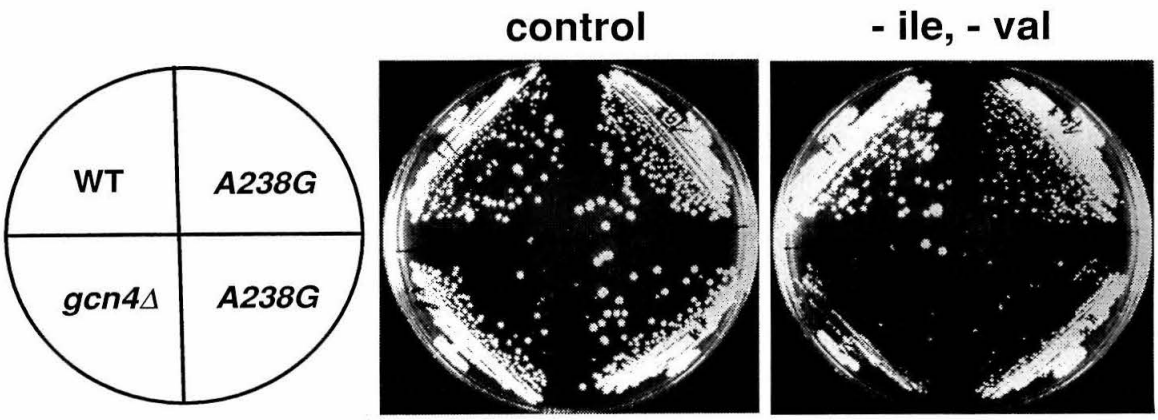

Figure 1. The A238G mutant of Gcn4 has diminished activity in vivo.

(*A*) To test the activities of various Gcn4 derivatives in our strains, we employed a starvation plate assay similar to that described by (Natarajan et al. 1999). Cells were streaked on a control plate with normal leucine supplement and a plate with excess leucine, which causes isoleucine (Ile) and valine (Val) starvation (Niederberger et al. 1981). This assay monitors Gcn4-dependent activation of Ile and Val biosynthetic genes. Specifically, Z719 (WT), *gcn4*^{mcy9}*::URA3* (*gcn4Δ*), and two isolates of *gcn4-A238G*^{myc9} (*A238G*) strains were grown on SD +leu (~0.23 mM) +his + ura medium (control) and the same medium supplemented with 40 mM leucine (-Ile, -Val) to induce starvation for isoleucine and valine. Plates were photographed after incubation at 30°C for 3 days. The *gcn4Δ* strain was used to control for loss of Gcn4 activity in vivo.

Fig. 2

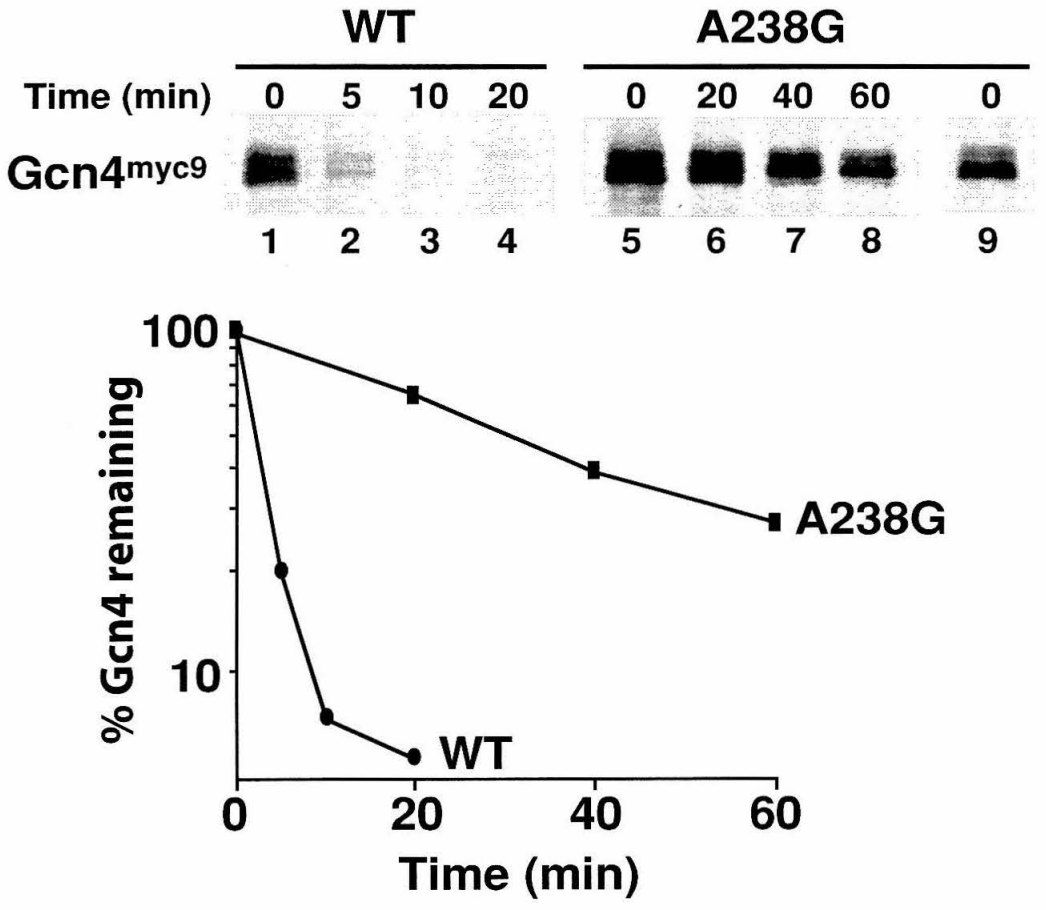
WT
A238G
Time (min)
0
5
10
20
0
20
40
60
0
Gcn4myc9
1
2
3
4
5
6
7
8
9
100
10
% Gcn4 remaining
A238G
WT
0
20
40
60
Time (min)

Figure 2. The A238G mutant of Gcn4 is stabilized in vivo.

Pulse-chase analysis of $Gcn4^{myc9}$ and $Gcn4\text{-}A238G^{myc9}$ stability was carried out as described in Materials and Methods. Quantitation of the experiment was shown on the bottom. Lane 9 is a light exposure (about three times less) of lane 1.

Fig. 3

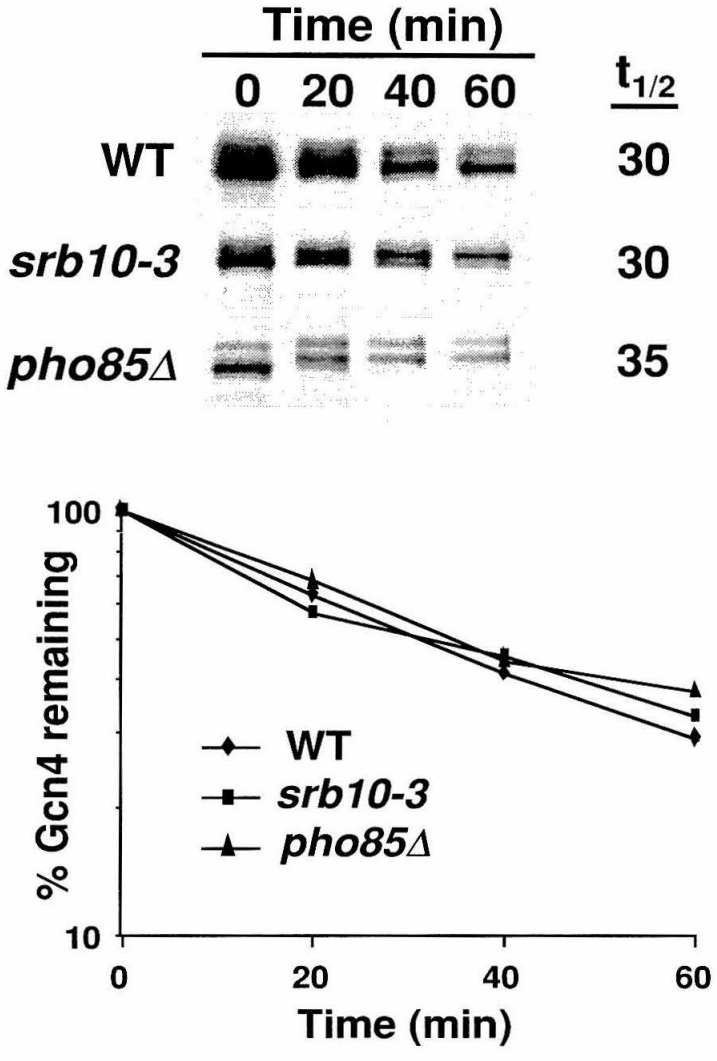
Time (min)
0
20
40
60
$t_{1/2}$
WT
30
srb10-3
30
pho85Δ
35
100
% Gcn4 remaining
10
WT
srb10-3
pho85Δ
0
20
40
60
Time (min)

Figure 3. Gcn4-A238G^{myc9} is not further stabilized in an *srb10-3* or a *pho85Δ* strain.

Same as Figure 2, except stability of Gcn4-A238G^{myc9} was measured in wild type, *srb10-3*, and *pho85Δ* cells. Estimated half-lives (t1/2) of the mutant protein were shown on the right, and quantitation of the experiment was shown on the bottom.

Fig. 4

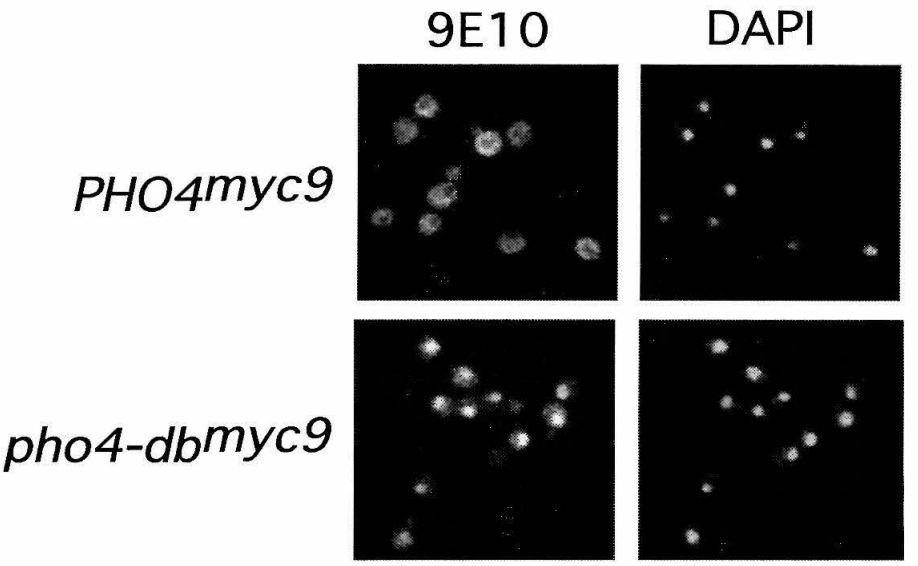

Figure 4. A DNA-binding mutant of Pho4 is defective in its nuclear exclusion.

Localization of Pho4^{myc9} and Pho4-db^{myc9} (H255A, E259A, and R263A) under phosphate-rich conditions by indirect immunofluorescence with 9E10 antibody. DAPI was used to stain cell nuclei.

Fig. 5

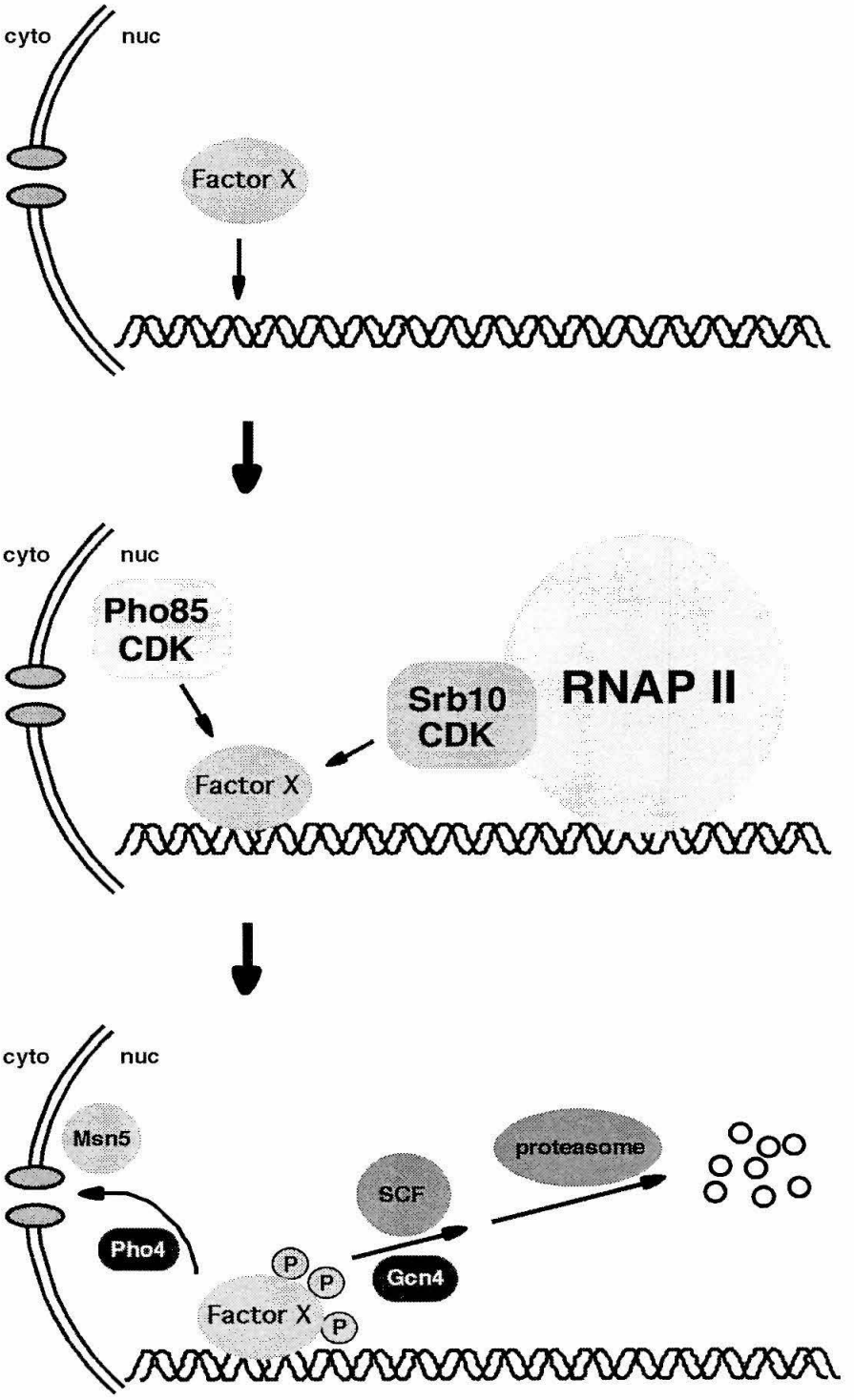
cyto
nuc
Factor X
cyto
nuc
Pho85
CDK
Srb10
CDK
RNAP II
Factor X
cyto
nuc
Msn5
proteasome
SCF
Pho4
P
P
Gcn4
Factor X
P

Figure 5. A model for the negative regulation of transcription activators.

An activator (factor X) enters the nucleus and binds to target DNA elements. DNA binding of the activator recruits the RNA polymerase II transcription initiation apparatus which contains the Srb10 CDK, and it may also become accessible to other kinases such as the Pho85 CDK complexes. Transcription of the target gene is activated, and meanwhile the activator is phosphorylated by one or more of the kinases. Specific phosphorylation of the activator leads to either its degradation by a ubiquitin-dependent pathway such as SCF (e.g. Gcn4) or its nuclear export by a nuclear transport receptor such as Msn5 (e.g. Pho4) (Kaffman et al. 1998).

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Appendix I. Polycation activation of Cdc34

Results and discussion

During the course of our investigation of Gcn4 ubiquitination in vitro, we identified an activity that stimulated Gcn4 ubiquitination in the absence of an E3 enzyme. This activity appeared to be heat-stable and fractionated in a low molecular weight range (~ 30 kDa). Since we used polyethyleneimine (PEI) in yeast extract preparations, we tested the trivial possibility that the stimulatory effect on Gcn4 ubiquitination might be provided by PEI.

We found that at low concentrations, PEI was indeed able to stimulate Gcn4 ubiquitination in the absence of an E3 enzyme (Fig. 1). Gcn4 was not modified detectably in the presence of E1, Cdc34, ATP, and ubiquitin (Fig. 6B, lane 2), but high-molecular-mass K48-linked multiubiquitin chains were assembled on Gcn4 upon inclusion of PEI (lanes 6,7). Thus, PEI acts like a classic E3. Together with the observation that polylysine activates the autoubiquitination of a C-terminally truncated form of Cdc34 (Seol et al. 1999), these results indicate that polycations can substitute for SCF.

The ability of polycations to substitute for SCF strongly suggests that an E3-linked ubiquitin thioester intermediate is not required to evoke the intrinsic ability of Cdc34 to assemble K48-linked multiubiquitin chains. It also sheds light on the potential mechanism of activation of Cdc34 by the Cdc53/Hrt1 module. We propose that Cdc53/Hrt1 stabilizes the oxyanion that is formed in the transition state as the carboxyl terminus of ubiquitin is transferred from Cdc34 to the ε-amino group of a substrate lysine. Cdc53/Hrt1 (or polycations) may directly provide a positive charge to neutralize

the oxyanion, or may elicit a favorable conformational change in Cdc34. Hrt1 binds both Cdc34 and components of the SCF complex directly, placing it in an optimal position to coordinate the transfer of ubiquitin from Cdc34 to substrate.

Materials and Methods

Ubiquitination of Gcn4 in vitro

Gcn4 was produced by in vitro translation in rabbit reticulocyte lysates and was partially purified by DEAE chromatography. Ubiquitination reaction was carried out in the presence of purified recombinant Uba1, Cdc34, wild-type ubiquitin (or His6-ubiquitin), or mutant ubiquitin derivative (His6-K48R ubiquitin) as previously described (Chi et al. 2001). Polyethyleneimine (PEI) was added to the final concentration of 0.0005% where indicated. Gcn4 ubiquitination was visualized by SDS-PAGE followed by autoradiography.

Fig. 1

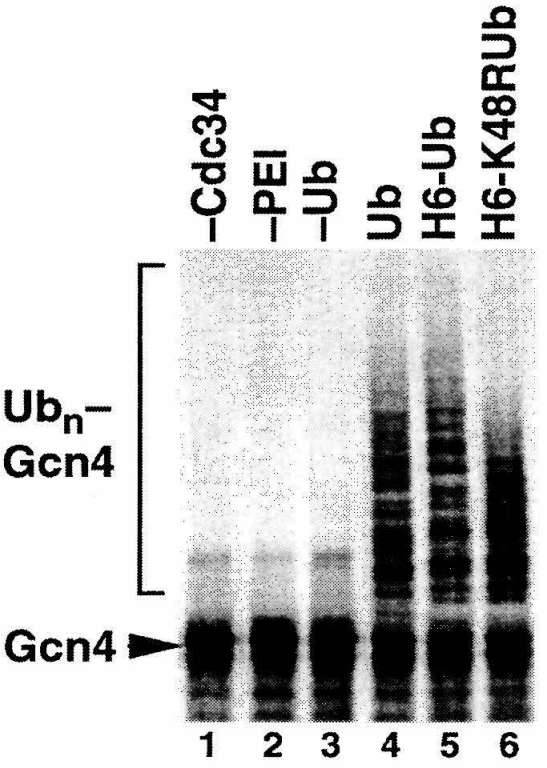

Figure 1. Polyethyleneimine is an E3 for Gcn4.

^{35}S-labeled Gcn4 produced by in vitro translation in reticulocyte lysate and enriched by DEAE chromatography was mixed with E1, Cdc34, ATP, the indicated ubiquitin derivatives (lanes 4-6; H6 stands for His6) and 0.0005% polyethyleneimine (PEI), and incubated at 25°C for 60 min. Reactions conducted in the absence of either Cdc34, PEI, or ubiquitin are depicted in lanes 1-3. Gcn4 was visualized by SDS-PAGE followed by autoradiography.

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Appendix II. Identification of Hrr25 as a Gcn4 kinase

This work was done in collaboration with Edwin Carmack from John R. Yates's lab, who carried out the protein identifications by mass spectrometry.

Results and discussion

To identify activities required for Gcn4 ubiquitination in vitro, we fractionated yeast extracts and discover a novel kinase activity that promoted Gcn4 ubiquitination in vitro. We attempted to purify this activity by conventional column chromatography. Our initial purification effort was partly guided by the premise that there is only one major Gcn4 kinase in the cell and this kinase promotes Gcn4 ubiquitination. Due to technical difficulties and reagent limitations, we only monitored the kinase activity by kinase assays using purified recombinant Gcn4 expressed in E. coli as substrate.

Following a purification scheme shown in Fig. 1, we were able to purify the kinase activity to near homogeneity and identify a protein band on a silver-stained SDS gel (Fig. 2A) that co-fractionated with Gcn4 kinase activity (Fig. 2C, D). This protein has a molecular weight (MW) of approximately 55-60 kDa as judged by its electrophoretic mobility on a SDS gel (Fig. 2A). The majority of the peak fractions from the Mono S column were pooled, concentrated, and fractionated by SDS PAGE. The gel was then stained with Coomassie Blue (Fig. 3). The Gcn4 kinase band (~ 1-2 μg) was excised and subjected to mass spectrometry (MS) analysis. The MS analysis showed that the majority of the peptides correspond to a yeast protein called Hrr25, which is a Casein kinase I isoform.

Hrr25 has a molecular mass of about 57 kDa, consistent with the size of the protein we purified. Furthermore, purified recombinant GST-Hrr25 expressed in E. coli was able to phosphorylate Gcn4 with reasonable efficiency (data not shown), confirming that the kinase activity we purified from yeast extracts was due to Hrr25.

To test if phosphorylation of Gcn4 by Hrr25 promotes its ubiquitination, we carried out Gcn4 ubiquitination using either ^{35}S-labeled Gcn4 or E. coli-expressed Gcn4 that was pre-phosphorylated with purified Hrr25 from yeast. In the presence of E1, Cdc34, and SCF (Cdc53/Cdc4/Skp1 triple infection), we did not observed any Gcn4 ubiquitination (data not shown). This suggests that phosphorylation of Gcn4 by Hrr25 is not sufficient to promote Gcn4 ubiquitination.

After the identification of Srb10 as a Gcn4 kinase and the availability of SCF preparations containing the Hrt1 subunit, we pre-phosphorylated E. coli-expressed Gcn4 using either immunopurified Srb10 (control) or recombinant GST-Hrr25 and carried out Gcn4 ubiquitination in the presence of purified SCF^{Cdc4} containing Hrt1. As shown in Fig. 4, Srb10-phosphorylated Gcn4 was ubiquitinated to high efficiency as evidenced by the accumulation of high MW ubiquitin conjugates and the disappearance of the input substrate (lane 2). In contrast, Hrr25-phosphorylated Gcn4 was only ubiquitinated to a small extent, and there is no apparent high MW ubiquitin conjugates (lane 4). It is also possible that these low level of ubiquitination of Gcn4 is independent of phosphorylation by Hrr25 because phosphorylation-dependence was not addressed in this assay. Nevertheless, it is clear that phosphorylation of Gcn4 by Hrr25 does not make it a efficient substrate for ubiquitination by $Cdc34/SCF^{Cdc4}$.

Hrr25 is a casein kinase I isoform that play multiple roles in cellular metabolism and regulation. It was originally identified as a kinase that might play a role in DNA damage repair (Hoekstra et al. 1991). It was later found to be a dual specificity kinase (Hoekstra et al. 1994). Although *HRR25* is not essential, *hrr25Δ* cells have extremely

slow growth under normal conditions in rich media, suggesting that Hrr25 is necessary for the normal rate of cell growth. To date, the exact functions of Hrr25 have not been elucidated, and genetic analyses have implicated several different functions of this protein. We identified Hrr25 as a potent Gcn4 kinase in yeast extracts. We do not yet know if Hrr25 is a physiological kinase for Gcn4 in vivo, and there is no genetic evidence linking Hrr25 to Gcn4. Determination of the potential role of Gcn4 phosphorylation by Hrr25 would require mapping and mutating Hrr25-dependent phosphorylation sites on Gcn4.

Although phosphorylation of Gcn4 by Hrr25 does not make Gcn4 a good substrate for SCF^{Cdc4} (Fig. 4), this demonstrates that the phosphorylation events that render Gcn4 competent for efficient ubiquitination by SCF^{Cdc4} is highly specific. In this case, CDK phosphorylations are likely the prominent signals that trigger efficient ubiquitination of Gcn4 by Cdc34/ SCF^{Cdc4}.

Methods

Purification of Gcn4 kinase activity

The purification scheme of the Gcn4 kinase activity is outlined in Fig. 1. 8 L of RJD 481 cells were grown to OD_{600} of 3-4 and harvested. Whole cell extract (WCE) was prepared as previously described (Chi et al. 2001). 150 mL of WCE (~ 2 g total protein) was first fractionated by ammonium sulfate precipitation. The 0-40% ammonium sulfate pellete was resuspended in 50 mL CWB (50 mM NaCl; 25 mM Tris-HCl, pH 7.6; 1 mM DTT) and dialyzed in the same buffer. The entire volume (~350 mg total protein) was then loaded onto a 10 mL phosphocellulose column. The kinase activities were eluted with a 0.2-0.5 M NaCl gradient and evaluated by Gcn4 kinase assay. Peak fractions were

pooled and dialyzed against CWB. The final pooled fraction (~15 mg total protein; 37 mL) was loaded onto a 3 mL GST-Gcn4 affinity column (prepared by binding of GST-Gcn4 to glutathione agarose beads at ~ 2mg/mL). Gcn4 kinase activities were eluted with a 0.1 M to 0.6 M NaCl gradient. Peak fractions were pooled and diluted with low salt buffer. The final fraction (~ 1 mg total protein; 10 mL) was loaded onto a 1 mL Mono Q column for FPLC (Pharmacia). Gcn4 kinase activities were eluted with a 0.15 M to 0.5 M NaCl gradient. Peak fractions (~100 μg total protein; 3 mL) from Mono Q were pooled, diluted with low salt buffer, and loaded onto a 0.1 mL Mono S PC column (Pharmacia). Gcn4 kinase activities were eluted with a 0.125 M to 0.55 M NaCl gradient. Most of the peak fractions were pooled, concentrated, and fractionated on SDS PAGE. The SDS gel was visualized by Coomassie and the kinase band was cut out for mass spectrometry analysis. One peak fraction was further fractionated on a Superdex 200 PC column (Pharmacia), and the co-fractionation of the kinase and the Gcn4 kinase activity was confirmed (Fig. 2).

Gcn4 kinase and ubiquitination assays

Gcn4 kinase and ubiquitination assays were carried as previously described (Chi et al. 2001). GST-Hrr25 was expressed in E. coli and purified by affinity chromatography using glutathione agarose (Sigma).

Fig. 1

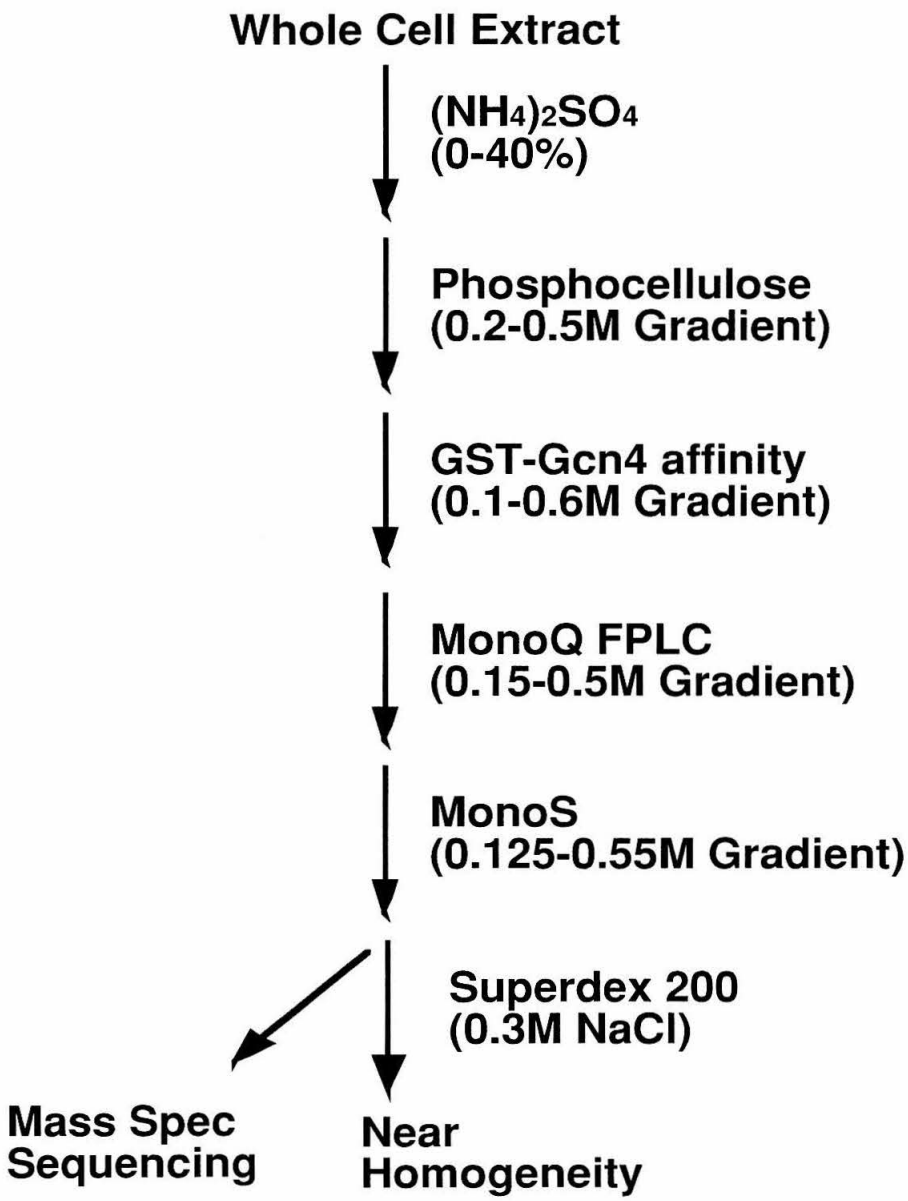
Whole Cell Extract
(NH4)2SO4
(0-40%)
Phosphocellulose
(0.2-0.5M Gradient)
GST-Gcn4 affinity
(0.1-0.6M Gradient)
MonoQ FPLC
(0.15-0.5M Gradient)
MonoS
(0.125-0.55M Gradient)
Superdex 200
(0.3M NaCl)
Mass Spec
Sequencing
Near
Homogeneity

Figure 1. Gcn4 kinase purification scheme.

See Methods for details.

Fig. 2

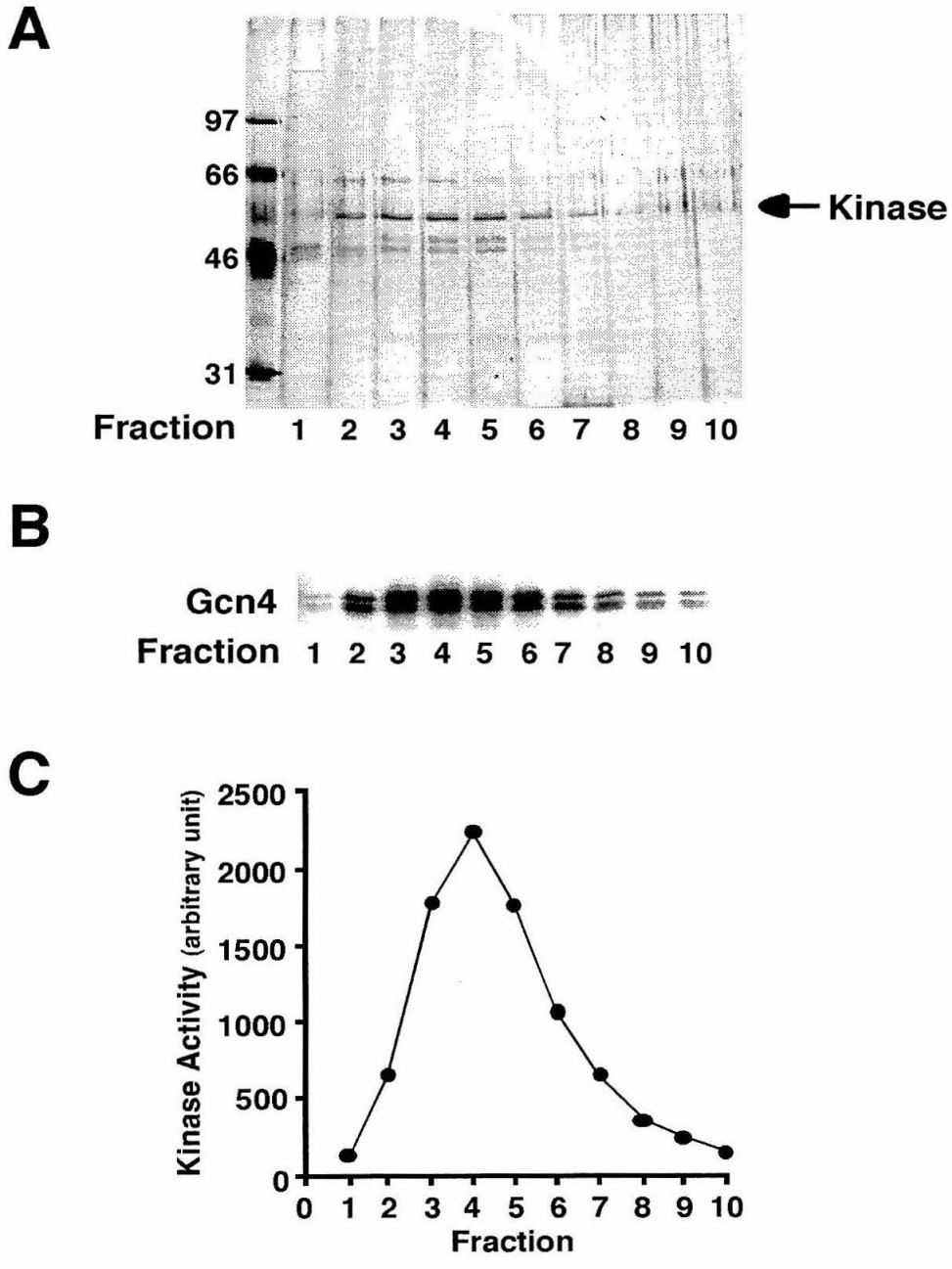
A
97
66
46
31
Kinase
Fraction 1 2 3 4 5 6 7 8 9 10
B
Gcn4
Fraction 1 2 3 4 5 6 7 8 9 10
C
Kinase Activity (arbitrary unit)
2500
2000
1500
1000
500
0
0 1 2 3 4 5 6 7 8 9 10
Fraction

Figure 2. Co-fractionation of the Gcn4 kinase with its activity.

(A) Silver staining of protein fractions from the Superdex 200 step in Fig 1. Note that the fractions were collected in the vicinity of the anticipated MW range of the kinase activity in this experiment. The arrow points at the protein band that correspond to the purified Gcn4 kinase.

(B) ^{32}P Gcn4 kinase assay was carried to measure the kinase activity of each of the fractions.

(C) Quantitation of the kinase activities by PhosphorImager.

Fig. 3

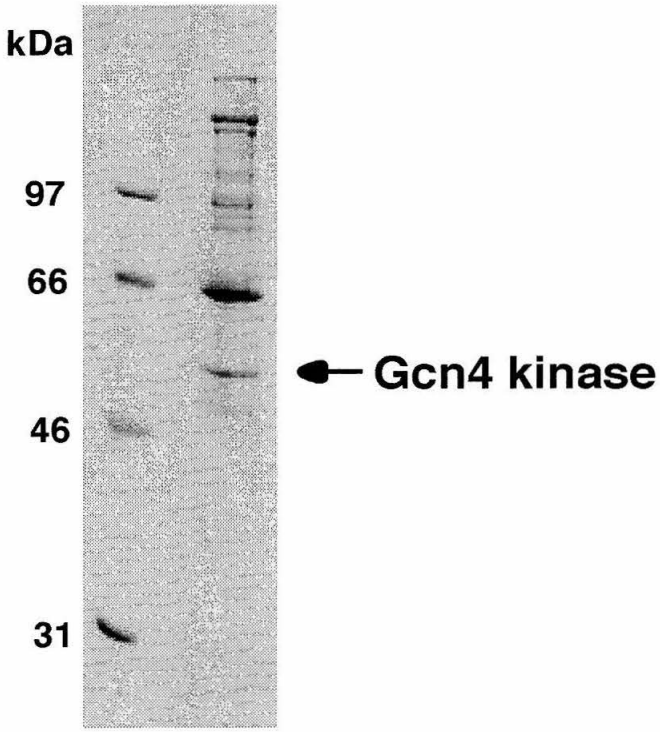

Figure 3. Coomassie staining of the purified Gcn4 kinase.

Most of the peak fractions of the Mono S step (Fig. 1) were pooled, concentrated, and loaded onto a large SDS gel. The protein bands were visualized by Coomassie staining. The band corresponding to the kinase was marked by the arrow.

Fig. 4

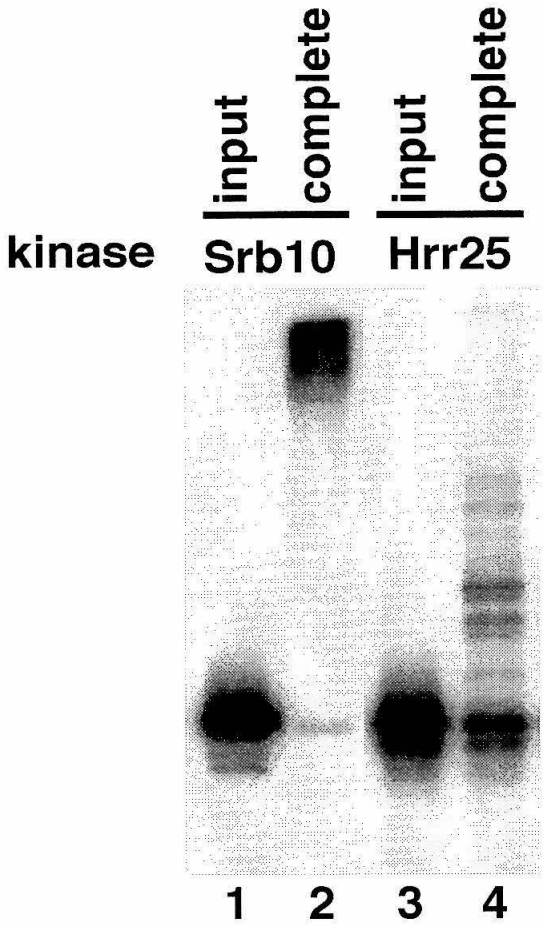

Figure 4. Ubiquitination of phosphorylated Gcn4.

Equal amount (~ 1 μg) of recombinant Gcn4 was first phosphorylated by either immunopurified Srb10 kinase or purified recombinant GST-Hrr25 expressed in E. coli. Phosphorylated Gcn4 (input; note the similar amount of ^{32}P incorporation) was then incubated with a ubiquitination reaction mix containing E1, Cdc34, ubiquitin and purified recombinant SCF^{Cdc4} complex (quadruple infection) (complete). Reactions were visualized by SDS PAGE followed by autoradiography.

References

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