FUNCTIONS OF THE UBIQUITIN-PROTEASOME SYSTEM IN *SACCHAROMYCES CEREVISIAE*: COTRANSLATIONAL PROTEIN DEGRADATION AND REGULATION OF THE UBR1 PATHWAY

Thesis by

Glenn Cameron Turner

In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

2000

(Defended May 15, 2000)

ACKNOWLEDGMENTS

I have had a wonderful time doing my thesis, and there are very many people who contributed to this experience. I would like to express my warm thanks to the following people:

1- Alex Varshavsky – without whom there would be no thesis, and especially no chapter two, which is my favorite part. I have never encountered anyone as creative as you - it was a pleasure to have been a part of this with you.

2- Chris Byrd – without whom there would be no chapters three and four. The science you did made my thesis possible, while the friendship and the conversation kept me going.

3- My thesis committee: Ray Deshaies, Bill Dunphy, Elliot Meyerowitz and Paul Sternberg – Thank you for your advice and support over the years.

4- Fangyong Du (& Ping Ren) – It was a privilege to collaborate with you, and a pleasure to share my time in the lab with you. I will always be grateful for your care when I broke my collarbone.

5- Nils Johnsson – without whom there, again, wouldn't have been a chapter two. You were a great teacher.

6- Ailsa Webster – for teaching me biochemistry. For being so supportive.

7- Ray Deshaies – It meant a lot to me to talk with you over lunch, and to know you'd take time out of your day whenever I came over. Thanks for being so excited about ubiquitin.

8- Tom Clandinin – I think you shaped me as a scientist more than anyone, partly because we understand each other so well, and partly because you are so incredibly good at science. I took part of your thesis as inspiration for my own, or at least the good parts of mine. Thank you for all this, and for driving me to the hospital after the infamous "vindaloo incident.."

9- Tina Iverson – Thanks for that bright cheery smile that always makes me so happy. Thanks for bringing out my goofy side. Thanks for the tremendous emotional and intellectual support behind this thesis, and the great editorial effort on it, and everything else I have written. Thanks for being such a wonderful part of my life in so many ways.

10- Anne Turner and Bryan Turner (aka Mom and Dad) – How could I possibly say how much you mean to me and how much you've done for me. Thanks.

I would also like to thank whoever is reading this. It's good to know that someone out there is interested.

ABSTRACT

The ubiquitin-proteasome system is the major pathway for protein degradation in the cytoplasm of eukaryotic cells. This pathway serves two main functions: protein quality control – removing damaged or misfolded proteins, and concentration control – regulating levels of the protein components of biochemical switches and oscillators.

Misfolded proteins expose hydrophobic patches that act as degradation signals recognized by the ubiquitin-proteasome system. Nascent proteins being synthesized by the ribosome expose similar patches that might also serve as degradation signals. I show here that nascent polypeptides carrying a strong degradation signal of the Ub-proteasome system experience a kinetic competition between degradation and biogenesis. These results suggest that there may be a proofreading pathway for protein folding that recognizes and degrades proteins that fail to fold correctly.

Levels of regulatory proteins must be adjusted in response to many different signals, both environmental and cell-intrinsic. I show here that the activity of a specific ubiquitin-protein ligase (E3), Ubr1, is allosterically regulated. Ubr1 regulates dipeptide uptake in *Saccharomyces cerevisiae* by controlling the degradation of Cup9, a homeodomain-containing repressor of the dipeptide transporter Ptr2. Ubr1 is allosterically activated by dipeptides bearing destabilizing residues according to the N-end rule. The import of these dipeptides stimulates Ubr1, increasing Cup9 degradation, thereby de-repressing Ptr2 expression. Thus, the expression of the machinery required for dipeptide uptake is coupled to the availability of dipeptides.

I also outline a novel pathway governing Ubr1 activity. Free amino acids induce Ptr2 expression via a signal transduction cascade containing Ssy1, a putative transmembrane amino acid receptor, and Ptr3, a novel downstream signaling component. One of the targets of this signal transduction pathway is Ubr1. Ubr1 is activated in the presence of amino acids, accelerating Cup9 degradation, thus inducing Ptr2.

TABLE OF CONTENTS

Chapter 1

The ubiquitin-proteasome system: target selectivity and regulation

Outline

Protein degradation by the ubiquitin (Ub)-proteasome system (Figure 1.1) serves two important functions: quality control – removing damaged and misfolded proteins, and concentration control – modulating levels of regulatory proteins.

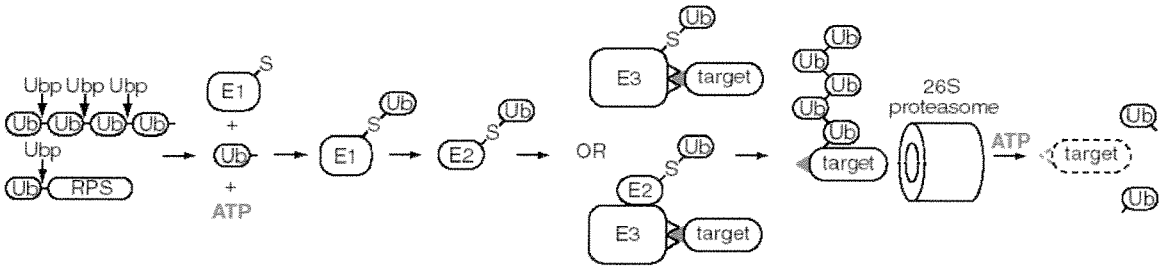


Figure 1.1) The Ub genes in *S. cerevisiae* encode fusion proteins in which Ub is fused to either multiple copies of itself (*UBI4*) or to ribosomal proteins L40 (*UBI1,2*) and S31 (*UBI3*)(26). Cleavage of these fusion proteins by Ub-specific proteases (UBPs) releases free Ub. ATP is utilized to enable Ub to be transferred to a target protein via a cascade of three enzymes: E1, E2 and E3. ATP is also utilized by the proteasome to degrade multiubiquitinated proteins selectively (see text for further details).

Targets of the Ub system are multiubiquitinated by a cascade of three enzymes: E1, E2 and E3 (reviewed in (45, 121)). E1 utilizes ATP to form a high-energy thioester with Ub, activating it for transfer to other proteins. E1 subsequently transfers this Ub thioester to an E2 **ub**iquitin **c**onjugating (Ubc) enzyme. Many E2s possess the capacity to synthesize a multiUb chain, but they generally lack target specificity (6). E3 Ub-protein ligases provide this specificity by directly recognizing the degradation signal carried by the target protein.

Thus, the energy of ATP hydrolysis endows the Ub system with selectivity. By activating Ub for transfer down the E1, E2, E3 cascade, ATP enables the molecular recognition properties of the different E3s to dictate which proteins are multiubiquitinated and which are not.

Multiubiquitinated proteins are degraded by the 26S proteasome (reviewed in (9, 18)). The proteasome consists of a hollow cylindrical core particle (CP or 20S core) containing the proteolytic active sites, capped on both ends by a multisubunit regulatory particle (RP or 19S caps). This self-compartmentalized structure sequesters the proteolytic sites from the cellular environment, preventing indiscriminate degradation of cytoplasmic proteins. Consequently, the RP caps must use ATP to unfold substrates and thread them into the CP cylinder, to the proteolytic active sites. Thus the ATP requirement for degradation by the proteasome also arises from the need for selectivity.

In this introduction I will detail the mechanisms by which the Ub-proteasome system achieves its remarkable specificity. I will argue that misfolded proteins are recognized because they display hydrophobic patches that are buried in their properly folded counterparts. I will discuss how the degradation of these misfolded proteins is coordinated with their refolding. I will argue that the degradation of regulatory components of biochemical switches and oscillators is essential for the function of these circuits. I will describe the regulatory mechanisms governing the degradation of these proteins, and how they link the lifespan of a protein to the appropriate environmental or cell-intrinsic signals.

Functions of Ub-dependent protein degradation

Although peptide bond hydrolysis is energetically favorable, depleting a cell of ATP causes some proteins that are normally rapidly degraded to suddenly become stable (46). A cell expends considerable energy to selectively degrade proteins for two important purposes:

1- Quality Control. Proteins that fold incorrectly are often degraded, thereby removing forms of a protein that are either simply useless, and therefore best recycled to amino acids, or that are hazardous, such as dominant negative or aggregation-inducing species. Misfolded proteins produced by either point mutation or incorporation of amino acid analogs are degraded in an ATP- and Ub-dependent manner (15, 47).

2- Concentration Control. Many key regulatory proteins are rapidly degraded, either constitutively or in response to certain signals. Such regulation serves two purposes:

i) Building a switch.

In order for changes in mRNA levels to be rapidly converted into changes in protein levels, the protein must be unstable. If transcription of a gene is repressed, but the protein encoded by the transcripts is stable, transcriptional regulation accomplishes little (especially if the cell in question is not growing and dividing). In order for these transcriptional changes to be manifest at the protein level, the protein must be short-lived.

ii) Building an oscillator

The cell cycle and circadian rhythms are two examples of biological processes driven by an underlying biochemical oscillator. Since an oscillator is really just a two-way switch, the components of that oscillator must be short-lived.

Recent theoretical and biochemical efforts to design genetic networks have shown that a two member closed loop network, where the first element activates the second, and the second represses the first, can form a simple transcriptional oscillator (Figure 1.2) (7, 23).

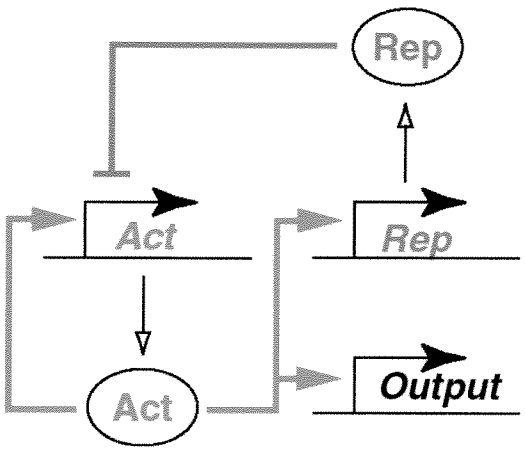


Figure 1.2) Simple oscillator consisting of an Activator (Act) that activates transcription of both itself and a Repressor (Rep). The Repressor accumulates with slow kinetics and shuts off Activator expression, thereby establishing an oscillatory level of Output expression.

One's intuition suggests that if activation of the two members of this network were coupled with a significant time lag, such that activation of Repressor transcription would lead to a slow accumulation of Repressor protein relative to Activator accumulation, the system would oscillate between high and low levels of Output. This time lag could be produced if the Activator drives transcription of both itself and Repressor at the same rate, but Repressor protein is highly unstable, while Activator is only modestly unstable. If there were no

time lag, the system would converge on a stable steady-state with constant, intermediate levels of both Activator and correspondingly stable Output.

This time lag can also be produced by highly cooperative kinetics of repression. In the two member network, fast but linearly building Activation, coupled to a slow but highly cooperative Repression, would produce oscillatory Output because during a given time in which Activator activity accumulated linearly, Repressor activity would become manifest with switch-like sigmoidal kinetics. In this way, Repressor and Activator activity could never come to a stable steady state, and Output would oscillate. However, to transition between the two stable states of the oscillation, it would be essential for both Activator and Repressor to be unstable proteins.

Mechanism of Ub-dependent protein degradation

Ubiquitination

The specificity of target selection by the Ub system resides in its E3 components, which recognize degradation signals of substrates. E3s direct the attachment of several Ub moieties to the target in the form of a multiUb chain (14). The chain is linked to the substrate via an isopeptide bond between the C-terminal carboxyl group of Ub and the amino group of the lysine side chain. Ub-Ub isopeptide linkages through lysine 48 of Ub form the chain itself (14). Multi-ubiquitination is required for substrate degradation because monoubiquitinated substrates synthesized *in vitro* using the chain-terminating variant $Ub^{Lys48 \rightarrow Arg}$ are stable, while an identical multiubiquitinated substrate is degraded (14).

The E3s identified so far can be classed into four groups: HECT domain E3s, SCF complexes, APC and Ubr1, described below. The mechanism of action of these different classes of E3s, and their regulation, demonstrated or only predicted, is discussed in this section.

HECT domain E3s

In association with the human papilloma virus (HPV) protein E6, E6-AP (E6-associated protein) targets p53 for degradation (100). This blocks the host cell from undergoing apoptosis, enabling the virus to exploit the host cell for maximal virus replication. E6-AP also acts as an E3 in the absence of E6 to target HHR23A, a protein involved in the DNA damage response (71). A 45 residue motif at the C-terminus of E6-AP, termed the HECT domain (Homology to E6-AP C-terminus) identifies a family of E3s, including:

1- Rsp5, which targets the carboxy-terminal domain of large subunit RNA pol II in *S. cerevisiae* in response to UV-mediated DNA damage (10, 55).

2- Ufd4, which targets reporter proteins engineered to carry an uncleavable Ub moiety linearly fused to the reporter (60). Endogenous substrates of the *S. cerevisiae* UFD (Ub fusion degradation) pathway remain to be identified.

The mechanism of action of this class of E3s was established using E6-AP (99). In the presence of E1 and E2, E6-AP forms a dithiothreitol-sensitive Ub thioester on a highly conserved cysteine residue in its HECT domain. Extract reactions demonstrate that E6-AP can directly transfer Ub to other proteins. E6-AP was first charged with radiolabeled Ub in a reaction containing E1 and E2, and then chased with cold Ub. The radiolabeled Ub was transferred to a variety

of other proteins in the extract, indicating that E6-AP directly ubiquitinates target proteins.

The structure of the E3-E2 complex E6-AP HECT domain-UbcH7 has been solved to 2.8Å resolution (54). The complex forms a U-shaped structure, with the active site cysteine of the HECT domain on one vertical wall of the U and the cysteine of UbcH7 on the opposite wall, separated by 41Å. Thus, the ubiquitination reaction probably involves a large conformational change to transfer the Ub-thioester from UbcH7 to the HECT domain cysteine. Consistent with this possibility, the HECT domain is organized into two hinged lobes, with the active site cysteine at the interface of the two lobes, so that it might be exposed by movement of the hinge. Co-crystallization of reaction cycle intermediates, as well as higher order complexes containing degradation signal-derived peptides, will be needed to understand the ubiquitination reaction in atomic detail.

The activity of the Ufd4 E3 is somewhat different from that of E6-AP. *In vitro* ubiquitination reactions consisting of E1, E2 and Ufd4 efficiently catalyze the transfer of one or two Ub moieties to the substrate, but do not form longer multiUb chains, even at extended periods of incubation (68). However, reactions containing a fourth protein, Ufd2, which is required for UFD pathway activity *in vivo* (60), yield long multiUb chains, not the smaller mono- and diubiquitinated conjugates. These results suggest that Ufd2 is doing more than simply stimulating activity of Ufd4. E1, E2 and E3 (Ufd4) efficiently initiate Ub chains; however, chain elongation requires E1, E2, E3 (Ufd4) and Ufd2. Consequently, Ufd2 has been dubbed E4 (68).

This mechanistic separation between chain initiation and elongation would help to explain the observation that there appears to be little specificity for the lysine on the substrate that is the site of Ub chain attachment, while Ub-Ub linkages within the chain are consistently through lysine 48 of Ub (14). These differences could reflect differences in the stringency of lysine selection at the initiation and elongation stages of the reaction. It will be interesting to determine whether the mechanics of multiubiquitination is split into chain building and chain elongation with E3s that function without E4s.

Regulation

The results with Ufd2 and Ufd4 raise the possibility that multiUb chain initiation and elongation are two separate steps that can be independently regulated. Mono- and diubiquitinated substrates produced by the action of E1, E2 and Ufd2 would probably be deubiquitinated by Ubps (Ub-specific proteases; also known as **de**u**b**iquitinating enzymes or Dubs) before they are degraded. Regulation of any HECT domain E3 has yet to be reported.

The separation between chain initiation and chain elongation may also play a role in the endocytosis of plasma membrane proteins. Attachment of mono- or diubiquitin triggers endocytosis of certain plasma membrane proteins, and their subsequent degradation in the vacuole (49, 50). The endocytosis of uracil permease (Fur4) requires the HECT domain E3 Rsp5 (32). Rsp5 mono- and diubiquitinates Fur4, forming lysine 63 diUb linkages (31). Interestingly, Rsp5 attaches lysine 48-linked multiUb chains to the large subunit of RNA pol II, a nuclear protein, to target it for degradation by the proteasome (10, 55). This ability to catalyze two different ubiquitination events may reflect an ability to

control chain initiation (mono- and diubiquitination) separately from chain elongation (multiubiquitination).

SCF complexes

SCF (Skp1 Cdc53/Cullin, F-box) complexes are multisubunit E3s composed of four subunits, one each of the Skp, Cullin, F-box and Rbx (RING box) families (4, 24, 105, 110, 111). The arrangement of the subunits in the complex is shown below:

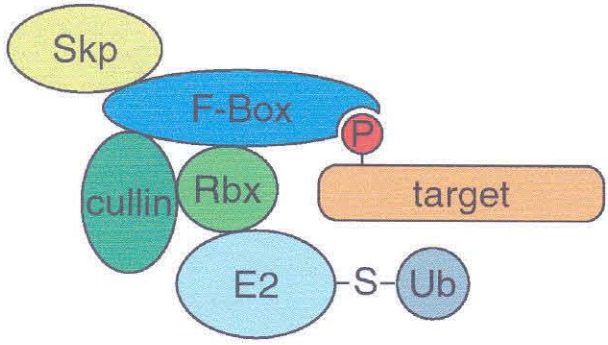


Figure 1.3) Arrangement of SCF complexes. Subunit interactions within SCF and interaction of SCF with a generic phosphorylated substrate are indicated by touching ovals.

F-box proteins bind directly to the target proteins, and thereby dictate the recognition specificity of an SCF complex. Thus, SCF complexes are modular, and their specificity can be altered by combining the same core components with different F-box proteins (110). Many different F-box proteins have been identified and their target specificities determined:

F-Box	Cdc4	β-TrCP	Grr1
Substrate	Sic1 CDK inhibitor (4, 24, 110)	IκBα β-catenin signal transduction (112, 132, 137)	Cln G1 cyclins (4, 110) Gcn4 transcription factor (81)

Table 1. 1: F-Box components of SCF E3s, and the substrates they recognize.

In vitro experiments have begun to elucidate the mechanism of SCF ubiquitination. SCF^{CDC4} targets the cyclin-dependent kinase inhibitor Sic1p in conjunction with the E2 Cdc34 (24). In the presence of E1, isolated Cdc34 autoubiquitinates itself, indicating that it possess the catalytic capacity to form a multiUb chain (6). Although the autoubiquitination reaction is probably not an intermediate step in the ubiquitination of SCF substrates, it can be used as a readout of E2 activity (105). SCF^{CDC4} stimulates E2 activity, with the cullin and RING box subunits being the most critical for stimulation. Sulfhydryl-alkylating agents do not inactivate this minimal E3, indicating that it does not form a Ub thioester. These experiments suggest that the function of SCF^{CDC4} is to recruit a substrate to an E2, and then stimulate E2 to form a multiUb chain on the substrate. The F-box subunit is responsible for recruiting the substrate, while the cullin and RING box subunits play the major role in stimulating E2 activity. This is supported by the observation that polycations, such as polylysine, can enable E2 to ubiquitinate substrate in the absence of SCF, suggesting that E2 activity is susceptible to acceleration by a simple electrostatic interaction.

Regulation

All SCF substrates identified so far carry phosphorylation-activated degradation signals, and the regulation of their degradation can be explained by the timing of phosphorylation (see **Regulated Degradation Signals**, p. 29). However, this does not exclude the possibility that SCF E3s may themselves be regulated. In fact, the F-box components of SCF complexes are short-lived *in vivo* (140). The degradation of the F-box subunits requires other components of SCF,

suggesting that the subunits are autoubiquitinated. This degradation likely plays an important role in changing the substrate specificity of SCF complexes by allowing different F-box subunits to be inserted during cell cycle progression or perhaps in response to environmental signals. Expression of a non-degradable form of Cdc4 prevents cell proliferation, and inhibits degradation of S-phase cyclins (Clns) that are recognized by the Grr1 F-Box protein, suggesting that the non-degradable Cdc4 titrates the available SCF.

Interestingly, the cullin components of SCF are modified, not by ubiquitination, but by the attachment of a Ub-like molecule (Ubl; this class of molecules includes Nedd8, Rub1 and SUMO) (73, 75, 85). This could also be an autocatalyzed reaction, since Skp1 is required for Ubl modification of Cdc53 in yeast (73). Similar modification of $SCF^{\beta TrCP}$ and SCF^{SKP2} activates these complexes for substrate ubiquitination *in vitro* (92, 93). The precise mechanism of this activation remains to be determined. It is also possible that Ubl modification enables SCF complexes to interact with the proteasome, much like the Ub-like domain of Rad23 supports its interaction with the proteasome (98). This may facilitate delivery of SCF substrates to the proteasome.

Anaphase Promoting Complex (APC)

APC degrades several proteins important for transit through mitosis, including mitotic cyclins and regulators of sister chromatic cohesion. At 16 subunits, APC is much larger than the SCF complex; however these E3s may actually be quite similar mechanistically. One APC subunit (APC11) possesses a RING box, and one (APC2) is a member of the cullin family (138, 139).

Regulation

Much like SCF, the substrate selectivity of APC can be programmed by the composition of its subunits, specifically the WD-40 repeat-containing proteins Cdc20 and Cdh1 (125). Cdc20-APC complexes target proteins carrying a degradation signal known as the D-box (consensus sequence RXXLSSSSN) (38, 65). Such targets include CyclinB, and Pds1, an important regulator of sister chromatid cohesion, whose degradation is required for transit through anaphase (135). Cdh1-APC targets KEN box (KENXXXN) containing substrates, including the Cdc20 subunit of Cdc20-APC (90). This is analogous to the swapping of F-box subunits that occurs in SCF complexes.

However, unlike SCF substrates, the degradation signals of APC substrates are constitutively active. Nevertheless, the substrates of APC must be degraded at different times in the cell cycle, so regulation of APC activity is extremely important for proper cell cycle progression. This is accomplished through the activity of several different phosphorylation pathways. Some of these pathways directly phosphorylate APC, while others act on the substrate-selection subunits Cdc20 and Cdh1, modulating their interaction with APC:

i) Polo-like kinase activates APC by phosphorylation, while cAMP-dependent protein kinase (PKA) phosphorylation inhibits APC (13, 39).

ii) S-phase cyclin-dependent kinase (MPF) phosphorylates Cdc20 and Cdh1, regulating their association with APC (69).

iii) The Cdc14 phosphatase dephosphorylates Cdh1, inactivating APC (1, 108, 124)

Ubr1 (N-Recognin)

Ubr1 is the E3 of the N-end rule pathway (8, 120). It recognizes proteins carrying a degradation signal called the N-degron, which consists of a destabilizing N-terminal residue, and an accessible lysine for multiUb chain attachment (see **Regulated Degradation Signals**, p. 29) (3). Ubr1 also controls dipeptide uptake by targeting Cup9, a homeodomain-containing repressor of the dipeptide transporter Ptr2.

Interestingly, Ubr1 exhibits characteristics of both SCF/APC and HECT domain classes of E3s. It has a substrate binding domain (126), an E2 (Rad6) interaction domain (22, 79), and a RING-H2 finger motif (8, 72, 133). This suggests that, similarly to SCF complexes, Ubr1 may act by bringing substrate and E2 into close proximity and stimulating activity of its associated E2. However, other features of Ubr1 suggest that it may be similar to HECT domain E3s. Mutation of two cysteine residues within the C-terminus of Ubr1 inactivates the enzyme (Fangyong Du, personal communication). However, the chemical modification of cysteine residues does not readily inactivate a mouse homolog of Ubr1, E3α, which raises the possibility that these cysteine residues are structurally important, but not catalytic (48).

Regulation

Ubr1 is the first example of an E3 regulated by an allosteric interaction (Chapter 3). Dipeptides with destabilizing residues activate Ubr1-dependent ubiquitination of Cup9, a repressor of the dipeptide permease Ptr2. This interaction creates a positive feedback loop. Imported peptides bind to Ubr1, accelerating Cup9 degradation, thus de-repressing the dipeptide transporter

Ptr2. This feedback loop couples the expression of the dipeptide pump to the availability of dipeptides in the environment.

Ubr1 is also regulated by an amino acid sensing pathway (Chapter 4). Certain amino acids activate a signal transduction pathway containing Ssy1, a protein with homology to amino acid transporters – a putative amino acid receptor – and Ptr3, a novel protein that is part of the signal transduction apparatus. Activation of this pathway stimulates Ubr1-dependent degradation of Cup9. Although Ubr1 is phosphorylated (Chapter 5), this phosphorylation is not strongly modulated by the presence of amino acids in the growth medium, suggesting that the Ssy1-Ptr3 signaling pathway activates Ubr1 by some other mechanism.

Degradation

Proteasome Structure and ATP utilization

The 26S proteasome utilizes ATP to break down multiubiquitinated target proteins into small peptides, and cleave the multiUb chain into free Ub monomers (9). Proteasomes are composed of two biochemically separable subparticles: the core particle (CP; also known as the 20S core), a multisubunit cylinder containing the proteolytic active sites, and the regulatory particle (RP; also known as the 19S cap or PA700) which caps the CP cylinder at both ends (Figure 1.4). X-ray crystallographic analysis of archeal (*Thermoplasma acidophilum*) proteasomes indicates that the proteolytic sites are located inside the CP cylinder, and are only accessible via two small (13Å diameter) pores at either end (76). Extended, flexible peptides that can pass through the pores are degraded by isolated CPs; however, peptides containing β–turns are too bulky to

degraded by isolated CPs; however, peptides containing β–turns are too bulky to enter, and remain intact (127). This observation suggests that proteins must be unfolded in order to be degraded. The RP caps serve this function.

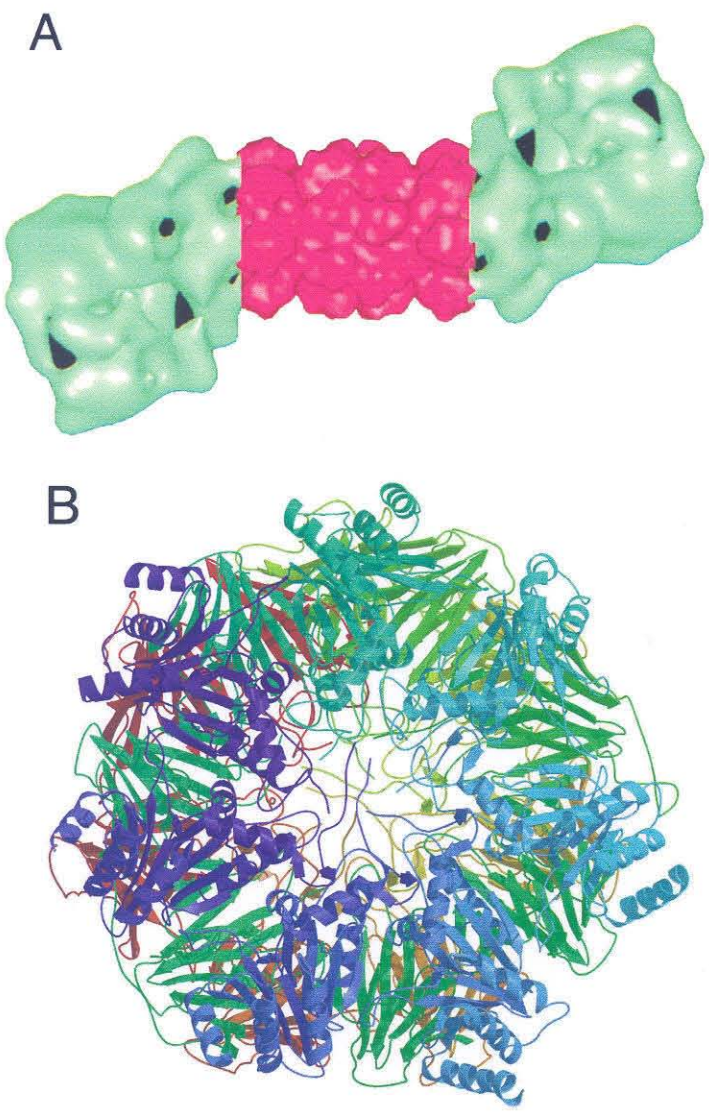


Figure 1.4A) The proteasome (modified from (9)). The central CP (red) is capped on either end by the RPs (green). Image is a hybrid of electron microscopy reconstructions of RP and crystal structure of CP. **B)** The core particle (CP) of the *S. cerevisiae* proteasome (41) viewed down the axis of the central channel in which the active sites are contained. Image constructed using Molscript and Raster3D (70, 82).

Each RP is composed of 17 subunits, although it interacts with other proteins, including one Ubp, which releases the multiUb chain from the substrate (88, 89). The RP can be dissociated into two subparticles *in vitro*, called the lid and the base (36). The lid contains eight subunits. The base contains nine subunits, including six ATPases, and connects the lid to the CP. The ATPase subunits are thought to perform two functions. ATP hydrolysis stimulates degradation of unstructured peptides by the eukaryotic proteasome (37) (78), suggesting that the RP ATPases gate the pores to the CP cylinder, allowing access to the proteolytic chamber. In fact, the pores are closed in the crystal structure of the *S. cerevisiae* CP (41), so pore opening by the RP must be important for protein degradation *in vivo*. The pores in the *Thermoplasma* CP are only 13 Å in diameter, so even in this case widening of the pores may facilitate protein degradation. In addition, ATP hydrolysis is likely to be required to unfold protein substrates and thread them into the proteolytic cavity. RPs have demonstrable protein-folding activity *in vitro*, suggesting that they can catalyze the opposite reaction *in vivo*, and unfold substrates (12, 113). Although the *in vitro* refolding reaction is ATP-independent, unfolding is very probably an endergonic process requiring ATP hydrolysis.

Substrate Recognition

How the proteasome selects targets for degradation remains unclear. If a single subunit of a multimeric complex carries a multiUb chain, this subunit is degraded by the proteasome, and the non-ubiquitinated subunits are spared (59) (123). Thus, a multiUb chain is a major specificity determinant for proteasome mediated degradation. Ub chains containing ≥ 4 Ub moieties bind to the Rpn10

component (also known as S5a/Mcb1) of the RP (20), suggesting that targets of the proteasome are recognized by their attached multiUb chain. Moreover, x-ray crystallographic analysis suggests that a multimer of Ub_4 possesses emergent qualities as a recognition signal (16). The arrangement of four Ub moieties brings certain hydrophobic residues on each Ub moiety into register, forming a strip of these residues down the Ub_4 chain, which is not present in smaller chains (Figure 1.5).

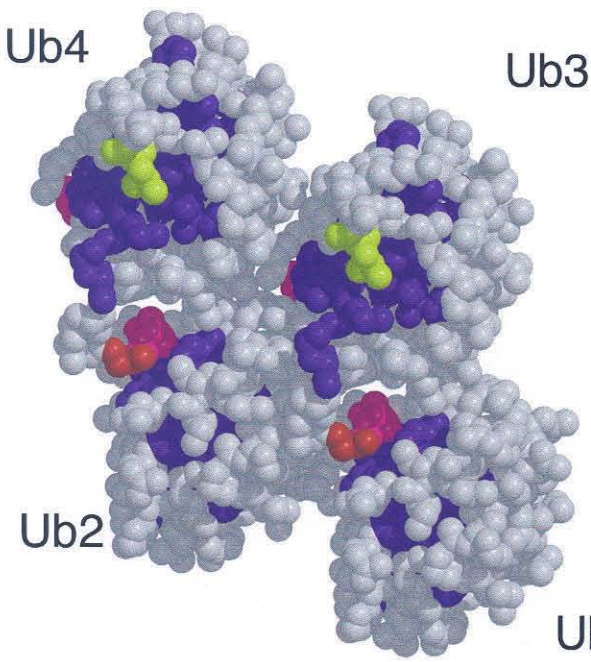


Figure 1.5) Structure of the Ub_4 chain (16). Lysine 48 and glycine 76 (positions of isopeptide bond) are shown in red. Hydrophobic residues are highlighted in blue. Leucine 8, an important residue in chain recognition by the proteasome, forms a repeating structure in Ub_4 but not Ub_3 or smaller chains. Image constructed using Molscript and Raster3D (70, 82).

Although appealing, experimental evidence for target recognition via a multiUb chain is less than compelling. The chain binding subunit Rpn10 is not

essential for Ub-dependent degradation (119). Additionally, ubiquitination of certain proteins is not essential for their degradation by the proteasome (84, 106). Ubr1, the E3 of the N-end rule pathway, interacts with multiple components of the RP cap, suggesting that E3s may directly deliver substrates to the proteasome (134). However, the consequences of preventing the Ubr1-proteasome interaction on substrate degradation remain to be tested.

On the other side of the argument, multiUb chains can act as competitive inhibitors of degradation *in vitro* (91, 117). However, it is not known whether this inhibition blocks the initial recognition and binding of multiubiquitinated substrates to the proteasome, or a subsequent step in their degradation, such as unfolding or threading into the CP. Interestingly, multiubiquitination of these particular substrates is not sufficient for degradation in this system unless multiUb chain breakdown by Ubps is inhibited (117). This indicates that substrate unfolding is rate limiting *in vitro*, despite the presence of the RP ATPases, which are thought to unfold substrates of the proteasome efficiently. The same substrates are degraded rapidly *in vivo* (57), suggesting that additional factors can accelerate unfolding and degradation of substrates.

Hydrolysis

The CP of eukaryotic proteasomes consists of fourteen different subunits, arranged in four stacked rings. Each ring is made up of seven different α-type subunits, or seven different β-type subunits, arranged in the form $\alpha_{1-7}\,\beta_{1-7}\,\beta_{1-7}\,\alpha_{1-7}$ (Figure 1.6) (9).

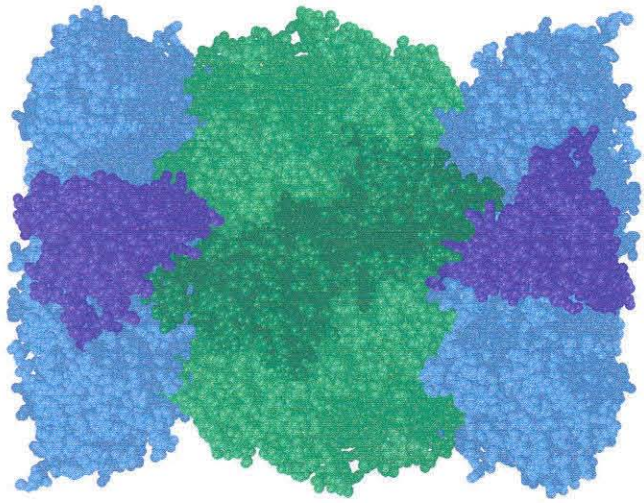


Figure 1.6) The CP viewed from the side (the axis of the channel is horizontal in the plane of the paper) (41). The α subunits (blue) and β subunits (green) are arranged in four stacked rings of the form $\alpha_{1-7}\,\beta_{1-7}\,\beta_{1-7}\,\alpha_{1-7}$. Individual α and β subunits are accented in darker hues. Images constructed using Molscript and Raster3D (70, 82).

Inhibitor studies show that three of the β subunit types are proteolytically active (25). Each carries an N-terminal threonine residue that is the active site for the hydrolysis reaction; however, each type has a distinct cleavage site specificity towards small peptides (76, 104). These activities are characterized as chymotrypsin-like (cleaves after large hydrophobic residues), trypsin-like (cleaves after basic residues) and caspase-like or post-glutamyl (cleaves after acidic residues) (9). Studies with peptides specifically hydrolyzed by each type of β subunit show that these active sites allosterically regulate one another, such that the proteasome degrades proteins in a well-ordered series of cleavages, not by stochastic hydrolysis (66). Peptide substrates of the chymotryptic subunit activate hydrolysis by the caspase-like site. However, occupancy of the caspase-

like subunit inhibits activity of the chymotryptic subunit. This suggests that protein substrates are initially cleaved into large peptides by the chymotryptic subunit, which activates the caspase-like subunit to degrade the initial cleavage products into smaller peptides. While hydrolyzing the initial cleavage products to smaller peptides, the caspase-like site suppresses activity of the chymotryptic subunit. This prevents the generation of more large cleavage products before the caspase-like site has hydrolyzed the previous such products. This cycle has been aptly termed the "bite and chew" mechanism of protein breakdown (66).

The end products of these hydrolytic events are peptides ranging from 4 to 25 residues in size (67). To be incorporated into new proteins, these peptides must be broken down to single amino acids. The tricorn protease appears to fulfill this function. Tricorn is composed of a hexamer of six identical subunits which forms a self-compartmentalized structure (116). This core structure hydrolyzes the peptide products of the proteasome into smaller peptides of 2 to 4 residues, which are then broken down to free amino acids by the action of three amino peptidases that interact with the tricorn core (115).

In mammals, the peptide breakdown products of the proteasome are utilized by the immune system (reviewed in (44)). Hydrolysis products of 8 to 11 residues are bound non-specifically by **m**ajor **h**istocompatibility **c**omplex (MHC) class I molecules for presentation to T cells, forming an integral part of the immune response to virally infected cells. In fact, the expression of certain β subunits of the proteasome is induced by γ–interferon during the immune response (28-30). The incorporation of these subunits into proteasomes is

thought to alter the identity or quantity of peptides they produce, thereby increasing the potency of the T cell response.

Non-degradative functions of the proteasome

The chaperone-like function of the RP (12, 113) may play a role in protein complex remodeling *in vivo*. Nucleotide excision repair (NER) requires the ATPase activity of the proteasome, but not its proteolytic activity, as defined by the inability of active site inhibitors of the proteasome to inhibit NER (95). Thus, the RP may directly remodel a protein complex involved in repairing DNA lesions, perhaps by breaking inter-subunit interactions, and removing or replacing subunits within the complex (95). This parallels observations that a protein required for error-free post-replication DNA repair directs the E2 Ubc13 to synthesize lysine 63-linked multiUb chains (52). These chains apparently do not target the attached protein for degradation (cited in (52)), which raises the possibility that lysine 63-linked multiUb chains direct the RP to remodel a protein complex involved in DNA damage repair. However, it is currently unclear whether NER and error-free post-replication DNA repair share any of the same protein components, so this is a very speculative connection. Moreover, the observation that proteasome inhibitors do not diminish NER activity should not be regarded as conclusive evidence that proteolytic activity is not required, since only one of the three proteasome active sites (chymotryptic) is effectively inactivated by the inhibitor used.

Quality Control

Degradation signals of misfolded proteins

How does the Ub-proteasome system recognize misfolded proteins but not their properly folded counterparts? The degree of unfolding, although influential, is not the only important variable. Only a weak correlation exists between half-life and melting temperature (T_M) for a variety of T4 lysozyme folding mutants (56). This suggests that misfolding reveals specific regions of a protein, probably hydrophobic patches that act as degradation signals when exposed, but are buried in a properly folded protein. Many globally misfolded mutants would expose these degradation signals in a manner correlated with their degree of unfolding, and therefore show a correlation between T_M and $t_{1/2}$. However, these signals may not be fully exposed in some misfolded conformations, or they may be so hydrophobic that their exposure causes the protein to precipitate into an undegradable aggregate.

To define such signals, it is necessary to disentangle regions of the protein that simply facilitate exposure of the cryptic degradation signal from those that actually constitute that signal. Thus, the best approach would be to isolate fragments of a misfolded protein that could physically bind to an E3. In this spirit, investigators have searched for sequences that confer rapid degradation on a reporter protein. One approach captured a small but highly diverse set of degradation signals from genomic DNA (35). These signals all possess strongly hydrophobic regions of 4-15 residues that are required for degradation. Unfortunately, all these signals are derived from fortuitously coding sequences in intergenic regions, not from open reading frames.

A second study built artificial degradation signals by randomizing sequences at the N-terminus of a reporter protein and screening for those that destabilize the reporter (96). The one degradation signal they successfully synthesized is predicted to form an amphipathic α-helix, again suggesting hydrophobic character can be an important determinant of a degradation signal. Measuring the half-life of reporter proteins carrying short runs of identical hydrophobic residues directly tests this possibility. Although some hydrophobic patches (e.g., Ile_5) function as degradation signals, the length and composition of these stretches is an important factor determining their efficiency, as Ile_9 and Trp_5 do not destabilize the reporter.

Thus, misfolded proteins are most likely recognized for degradation via specific hydrophobic patches. Misfolding can expose these patches to the ubiquitination machinery, while proper folding buries them within the hydrophobic core of the protein. However, this must be an over-simplified view, because only some hydrophobic stretches are effective degradation signals. Identifying specific E3s that target misfolded proteins would help to explain more precisely the role exposed hydrophobic residues play in forming a degradation signal.

Coordinating protein folding and degradation

Misfolded proteins may undergo refolding to return to their native conformations, or removal from the cell by degradation (40) (131). There are two

ways the competing processes of refolding and degradation might be coordinated:

1- A misfolded protein undergoes stochastic kinetic partitioning between refolding by the chaperones and degradation by the Ub-proteasome system. There is a particular probability the protein is recognized by chaperones and a probability it is recognized by the Ub-proteasome system, but there is no interaction between the two processes.

2- The chaperones directly deliver misfolded proteins to the Ub-proteasome system. This may be a stochastic process, in which the chaperones release the protein to refold with one probability, and deliver the protein to the degradation machinery with another probability. Alternatively, when a chaperone interacts with a protein it cannot refold, the chaperone may undergo some alteration, perhaps a conformational change, so that it interacts with the degradation machinery, delivering the substrate directly.

It is currently unclear which components of the Ub-proteasome system, the E3s or the proteasome itself, recognize misfolded proteins. The observation that E1 inactivation stabilizes misfolded proteins (15) suggests that E3s recognize these substrates. Additionally, specific E2 proteins are required for degradation of reporter proteins carrying hydrophobic stretches (35, 96); however, the reporter proteins are themselves folded properly, so they may not be degraded in the same manner as a truly misfolded protein. In support of the possibility that the proteasome directly recognizes misfolded proteins, the RP caps of the proteasome directly interact with certain damaged proteins (12, 113). In addition, proteins that are sufficiently unfolded to enter the CP cylinder of the

proteasome are readily degraded (127, 128), so degradation of some misfolded proteins may be ubiquitin-independent.

Experiments in prokaryotes suggest that there is stochastic partitioning between refolding and degradation, but also that chaperones can directly deliver substrates to the degradation machinery (i.e., model (2) above) (86). The *E. coli* protein ClpA is a member of the Clp/Hsp100 family of chaperones (40). ClpA forms a hexameric ring that recognizes the transcription factor RepA and conformationally remodels it from an inactive dimer into two active RepA monomers (130) (87). ClpA hexamers also stack onto a heptameric ring of ClpP proteolytic subunits to form the ClpAP protease (11, 64). ClpA is capable of acting as a chaperone within the ClpAP complex. Substrates bound to ClpA undergo either conformational remodeling and release, or translocation into the ClpP heptamer, and degradation (53) (86). Thus, each cycle of substrate binding to the ClpA chaperone is stochastically partitioned between two possible outcomes, refolding or delivery to the ClpP protease. It is thought that the probability of refolding is high relative to the probability of delivery to ClpP, which ensures that, on average, a protein has several opportunities to refold before it is degraded. However, the fate of the protein at any given cycle would not be influenced by its fate in a previous cycle. Some proteins might be immediately degraded and not given an opportunity to refold, while non-refoldable proteins might undergo many futile cycles of release before they are finally degraded.

Interestingly, the RP of the proteasome can act as a chaperone *in vitro* (12, 113). As described above, evidence suggests that, *in vivo*, the RP can remodel a

protein complex that repairs DNA lesions (95). Such a function would be closely analogous to the remodeling of inactive RepA dimers into active RepA monomers by ClpAP (86). Thus it is possible that the RP directly recognizes misfolded proteins and refolds or degrades them based on a stochastic mechanism similar to that operating in ClpAP.

Clp/Hsp100 chaperones are found in all organisms (40), suggesting that chaperones other than the RP caps may deliver substrates to the proteasome or the ubiquitination machinery in eukaryotes. Chaperones and proteases do interact in a variety of systems. Eukaryotic Hsp70 interacts with the proteasome through the bridging Ub-like molecule BAG-1 (77). In prokaryotes, the misfolded protein PhoA61 interacts with the chaperone DnaK and the protease La (*Lon*), and furthermore, the amount of PhoA61-DnaK complexes correlates with the degree of PhoA61 degradation (107). However, such observations are strictly correlative, and cannot make an effective argument for a functional connection between chaperone activity and degradation.

More direct experiments involve ansamycins, small molecule inhibitors of Hsp90. Ansamycins stabilize the interaction of Hsp90 with misfolded substrates (101). When ansamycin-stabilized Hsp90-substrate complexes were isolated and added back to degradation competent lysate, the substrate was degraded rapidly, in comparison to unmodified Hsp90-substrate complexes (101). This suggests a model in which proteins that are difficult to refold, and therefore spend a long time bound to Hsp90, are somehow transferred directly to the degradation machinery. However, there is currently no evidence that refoldable and non-refoldable proteins bind to chaperones differently (40). Additionally, it is possible that the misfolded protein occasionally assumes a non-degradable

conformation when released from Hsp90. In this case, the fewer cycles of binding and release the substrate experiences, the more likely it would be to remain competent for degradation.

In summary, it seems possible that chaperone proteins, either those of the RP cap or traditional chaperones, recognize misfolded proteins and either refold them or feed them into the proteasome in a stochastically determined process. However, since the misfolded proteins examined so far are degraded in a Ub-dependent manner, it is more likely that a kinetic competition between chaperone binding and E3 recognition dictates the fate of the substrate (131). Chaperones may indirectly cooperate with E3s by maintaining substrates in a soluble, degradation competent conformation (63), and perhaps directly by presenting them to an E3 in some cases, as the results with Hsp90 suggest (101). Substrates carrying an N-terminal degradation signal termed the N-degron become resistant to degradation 20 minutes after their synthesis, indicating that either they slowly adopt a soluble conformation that is resistant to degradation, or their N-terminal degradation signal is cleaved off by a peptidase (114). Chapter 5 describes a preliminary investigation into the role of Hsp70 chaperones in the recognition of an N-degron, suggesting that Hsp70 proteins present substrates to the E3 of the N-end rule pathway.

Interestingly, when nascent polypeptides that are in the process of being synthesized are prematurely released from the ribosome by treatment with the polypeptide chain-terminating drug puromycin, the released fragments are rapidly degraded in a Ub-dependent manner (47). This suggests that polypeptides that are in the process of folding display unfolded regions similar to the degradation signals recognized in mature misfolded proteins. Chapter 2

describes a series of experiments which demonstrate that a nascent polypeptide carrying a degradation signal does in fact experience a kinetic competition between folding and degradation while being synthesized by the ribosome. Recent work suggests that the immune system exploits this inherent inefficiency in protein biogenesis (94, 102). Cotranslational degradation may generate peptides from post-translationally stable viral proteins, which are then loaded onto MHC class I molecules for presentation to T cells (44). This process ensures that metazoans can always mount an immune response to virally infected cells, regardless of the posttranslational half-life of the viral proteins.

Concentration Control

Regulated Degradation Signals

Many regulatory proteins that are components of biochemical switches or oscillators are degraded by the Ub-proteasome system. These proteins carry specific degradation signals comprising a sequence or structural determinant that is directly recognized by an E3, and a lysine residue(s) that is the site of multiUb chain attachment (3, 65).

A priori, there is no reason why proteins like transcription factors, cyclins, and cyclin-dependent kinase inhibitors are not recognized in the same way as misfolded proteins – their degradation signals could really be unfolding signals. In fact, degradation of the α2 transcription factor is controlled by a degradation signal that is an amphipathic α-helix (62), suggesting that regulated degradation signals are not necessarily different from those in misfolded proteins.

Mat α2p

The yeast mating type regulator α2 is short-lived in haploid cells (51). This ensures that when a *MATα* mother cell switches mating type, shutting off *MATα2* transcription, the preexisting α2 protein rapidly decays, enabling *MAT**a*** specific genes to be expressed. The **a**1 transcription factor is also short-lived in *MAT**a*** cells (62). However, in α/a diploids, α2 and **a**1 form a heterodimer that represses both sets of haploid specific genes. The interaction of α2 and **a**1 within the heterodimer metabolically stabilizes both proteins (62). Yeast spend most of their natural life cycle in the diploid state, continuously repressing haploid-specific genes, so this stabilization avoids needless degradation.

The α2 degradation signal maps to a hydrophobic patch on one side of a leucine zipper involved in the α2 – **a**1 interaction (62). Mutations in the hydrophobic face of this amphipathic α-helix stabilize α2 in haploids, and simultaneously interfere with its ability to interact with and stabilize **a**1 in diploids. Thus the physical association of α2 and **a**1 regulates their degradation. In haploid *MATα* cells, which do not express **a**1, this region of α2 is free to act as a degradation signal. Consequently, when a *MATα* cell switches mating type, α2 decays rapidly, releasing **a**-specific genes from repression. In a diploid, α2 – **a**1 heterodimerization buries this hydrophobic patch, and both α2 and **a**1 are stable. This meets the need for steady α2 – **a**1 mediated repression of haploid-specific genes in diploids.

Many other protein interaction domains may act as hydrophobic degradation signals when the interacting partner is absent. Sometimes, as with

the degradation of an artificially over-produced subunit of the ribosome, this process is labeled protein quality control (27). However, regulating the abundance or binding competence of proteins that interact with such a domain thereby regulates degradation of the target protein. Thus, quality control and concentration control may be mechanistically identical in some cases; the difference is in the function the degradation serves. Attempts to map the degradation signal of the yeast HMG-CoA reductase-2 further illustrate this point.

HMG-CoA reductase-2

HMG-CoA reductase-2 (Hmg2) is a key enzyme in the sterol biosynthesis pathway, and is subject to multiple forms of feedback regulation by the metabolites of this pathway (43). Hmg2 resides in the endoplasmic reticulum (ER) membrane, and when the end-products of sterol biosynthesis accumulate, they specifically induce retrotranslocation of Hmg2 out of the ER, into the cytosol, where it is degraded by the Ub-proteasome system (42).

In an attempt to locate sequences required for Hmg2 degradation, large fragments of Hmg2 were replaced with the corresponding regions of the metabolically stable Hmg1 isozyme (33, 34). This analysis showed that two specific lysines at positions 6 and 357 are essential for Hmg2 degradation. However numerous regions of the transmembrane domain are also important for properly regulated degradation. This observation cannot be explained by a model in which the transmembrane segments each function as independent hydrophobic patch degradation signals, because transplanting of one of these regions from Hmg2 to Hmg1 does not destabilize Hmg1 (33). The folding of the

entire transmembrane domain may bring together multiple parts of Hmg2 to form a composite surface that acts as a degradation signal. This would account for the observation that transplanting a very large region of Hmg2 is required to destabilize Hmg1 (33). It remains to be determined whether Hmg2 folding forms a composite signal, possibly bringing together Lys 6 and Lys 357, or if a single region of the protein is merely easily perturbed by changes in the transmembrane domain. Isolation of the E3 responsible for Hmg2 degradation would help distinguish these possibilities.

In either case, it appears likely that global folding of Hmg2 is intimately linked to the presentation of its degradation signal. Therefore, the end-products of sterol biosynthesis could regulate Hmg2 degradation by simply inducing it to misfold. Thus, Hmg2 may be a specific example of a protein carrying a degradation signal that is really an unfolding signal.

The N-degron

An N-degron is a degradation signal that consists of a destabilizing N–terminal residue and an accessible lysine that serves as a site of multiUb chain attachment (2, 3). Ubr1, the E3 of the N-end rule pathway, directly recognizes these residues (Figure 1.7) (8). A complex of Ubr1 and the E2 Rad6 multiubiquitinates the substrate on an accessible lysine (22, 79).

The N-end rule pathway was discovered through the use of artificial reporter proteins (2). Due to the constraints of the genetic code, protein synthesis initiates with methionine, a stabilizing residue. The specificity of methionine amino peptidase, which removes the N-terminal methionine, is such that it does not expose a destabilizing N-terminal residue. To study the N-end rule pathway,

standard cytoplasmic N-terminal processing is bypassed by synthesizing a reporter protein as a linear fusion protein with an N-terminal Ub moiety, i.e., Ub-X-reporter, where X is any amino acid residue desired. Ub specific proteases (Ubps) cleave such linear fusions after the C-terminal residue of Ub, creating a new, engineered, N-terminal residue of choice (X) on the reporter protein.

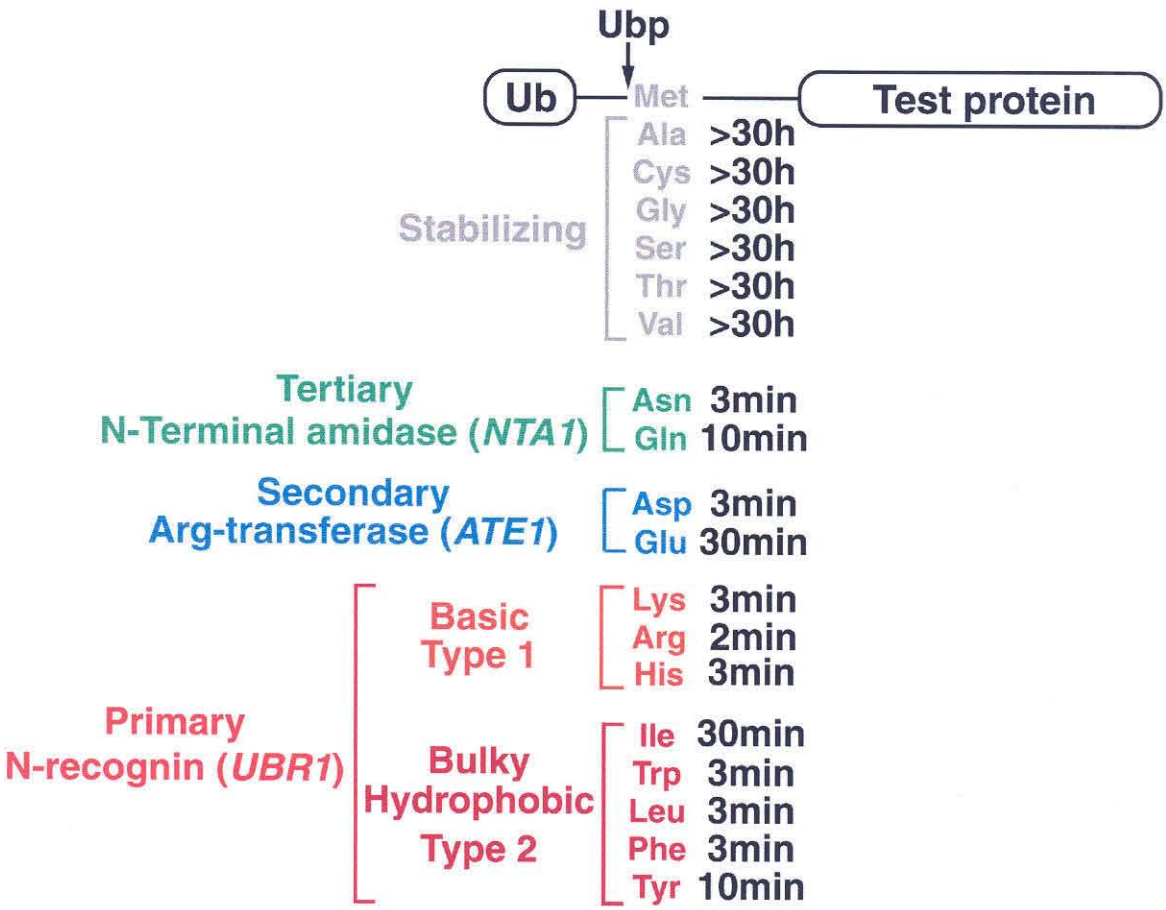


Figure 1.7) The N-end rule pathway in *S. cerevisiae*. Reporter proteins bearing any residue of choice (except proline) are generated by Ubp cleavage of linear Ub fusion proteins. Ubr1, the E3 of the N-end rule pathway, does not recognize stabilizing residues. Destabilizing residues fall into three general classes. Tertiary destabilizing residues are converted into secondary destabilizing residues by a deamidation reaction catalyzed by Nta1 (N-terminal amidase).

Secondary residues receive an N-terminal Arg residue, in a reaction catalyzed by Ate1 (Arg-transferase). Primary destabilizing residues (including Arg) are directly recognized by Ubr1. Dipeptide inhibition studies have defined two distinct N-terminal residue binding sites on Ubr1, one for basic residues (type 1) and one for bulky hydrophobic residues (type 2).

Thus, some endoprotease or peptidase is required to expose destabilizing residues under physiological circumstances. For example, Sindbis virus RNA polymerase nsP4 is synthesized as a polyprotein precursor, which is autocatalytically cleaved to expose a destabilizing residue (17). Another physiological substrate, the p60 protein of the intracellular bacterial parasite *Listeria monocytogenes*, is short-lived in the cytosol of a host cell that *Listeria* penetrates. p60 acquires a destabilizing N-terminal residue through cleavage by signal peptidase during its export from *Listeria* (109). On one level, the degradation of nsP4 and p60 is quality control, because the host cell is removing a foreign protein from its cytoplasm. However, it is also more than just quality control, because the degradation of p60 (and probably also nsP4) generates peptides for presentation to the immune system on MHC class I molecules (109).

Scc1, a subunit of the sister chromatid cohesion complex (cohesin), is cleaved during anaphase to permit separation of the chromatids and their subsequent segregation to opposite poles of the mitotic spindle (118). This cleavage exposes an N-degron on one of the fragments of Scc1, targeting it for degradation by the N-end rule pathway (Hai Rao, personal communication), thereby removing pieces of Scc1 that could interfere with sister chromatid cohesion on the next round of the cell cycle.

Thus, the N-end rule pathway performs both quality control and regulatory functions. The N-degron, and the other degradation signals described above, all constitute a structural feature that would also present on damaged or miscompartmentalized proteins. However, a protein need only be recognized by an E3 to be degraded, and there are many potential protein-protein recognition paradigms.

Phosphorylation plays a key role in the regulation of many protein degradation signals. It is possible that in some cases phosphorylation regulates protein folding, and that there is mechanistic overlap with the protein quality control pathways. However, IκBα and β–catenin (described below) carry degradation signals that can be specifically recognized as phosphorylated peptides of ten residues. Such a small peptide is unlikely to possess significant structure on its own. Moreover, these peptides are extremely hydrophilic. Thus, although phosphorylation of these signals may influence folding of the substrate, the exposure of hydrophobic regions of these target proteins is not required for E3 recognition.

IκBα and β–catenin

IκBα forms a complex with the transcription factor NFκB, a key regulator of the inflammatory and immune responses. Under normal conditions, interaction with IκBα shields the nuclear localization signal on NFκB, sequestering NFκB in a complex in the cytoplasm. Inflammation initiates a signal transduction cascade that activates IκBα degradation, freeing NFκB to travel to the nucleus and elicit the appropriate changes in gene expression.

This signal transduction cascade phosphorylates IκBα on two residues, serines 32 and 36 (21) (129). IκBα is ubiquitinated on either lysine 21 or 22 (5), strongly suggesting that the IκBα degradation signal is contained within a small N-terminal region. These observations were used to derive a ten residue peptide (DRH**DSGLDS**M), that acts as a competitive inhibitor of IκBα degradation *in vitro*, but only when appropriately phosphorylated on the two serine residues (136).

A very similar sequence (**DSGIHS**) is found near the N-terminus of β–catenin, a component of the Wingless/Wnt signal transduction pathway (80). In fact, using the IκBα-derived phosphopeptide as a ligand for affinity chromatography identified an E3 that also recognizes β–catenin (132) (112, 137). The phosphopeptide is directly recognized by β–TrCP, an F-box protein. F-box proteins are the substrate-recognition components of the multisubunit SCF family of E3s (4, 110) (24).

The small size of the recognition signals in IκBα and β–catenin raises some important questions (74). How can these proteins be specifically recognized by such a small, apparently unstructured signal? The answer may be that the degradation signal is also negatively regulated. Unphosphorylated IκBα is conjugated to a Ub-like molecule (Ubl, SUMO-1 in this case) at the same lysine to which $SCF^{\beta TrCP}$ attaches a multiUb chain (19). Ubls are conjugated to proteins using homologous machinery to the E1, E2, E3 cascade for Ub conjugation (58, 61). Presumably Ubl modification of IκBα simply blocks any possible multiUb chain attachment, preventing inappropriate degradation of unphosphorylated IκBα.

The hydrophilic nature of the IκBα and β–catenin degradation signals sharply contrasts with the hydrophobic character of protein quality control degradation signals. This may reflect a need to recognize certain proteins when they are part of a multisubunit complex, as part of complex remodeling. Such targets would have to carry surface-exposed hydrophilic degradation signals. IκBα and β–catenin are both components of multisubunit complexes. IκBα is recognized while bound to NFκB, and in fact the purpose of degrading IκBα is to release NFκB from this interaction. The CDK inhibitor Sic1 is another example of a protein that must be degraded while part of a multisubunit complex.

Sic1p

Sic1 binds to and inhibits the S-phase promoting CDK Clb5-Cdc28. As cells pass from G1 into S-phase, G1 CDK Cln-Cdc28 phosphorylates Sic1, triggering its degradation, releasing Clb5-Cdc28 to drive the cell into S-phase (24, 103, 122).

Mass spectrometry analysis shows that Sic1 is phosphorylated on at least six serine/threonine residues *in vivo* (122). Point mutagenesis shows that these phosphorylated residues act additively to target the protein for ubiquitination *in vitro*. Single mutants have very modest defects in ubiquitination, double mutants show more pronounced effects, and ubiquitination is essentially abolished in triple mutants. Although the number of phosphorylated residues is important, the identities of the residues have little influence on the extent of ubiquitination.

This result suggests that charge plays an important role in E3 recognition of Sic1, while specific structural determinants are less important. Interestingly, the acidic activation domains of many transcription factors also act as degradation signals (83, 97). Acidic activation domains have little structure in isolation, but they may assume specific conformations upon interaction with components of the basal transcription machinery. The phosphorylation based degradation signal of Sic1 and the activation domain degron may be closely analogous. Both may be initially recognized based on their negative charge, with the specificity of recognition refined during interaction with the E3.

Summary

The Ub-proteasome system utilizes the energy of ATP hydrolysis to degrade proteins specifically. The E1 component of the E1, E2, E3 cascade hydrolyzes ATP to activate Ub for transfer to target proteins in the form of a multiUb chain. At least in some cases, a multiUb chain is a prerequisite for target degradation. The E3s of the Ub system recognize target proteins via the degradation signals they carry. The sequence diversity of E3s may represent an underlying variety of mechanisms of multiUb chain formation. HECT domain E3s directly participate in the cascade of Ub-thioester transfers from E1 to E2 to E3 to substrate. One HECT domain protein (Ufd4) is only capable of attaching mono or di-ubiquitin to the substrate, and requires an additional factor (E4; Ufd2) to synthesize longer multiUb chains. SCF and APC are modular multisubunit E3s, with one subunit, the F-box protein of SCF or the WD-repeat proteins of APC, directly recognizing the substrate. SCF complexes do not contain an active site cysteine for Ub-thioesterification. Instead, the cullin and

RING box subunits of SCF are proposed to stimulate an associated E2 to synthesize a multiUb chain on the substrate. The Ubr1 family of E3s shares features of both HECT domain and SCF/APC E3s.

Multiubiquitinated proteins are degraded by the proteasome. The proteasome is a self-compartmentalized protease; its active sites are contained within its hollow cylindrical core particle. The regulatory particle of the proteasome sits at either end of this core cylinder and uses the energy of ATP hydrolysis to regulate the opening of a channel to the proteolytic sites in the core, and to unfold target proteins and thread them into this channel.

The targets of the Ub-proteasome system are typically components of biochemical switches and oscillators. In many cases, the destruction of such proteins is regulated by modulating their degradation signals. This can be accomplished in a variety of ways:

i) **Phosphorylation**. Proteins that are degraded as part of multisubunit complexes typically carry hydrophilic, phosphorylation-regulated degradation signals. However, the small size of these degradation signals, and their electrostatic nature make specificity of recognition hard to attain. In one case, specificity is refined by modifying the unphosphorylated target with a Ub-like molecule (Ubl) at the same site as multiUb chain attachment, thereby blocking accidental ubiquitination of an unphosphorylated target (19).

ii) **Protein-protein interactions** Some proteins are stable when part of a multisubunit complex, but rapidly degraded as isolated subunits. These proteins carry hydrophobic degradation signals that overlap with a protein-protein interaction domain (62). Although the target is rapidly degraded when free, complex formation buries its degradation signal, stabilizing the protein.

Controlling the activity of the ubiquitination machinery also regulates degradation. As E3s are the specificity determinants for ubiquitination, they are the most common targets of regulation. The following mechanisms regulate the activity and/or specificity of E3s:

i) **Degradation**. Both SCF and APC E3s are large multisubunit complexes with specific subunits that recognize substrates: the F-box components of SCF and the WD40-repeat proteins (Cdc20 and Cdh1) of APC. These substrate-recognizing components are degraded, possibly by autoubiquitination events, which enables new substrate-selecting modules to associate with the catalytic cores of these E3s.

ii) **Phosphorylation**. A complex network of phosphorylation regulates APC both positively and negatively. Phosphorylation regulates both the catalytic activity of APC, and the association of the substrate-recognition components (Cdc20 and Cdh1).

iii) **Ubl modification**

The cullin component of SCF is modified by Ubl attachment. This modification is required for efficient ubiquitination of IκBα by $SCF^{\beta TrCP}$, and $p27^{Kip1}$ by SCF^{Skp2}; however, the mechanism of this activation is not currently understood (92, 93).

Thesis Overview

The ability of the Ub-proteasome system to selectively target misfolded proteins raises the question of what happens to proteins before they fold, as they

are being synthesized by the ribosome. Chapter 2 demonstrates that nascent proteins are in fact susceptible to degradation. I discuss some preliminary work, and propose some experiments, which address the coordination of protein folding and degradation in Chapter 5. In the Appendix, I describe the identification of a target of the Ubr1 pathway in *S. cerevisiae*, namely Cup9, a repressor of the dipeptide transporter Ptr2. In Chapters 3 and 4, I discuss how the Ubr1-dependent degradation of this target is regulated. In Chapter 3, I show that Ubr1 acts as an allosteric sensor of dipeptide concentrations, coupling expression of the dipeptide transporter (Ptr2) to the availability of dipeptides by regulating degradation of the Cup9 repressor. In Chapter 4, I show that certain amino acids induce Ptr2 expression by regulating Ubr1-dependent degradation of Cup9 through a signal transduction pathway containing novel components. Chapter 5 describes proposed experiments designed to investigate the regulation of Ubr1 more closely.

REFERENCES

1. **Alexandru, G., W. Zachariae, A. Schleiffer, and K. Nasmyth.** 1999. Sister chromatid separation and chromosome re-duplication are regulated by different mechanisms in response to spindle damage. EMBO J. **18:**2707-2721.

2. **Bachmair, A., D. Finley, and A. Varshavsky.** 1986. *In vivo* half-life of a protein is a function of its amino-terminal residue. Science **234:**179-186.

3. **Bachmair, A., and A. Varshavsky.** 1989. The degradation signal in a short-lived protein. Cell **56:**1019-1032.

4. **Bai, C., P. Sen, K. Hofman, L. Ma, M. Goebl, J. W. Harper, and S. J. Elledge.** 1996. *SKP1* connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. Cell **86:**263-274.

5. **Baldi, L., K. Brown, G. Franzoso, and U. Siebenlist.** 1996. Critical role for lysines 21 and 22 in signal-induced, ubiquitin-mediated proteolysis of IκBα. J. Biol. Chem. **271:**376-379.

6. **Banerjee, A., L. Gregori, Y. P. Xu, and V. Chau.** 1993. The bacterially expressed yeast Cdc34 gene-product can undergo autoubiquitination to form a multiubiquitin chain-linked protein. J. Biol. Chem. **268:**5668-5675.

7. **Barkai, N., and S. Leibler.** 2000. Biological rhythms - circadian clocks limited by noise. Nature **403:**267-268.

8. **Bartel, B., I. Wünning, and A. Varshavsky.** 1990. The recognition component of the N-end rule pathway. EMBO J. **9:**3179-3189.

9. **Baumeister, W., J. Walz, F. Zühl, and E. Seemüller.** 1998. The proteasome: paradigm of a self-compartmentalizing protease. Cell **92:**367-380.

10. **Beaudenon, S. L., M. R. Huacani, G. L. Wang, D. P. McDonnell, and J. M. Huibregtse.** 1999. Rsp5 ubiquitin-protein ligase mediates DNA damage-

induced degradation of the large subunit of RNA polymerase II in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **19:**6972-6979.

11. **Beuron, F., M. R. Maurizi, D. M. Belnap, E. Kocsis, F. P. Booy, M. Kessel, and A. C. Steven.** 1998. At sixes and sevens: characterization of the symmetry mismatch of the ClpAP chaperone-assisted protease. J. Struct. Biol. **123:**248-259.

12. **Braun, B. C., M. Glickman, R. Kraft, B. Dahlmann, P. M. Kloetzel, D. Finley, and M. Schmidt.** 1999. The base of the proteasome regulatory particle exhibits chaperone-like activity. Nat. Cell Biol. **1:**221-226.

13. **Charles, J. F., S. L. Jespersen, R. L. Tinker-Kulberg, L. Hwang, A. Szidon, and D. O. Morgan.** 1998. The Polo-related kinase Cdc5 activates and is destroyed by the mitotic cyclin destruction machinery in *S. cerevisiae*. Curr. Biol. **8:**497-507.

14. **Chau, V., J. W. Tobias, A. Bachmair, D. Marriott, D. J. Ecker, D. K. Gonda, and A. Varshavsky.** 1989. A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. Science **243:**1576-1583.

15. **Ciechanover, A., D. Finley, and A. Varshavsky.** 1984. Ubiquitin dependence of selective protein degradation demonstrated in the mammalian cell cycle mutant ts85. Cell **37:**57-66.

16. **Cook, W. J., L. C. Jeffrey, E. Kasperek, and C. M. Pickart.** 1994. Structure of tetraubiquitin shows how multiubiquitin chains can be formed. J. Mol. Biol. **236:**601-609.

17. **deGroot, R. J., T. Rümenapf, R. J. Kuhn, and J. H. Strauss.** 1991. Sindbis virus RNA polymerase is degraded by the N-end rule pathway. Proc. Natl. Acad. Sci. USA **88:**8967-8971.

18. **DeMartino, G. N., and C. A. Slaughter.** 1999. The proteasome, a novel protease regulated by multiple mechanisms. J. Biol. Chem. **274:**22123-22126.

19. **Desterro, J. M. P., M. S. Rodriguez, and R. T. Hay.** 1998. SUMO-1 modification of IκBα inhibits NF-κB activation. Mol. Cell **2:**233-239.

20. **Deveraux, Q., R. Wells, and M. Rechsteiner.** 1990. A 26S protease subunit that binds ubiquitin conjugates. J. Biol. Chem. **269:**7059-7061.

21. **DiDonato, J., F. Mercurio, C. Rosette, J. WuLi, H. Suyang, S. Ghosh, and M. Karin.** 1996. Mapping of the inducible IκBα phosphorylation sites that signal its ubiquitination and degradation. Mol. Cell. Biol. **16:**1295-1304.

22. **Dohmen, R. J., K. Madura, B. Bartel, and A. Varshavsky.** 1991. The N-end rule is mediated by the UBC2(RAD6) ubiquitin-conjugating enzyme. Proc. Natl. Acad. Sci. USA **88:**7351-5.

23. **Elowitz, M. B., and S. Leibler.** 2000. A synthetic oscillatory network of transcriptional regulators. Nature **403:**335-338.

24. **Feldman, R. M. R., C. C. Correll, K. B. Kaplan, and R. J. Deshaies.** 1997. A complex of Cdc4p, Skp1p, and Cdc53p/cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sic1p. Cell **91:**221-230.

25. **Fenteany, G., R. F. Standaert, W. S. Lane, S. Choi, E. J. Corey, and S. L. Schreiber.** 1995. Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. Science **268:**726-731.

26. **Finley, D., B. Bartel, and A. Varshavsky.** 1989. The tails of ubiquitin precursors are ribosomal proteins whose fusion to ubiquitin facilitates ribosome biogenesis. Nature **338:**394-401.

27. **Finley, D., and V. Chau.** 1991. Ubiquitination. Annu. Rev. Cell Biol. **7:**25-69.

28. **Fruh, K., M. Gossen, K. N. Wang, H. Bujard, P. A. Peterson, and Y. Yang.** 1994. Displacement of housekeeping proteasome subunits By MHC-Encoded LMPs - a newly discovered mechanism for modulating the multicatalytic proteinase complex. EMBO J. **13:**3236-3244.

29. **Gaczynska, M., A. L. Goldberg, K. Tanaka, K. B. Hendil, and K. L. Rock.** 1996. Proteasome subunits X and Y alter peptidase activities in opposite ways to the γ-interferon-induced subunits LMP2 and LMP7. J. Biol. Chem. **271:**17275-17280.

30. **Gaczynska, M., K. L. Rock, and A. L. Goldberg.** 1993. γ-Interferon and expression of MHC genes regulate peptide hydrolysis by proteasomes. Nature **365:**264-267.

31. **Galan, J. M., and R. HaguenauerTsapis.** 1997. Ubiquitin Lys63 is involved in ubiquitination of a yeast plasma membrane protein. EMBO J. **16:**5847-5854.

32. **Galan, J. M., V. Moreau, B. Andre, C. Volland, and R. Haguenauer-Tsapis.** 1996. Ubiquitination mediated by the Npi1p/Rsp5b ubiquitin-protein ligase is required for endocytosis of the yeast uracil permease. J. Biol. Chem. **271:**10946-10952.

33. **Gardner, R., S. Cronin, B. Leder, J. Rine, and R. Hampton.** 1998. Sequence determinants for regulated degradation of yeast 3-hydroxy-3-methylglutaryl-CoA reductase, an integral endoplasmic reticulum membrane protein. Mol. Biol. Cell **9:**2611-2626.

34. **Gardner, R. G., and R. Y. Hampton.** 1999. A 'distributed degron' allows regulated entry into the ER degradation pathway. EMBO J. **18:**5994-6004.

35. **Gilon, T., O. Chomsky, and R. G. Kulka.** 1998. Degradation signals for ubiquitin system proteolysis in *Saccharomyces cerevisiae*. EMBO J. **17:**2759-2766.

36. **Glickman, M. H., D. M. Rubin, O. Coux, I. Wefes, G. Pfeifer, Z. Cjeka, W. Baumeister, V. A. Fried, and D. Finley.** 1998. A subcomplex of the proteasome regulatory particle required for ubiquitin-conjugate degradation and related to the COP9-signalosome and eIF3. Cell **94:**615-623.

37. **Glickman, M. H., D. M. Rubin, V. A. Fried, and D. Finley.** 1998. The regulatory particle of the *Saccharomyces cerevisiae* proteasome. Mol. Cell. Biol. **18:**3149-62.

38. **Glotzer, M., A. W. Murray, and M. W. Kirschner.** 1991. Cyclin is degraded by the ubiquitin pathway. Nature **349:**132-138.

39. **Glover, D. M., I. M. Hagan, and A. A. M. Tavares.** 1998. Polo-like kinases: a team that plays throughout mitosis. Genes Dev. **12:**3777-3787.

40. **Gottesman, S., S. Wickner, and M. R. Maurizi.** 1997. Protein quality control: triage by chaperones and proteases. Genes Dev. **11:**815-823.

41. **Groll, M., L. Ditzel, J. Löwe, D. Stock, M. Bochtler, H. D. Bartunik, and R. Huber.** 1997. Structure of 20S proteasome from yeast at 2.4 Å resolution. Nature **386:**463-471.

42. **Hampton, R. Y., R. G. Gardner, and J. Rine.** 1996. Role of 26S proteasome and HRD genes in the degradation of 3-hydroxy-3-methylglutaryl-CoA reductase, an integral endoplasmic reticulum membrane protein. Mol. Biol. Cell **7:**2029-2044.

43. **Hampton, R. Y., and J. Rine.** 1994. Regulated degradation of Hmg-CoA reductase, an integral membrane-protein of the endoplasmic reticulum, in yeast. J. Cell Biol. **125:**299-312.

44. **Heemels, M. T., and H. Ploegh.** 1995. Generation, translocation, and presentation of MHC class I-restricted peptides. Ann. Rev. Biochem. **64:**463-491.

45. **Hershko, A., and A. Ciechanover.** 1998. The ubiquitin system. Annu. Rev. Biochem. **76:**425-479.

46. **Hershko, A., A. Ciechanover, and I. A. Rose.** 1979. Resolution of the ATP-dependent proteolytic system from reticulocytes: a component that interacts with ATP. Proc. Natl. Acad. Sci. USA **76:**3107-10.

47. **Hershko, A., E. Eytan, A. Ciechanover, and A. L. Haas.** 1982. Immunochemical analysis of the turnover of ubiquitin-protein conjugates in intact cells. Relationship to the breakdown of abnormal proteins. J. Biol. Chem. **257:**13964-70.

48. **Hershko, A., H. Heller, S. Elias, and A. Ciechanover.** 1983. Components of ubiquitin-protein ligase system. Resolution, affinity purification, and role in protein breakdown. J. Biol. Chem. **258:**8206-14.

49. **Hicke, L.** 1997. Ubiquitin-dependent internalization and down-regulation of plasma membrane proteins. FASEB J. **11:**1215-1226.

50. **Hicke, L., and H. Riezman.** 1996. Ubiquitination of a yeast plasma membrane receptor signals its ligand-stimulated endocytosis. Cell **84:**277-287.

51. **Hochstrasser, M., and A. Varshavsky.** 1990. *In vivo* degradation of a transcriptional regulator: the yeast Matα2 repressor. Cell **61:**697-708.

52. **Hofmann, R. M., and C. M. Pickart.** 1999. Noncanonical MMS2-encoded ubiquitin-conjugating enzyme functions in assembly of novel polyubiquitin chains for DNA repair. Cell **96:**645-653.

53. **Hoskins, J. R., M. Pak, M. R. Maurizi, and S. Wickner.** 1998. The role of the ClpA chaperone in proteolysis by ClpAP. Proc. Natl. Acad. Sci. USA **95:**12135-12140.

54. **Huang, L., E. Kinnucan, G. L. Wang, S. Beaudenon, P. M. Howley, J. M. Huibregtse, and N. P. Pavletich.** 1999. Structure of an E6AP-UbcH7 complex: insights into ubiquitination by the E2-E3 enzyme cascade. Science **286:**1321-1326.

55. **Huibregtse, J. M., J. C. Yang, and S. L. Beaudenon.** 1997. The large subunit of RNA polymerase II is a substrate of the Rsp5 ubiquitin-protein ligase. Proc. Natl. Acad. Sci. USA **94:**3656-3661.

56. **Inoue, I., and M. Rechsteiner.** 1994. On the relationship between the metabolic and thermodynamic stabilities of T4 lysozymes - measurements in eukaryotic cells. J. Biol. Chem. **269:**29247-29251.

57. **Johnson, E. S., B. Bartel, W., and A. Varshavsky.** 1992. Ubiquitin as a degradation signal. EMBO J. **11:**497-505.

58. **Johnson, E. S., and G. Blobel.** 1997. Ubc9p is the conjugating enzyme for the ubiquitin-like protein Smt3p. J. Biol. Chem. **272:**26799-26802.

59. **Johnson, E. S., D. K. Gonda, and A. Varshavsky.** 1990. *Cis-trans* recognition and subunit-specific degradation of short-lived proteins. Nature **346:**287-291.

60. **Johnson, E. S., P. C. Ma, I. M. Ota, and A. Varshavsky.** 1995. A proteolytic pathway that recognizes ubiquitin as a degradation signal. J. Biol. Chem. **270:**17442-17456.

61. **Johnson, E. S., I. Schwienhorst, R. J. Dohmen, and G. Blobel.** 1997. The ubiquitin-like protein Smt3p is activated for conjugation to other proteins by an Aos1p/Uba2p heterodimer. EMBO J. **16:**5509-5519.

62. **Johnson, P. R., R. Swanson, L. Rakhilina, and M. Hochstrasser.** 1998. Degradation signal masking by heterodimerization of MATα2 and MATa1 blocks their mutual destruction by the ubiquitin-proteasome pathway. Cell **94:**217-27.

63. **Jubete, Y., M. R. Maurizi, and S. Gottesman.** 1996. Role of the heat shock protein DnaJ in the Lon-dependent degradation of naturally unstable proteins. J. Biol. Chem. **271:**30798-30803.

64. **Kessel, M., M. R. Maurizi, B. Kim, E. Kocsis, B. L. Trus, S. K. Singh, and A. C. Steven.** 1995. Homology in structural organization between *Escherichia coli* ClpAP protease and the eukaryotic 26S proteasome. J. Mol. Biol. **250:**587-594.

65. **King, R. W., M. Glotzer, and M. W. Kirschner.** 1996. Mutagenic analysis of the destruction signal of mitotic cyclins and structural characterization of ubiquitinated intermediates. Mol. Biol. Cell **7:**1343-1357.

66. **Kisselev, A. F., T. N. Akopian, V. Castillo, and A. L. Goldberg.** 1999. Proteasome active sites allosterically regulate each other, suggesting a cyclical bite-chew mechanism for protein breakdown. Mol. Cell **4:**395-402.

67. **Kisselev, A. F., T. N. Akopian, K. M. Woo, and A. L. Goldberg.** 1999. The sizes of peptides generated from protein by mammalian 26S and 20S proteasomes - implications for understanding the degradative mechanism and antigen presentation. J. Biol. Chem. **274:**3363-3371.

68. **Koegl, M., T. Hoppe, S. Schlenker, H. D. Ulrich, T. U. Mayer, and S. Jentsch.** 1999. A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly. Cell **96:**635-644.

69. **Kotani, S., H. Tanaka, H. Yasuda, and K. Todokoro.** 1999. Regulation of APC activity by phosphorylation and regulatory factors. J. Cell Biol. **146:**791-800.

70. **Kraulis, P. J.** 1991. MOLSCRIPT - a program to produce both detailed and schematic plots of protein structures. J. Appl. Cryst. **24:**946-950.

71. **Kumar, S., A. L. Talis, and P. M. Howley.** 1999. Identification of HHR23A as a substrate for E6-associated protein-mediated ubiquitination. J. Biol. Chem. **274:**18785-18792.

72. **Kwon, Y. T., Y. Reiss, V. A. Fried, A. Hershko, J. K. Yoon, D. K. Gonda, P. Sangan, N. G. Copeland, N. A. Jenkins, and A. Varshavsky.** 1998. The mouse and human genes encoding the recognition component of the N-end rule pathway. Proc. Natl. Acad. Sci. USA **95:**7898-7903.

73. **Lammer, D., N. Mathias, J. M. Laplaza, W. D. Jiang, Y. Liu, J. Callis, M. Goebl, and M. Estelle.** 1998. Modification of yeast Cdc53p by the ubiquitin-related protein Rub1p affects function of the SCF^{Cdc4} complex. Genes Dev. **12:**914-926.

74. **Laney, J. D., and M. Hochstrasser.** 1999. Substrate targeting in the ubiquitin system. Cell **97:**427-30.

75. **Liakopoulos, D., G. Doenges, K. Matuschewski, and S. Jentsch.** 1998. A novel protein modification pathway related to the ubiquitin system. EMBO J. **17:**2208-2214.

76. **Lowe, J., D. Stock, R. Jap, P. Zwickl, W. Baumeister, and R. Huber.** 1995. Crystal structure of the 20S proteasome from the archaeon *T. acidophilum* at 3.4Å resolution. Science **268:**533-539.

77. **Luders, J., J. Demand, and J. Hohfeld.** 2000. The ubiquitin-related BAG-1 provides a link between the molecular chaperones Hsc70/Hsp70 and the proteasome. J. Biol. Chem. **275:**4613-4617.

78. **Ma, C. P., J. H. Vu, R. J. Proske, C. A. Slaughter, and G. N. Demartino.** 1994. Identification, purification, and characterization of a high-molecular-weight, ATP-dependent activator (PA700) of the 20S proteasome. J. Biol. Chem. **269:**3539-3547.

79. **Madura, K., R. J. Dohmen, and A. Varshavsky.** 1993. N-recognin/Ubc2 interactions in the N-end rule pathway. J. Biol. Chem. **268:**12046-12054.

80. **Maniatis, T.** 1999. A ubiquitin ligase complex essential for the NF-κB, Wnt/Wingless, and Hedgehog signaling pathways. Genes Dev. **13:**505-510.

81. **Meimoun, A., T. Holtzman, Z. Weissman, H. J. McBride, D. J. Stillman, G. R. Fink, and D. Kornitzer.** 2000. Degradation of the transcription factor Gcn4 requires the kinase Pho85 and the SCF^{CDC4} ubiquitin-ligase complex. Mol. Biol. Cell **11:**915-927.

82. **Merritt, E. A., and M. E. P. Murphy.** 1994. Raster3D version 2.0 - a program for photorealistic molecular graphics. Acta Cryst. **D50:**869-873.

83. **Molinari, E., M. Gilman, and S. Natesan.** 1999. Proteasome-mediated degradation of transcriptional activators correlates with activation domain potency *in vivo*. EMBO J. **18:**6439-6447.

84. **Murakami, Y., S. Matsufuji, T. Kameji, S. Hayashi, K. Igarashi, T. Tamura, K. Tanaka, and A. Ichihara.** 1992. Ornithine decarboxylase is degraded by the 26S proteasome without ubiquitination. Nature **360:**597-599.

85. **Osaka, F., H. Kawasaki, N. Aida, M. Saeki, T. Chiba, S. Kawashima, K. Tanaka, and S. Kato.** 1998. A new NEDD8-ligating system for cullin-4A. Genes Dev. **12:**2263-2268.

86. **Pak, M., J. R. Hoskins, S. K. Singh, M. R. Maurizi, and S. Wickner.** 1999. Concurrent chaperone and protease activities of ClpAP and the requirement for the N-terminal ClpA ATP binding site for chaperone activity. J. Biol. Chem. **274:**19316-19322.

87. **Pak, M., and S. Wickner.** 1997. Mechanism of protein remodeling by ClpA chaperone. Proc. Natl. Acad. Sci. USA **94:**4901-4906.

88. **Papa, F., and M. Hochstrasser.** 1993. The yeast *DOA4* gene encodes a deubiquitinating enzyme related to a product of the human *tre-2* oncogene. Nature **366:**313-319.

89. **Papa, F. R., A. Y. Amerik, and M. Hochstrasser.** 1999. Interaction of the Doa4 deubiquitinating enzyme with the yeast 26S proteasome. Mol. Biol. Cell **10:**741-56.

90. **Pfleger, C. M., and M. W. Kirschner.** 2000. The KEN box: an APC recognition signal distinct from the D box targeted by Cdh1. Genes Dev. **14:**655-665.

91. **Piotrowski, J., R. Beal, L. Hoffman, K. D. Wilkinson, R. E. Cohen, and C. M. Pickart.** 1997. Inhibition of the 26S proteasome by polyubiquitin chains synthesized to have defined lengths. J. Biol. Chem. **272:**23712-23721.

92. **Podust, V. N., J. E. Brownell, T. B. Gladysheva, R.-S. Luo, C. Wang, M. B. Coggins, J. W. Pierce, E. S. Lightcap, and V. Chau.** 2000. A Nedd8 conjugation pathway is essential for proteolytic targeting of $p27^{Kip1}$ by ubiquitination. Proc. Natl. Acad. Sci. USA **97:**4579-4584.

93. **Read, M. A., J. E. Brownell, T. B. Gladysheva, M. Hottelet, L. A. Parent, M. B. Coggins, J. W. Pierce, V. N. Podust, R. S. Luo, V. Chau, and V. J. Palombella.** 2000. Nedd8 modification of Cul-1 activates $SCF^{\beta TrCP}$-dependent ubiquitination of IκBα. Mol. Cell. Biol. **20:**2326-2333.

94. **Reits, E. A. J., J. C. Vos, M. Gromme, and J. Neefjes.** 2000. The major substrates for TAP *in vivo* are derived from newly synthesized proteins. Nature **404:**774-778.

95. **Russell, S. J., S. H. Reed, W. Y. Huang, E. C. Friedberg, and S. A. Johnston.** 1999. The 19S regulatory complex of the proteasome functions independently of proteolysis in nucleotide excision repair. Mol. Cell **3:**687-695.

96. **Sadis, S., C. Atienza, and D. Finley.** 1995. Synthetic signals for ubiquitin-dependent proteolysis. Mol. Cell. Biol. **15:**4086-94.

97. **Salghetti, S. E., M. Muratani, H. Wijnen, B. Futcher, and W. P. Tansey.** 2000. Functional overlap of sequences that activate transcription and signal ubiquitin-mediated proteolysis. Proc. Natl. Acad. Sci. USA **97:**3118-3123.

98. **Schauber, C., L. Chen, P. Tongaonkar, I. Vega, D. Lambertson, W. Potts, and K. Madura.** 1998. Rad23 links DNA repair to the ubiquitin/proteasome pathway. Nature **391:**715-718.

99. **Scheffner, M., U. Nuber, and J. M. Huibregtse.** 1995. Protein ubiquitination involving an E1-E2-E3 enzyme ubiquitin thioester cascade. Nature **373:**81-83.

100. **Scheffner, M., B. A. Werness, J. M. Huibregtse, A. J. Levine, and P. M. Howley.** 1990. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell **63:**1129-1136.

101. **Schneider, C., L. Sepp-Lorenzino, E. Nimmesgern, O. Ouerfelli, S. Danishefsky, N. Rosen, and F. U. Hartl.** 1996. Pharmacologic shifting of a balance between protein refolding and degradation mediated by Hsp90. Proc. Natl. Acad. Sci. USA **93:**14536-14541.

102. **Schuberg, U., L. C. Anton, J. Gibbs, C. C. Norbury, J. W. Yewdell, and J. R. Bennink.** 2000. Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. Nature **404:**770-774.

103. **Schwob, E., T. Bohm, M. D. Mendenhall, and K. Nasmyth.** 1994. The B-type cyclin kinase inhibitor p40 (Sic1) controls the G1 to S transition in *Saccharomyces cerevisiae*. Cell **79:**233-244.

104. **Seemüller, E., A. Lupas, D. Stock, J. Lowe, R. Huber, and W. Baumeister.** 1995. Proteasome from *Thermoplasma acidophilum* - a threonine protease. Science **268:**579-582.

105. **Seol, J. H., R. M. Feldman, W. Zachariae, A. Shevchenko, C. C. Correll, S. Lyapina, Y. Chi, M. Galova, J. Glaypool, S. Sanmeyer, K. Nasmyth, and R. J. Deshaies.** 1999. Cdc53/cullin and the essential Hrt1 RING-H2 subunit of SCF define a ubiquitin ligase module that activates the E2 enzyme Cdc34. Genes Dev. **13:**1614-1626.

106. **Sheaff, R. J., J. D. Singer, J. Swanger, M. Smitherman, J. M. Roberts, and B. E. Clurman.** 2000. Proteasomal turnover of p21(Cip1) does not require p21(Cip1) ubiquitination. Mol. Cell **5:**403-410.

107. **Sherman, M. Y., and A. L. Goldberg.** 1992. Involvement of the chaperonin DnaK in the rapid degradation of a mutant protein in *Escherichia coli*. EMBO J. **11:**71-77.

108. **Shou, W. Y., J. H. Seol, A. Shevchenko, C. Baskerville, D. Moazed, Z. W. S. Chen, J. Jang, H. Charbonneau, and R. J. Deshaies.** 1999. Exit from mitosis is triggered by Tem1-dependent release of the protein phosphatase Cdc14 from nucleolar RENT complex. Cell **97:**233-244.

109. **Sijts, A. J., I. Pilip, and E. G. Pamer.** 1997. The *Listeria monocytogenes*-secreted p60 protein is an N-end rule substrate in the cytosol of infected cells. Implications for major histocompatibility complex class I antigen processing of bacterial proteins. J. Biol. Chem. **272:**19261-19268.

110. **Skowyra, D., K. L. Craig, M. Tyers, S. J. Elledge, and J. W. Harper.** 1997. F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. Cell **91:**209-219.

111. **Skowyra, D., D. M. Koepp, T. Kamura, M. N. Conrad, R. C. Conaway, J. W. Conaway, S. J. Elledge, and J. W. Harper.** 1999. Reconstitution of G1 cyclin ubiquitination with complexes containing SCF^{Grr1} and Rbx1. Science **284:**662-5.

112. **Spencer, E., J. Jiang, and Z. J. J. Chen.** 1999. Signal-induced ubiquitination of IκBα by the F-box protein Slimb/β-TrCP. Genes Dev. **13:**284-294.

113. **Strickland, E., K. Hakala, P. J. Thomas, and G. N. DeMartino.** 2000. Recognition of misfolding proteins by PA700, the regulatory subcomplex of the 26S proteasome. J. Biol. Chem. **275:**5565-5572.

114. **Suzuki, T., and A. Varshavsky.** 1999. Degradation signals in the lysine-asparagine sequence space. EMBO J. **18:**6017-6026.

115. **Tamura, N., F. Lottspeich, W. Baumeister, and T. Tamura.** 1998. The role of Tricorn protease and its aminopeptidase-interacting factors in cellular protein degradation. Cell **95:**637-648.

116. **Tamura, T., N. Tamura, Z. Cejka, R. Hegerl, F. Lottspeich, and W. Baumeister.** 1996. Tricorn protease - the core of a modular proteolytic system. Science **274:**1385-1389.

117. **Thrower, J. S., L. Hoffman, M. Rechsteiner, and C. M. Pickart.** 2000. Recognition of the polyubiquitin proteolytic signal. EMBO J. **19:**94-102.

118. **Uhlmann, F., F. Lottspeich, and K. Nasmyth.** 1999. Sister-chromatid separation at anaphase onset is promoted by cleavage of the cohesin subunit Scc1. Nature **400:**37-42.

119. **van Nocker, S., S. Sadis, D. M. Rubin, M. Glickman, H. Fu, O. Coux, I. Wefes, D. Finley, and R. D. Vierstra.** 1996. The multiubiquitin-chain-binding protein Mcb1 is a component of the 26S proteasome in *Saccharomyces cerevisiae* and plays a nonessential, substrate-specific role in protein turnover. Mol. Cell. Biol. **16:**6020-6028.

120. **Varshavsky, A.** 1997. The N-end rule pathway of protein degradation. Genes Cells **2:**13-28.

121. **Varshavsky, A.** 1997. The ubiquitin system. Trends Biochem. Sci. **22:**383-387.

122. **Verma, R., R. S. Annan, M. J. Huddleston, S. A. Carr, G. Reynard, and R. J. Deshaies.** 1997. Phosphorylation of Sic1p by G(1) Cdk required for its degradation and entry into S phase. Science **278:**455-460.

123. **Verma, R., R. J. Dohmen, and R. J. Deshaies.** in review. Activation of S-Cdk by subunit-selective degradation of ubiquitinated Cdk inhibitor by purified 26S proteasomes.

124. **Visintin, R., E. S. Hwang, and A. Amon.** 1999. Cfi1 prevents premature exit from mitosis by anchoring Cdc14 phosphatase in the nucleolus. Nature **398:**818-823.

125. **Visintin, R., S. Prinz, and A. Amon.** 1997. *CDC20* and *CDH1*: A family of substrate-specific activators of APC-dependent proteolysis. Science **278:**460-463.

126. **Webster, A., M. Ghislain, and A. Varshavsky.** manuscript in preparation.

127. **Wenzel, T., and W. Baumeister.** 1995. Conformational constraints in protein degradation by the 20S proteasome. Nat. Struct. Biol. **2:**199-204.

128. **Wenzel, T., and W. Baumeister.** 1993. *Thermoplasma acidophilum* proteasomes degrade partially unfolded and ubiquitin-associated proteins. FEBS Lett. **326:**215-218.

129. **Whiteside, S. T., M. K. Ernst, O. Lebail, C. Laurentwinter, N. Rice, and A. Israel.** 1995. N-Terminal and C-terminal sequences control degradation of Mad3/IκBα in response to inducers of NF-κB activity. Mol. Cell. Biol. **15:**5339-5345.

130. **Wickner, S., S. Gottesman, D. Skowyra, J. Hoskins, K. McKenney, and M. R. Maurizi.** 1994. A molecular chaperone, ClpA, functions like DnaK and DnaJ. Proc. Natl. Acad. Sci. USA **91:**12218-12222.

131. **Wickner, S., M. R. Maurizi, and S. Gottesman.** 1999. Posttranslational quality control: Folding, refolding, and degrading proteins. Science **286:**1888-1893.

132. **Winston, J. T., P. Strack, P. Beer-Romero, C. Y. Chu, S. J. Elledge, and J. W. Harper.** 1999. The SCF$^{\beta\text{-TRCP}}$-ubiquitin ligase complex associates specifically with phosphorylated destruction motifs in IκBα and β-catenin and stimulates IκBα ubiquitination *in vitro*. Genes Dev. **13:**270-83.

133. **Xie, Y. M., and A. Varshavsky.** 1999. The E2-E3 interaction in the N-end rule pathway: the RING-H2 finger of E3 is required for the synthesis of multiubiquitin chain. EMBO J. **18:**6832-6844.

134. **Xie, Y. M., and A. Varshavsky.** 2000. Physical association of ubiquitin ligases and the 26S proteasome. Proc. Natl. Acad. Sci. USA **97:**2497-2502.

135. **Yamamoto, A., V. Guacci, and D. Koshland.** 1996. Pds1p, an inhibitor of anaphase in budding yeast, plays a critical role in the APC and checkpoint pathway(s). J. Cell Biol. **133:**99-110.

136. **Yaron, A., H. Gonen, I. Alkalay, A. Hatzubai, S. Jung, S. Beyth, F. Mercurio, A. M. Manning, A. Ciechanover, and Y. Ben-Neriah.** 1997. Inhibition of NF-κB cellular function via specific targeting of the IκBα ubiquitin ligase. EMBO J. **16:**6486-94.

137. **Yaron, A., A. Hatzubai, M. Davis, I. Lavon, S. Amit, A. M. Manning, J. S. Andersen, M. Mann, F. Mercurio, and Y. Ben-Neriah.** 1998. Identification of the receptor component of the IκBα ubiquitin ligase. Nature **396:**590-594.

138. **Yu, H., J. M. Peters, R. W. King, A. M. Page, P. Hieter, and M. W. Kirschner.** 1998. Identification of a cullin homology region in a subunit of the anaphase-promoting complex. Science **279:**1219-1222.

139. **Zachariae, W., A. Shevchenko, P. D. Andrews, R. Ciosk, M. Galova, J. R. Stark, M. Mann, and K. Nasmyth.** 1998. Mass spectrometric analysis of the anaphase-promoting complex from yeast: identification of a subunit related to cullins. Science **279:**1216-1219.

140. **Zhou, P. B., and P. M. Howley.** 1998. Ubiquitination and degradation of the substrate recognition subunits of SCF ubiquitin-protein ligases. Mol. Cell **2:**571-580.

Chapter 2

Detecting and measuring cotranslational protein degradation *in vivo*

Glenn C. Turner and Alexander Varshavsky

(*Science* under review)

Abstract

Nascent polypeptides emerging from the ribosome and not yet folded may at least transiently present degradation signals similar to those recognized by the ubiquitin system in misfolded proteins. Thus, the folding of nascent proteins, including abnormal ones, may be in kinetic competition with pathways that target these proteins for degradation cotranslationally. Here we describe the ubiquitin sandwich technique that allows the detection and measurement of cotranslational protein degradation in living cells. We use this method to demonstrate that more than 50% of nascent protein molecules bearing an N-terminal degradation signal can be degraded cotranslationally, never reaching their mature size before their destruction by processive proteolysis.

Introduction, Results and Discussion

Nascent (being synthesized) polypeptides emerging from the ribosome may, in the process of folding, present hydrophobic patches and other structural features that serve as degradation signals similar to those recognized by the ubiquitin system in misfolded or otherwise damaged proteins (*1*). It has been a long-standing question whether a significant fraction of nascent polypeptides is cotranslationally degraded (*2*). Determining whether nascent polypeptides are actually degraded *in vivo* has been difficult because at any given time the nascent chains of a particular protein species are of different sizes, and therefore would not form a band upon electrophoresis

in a conventional pulse-chase assay (*3*). The ubiquitin (Ub) sandwich technique makes it possible to detect cotranslational protein degradation by measuring the steady-state ratio of two reporter proteins whose relative abundance is established cotranslationally.

The polypeptide to be examined for cotranslational degradation, termed **B**, is sandwiched between two stable reporter domains **A** and **C** in a linear fusion protein. The three polypeptides are connected via Ub moieties to create a fusion protein of the form **A**Ub-**B**Ub-**C**Ub (Fig. 1A). Ub-specific processing proteases (UBPs) (*4*) cotranslationally cleave such linear Ub fusions at the C-terminal residue of Ub (*5-8*). The independent polypeptides **A**Ub, **B**Ub and **C**Ub that result from the cleavage of **A**Ub-**B**Ub-**C**Ub are called modules below.

UBP-mediated cleavage establishes a kinetic competition between two mutually exclusive events during the synthesis of the **A**Ub-**B**Ub-**C**Ub fusion: cotranslational UBP cleavage at the **B**Ub-**C**Ub junction to release the long-lived **C**Ub module or, alternatively, cotranslational degradation of the entire **B**Ub-**C**Ub nascent chain by the 26S proteasome (*9*) (Fig. 1B). In the latter case, the processivity of proteasome-mediated degradation results in the destruction of the Ub moiety between **B** and **C** *before* it can be recognized by UBPs. The resulting drop in levels of the **C**Ub module relative to levels of **A**Ub, referred to as the C/A ratio, reflects the cotranslational degradation of domain **B** (Fig. 1B). This measurement provides a *minimal* estimate of the total amount of cotranslational degradation, because non-processive

cotranslational degradation events that do not extend into the **C** domain are not detected.

It should be noted that the Ub moieties of the fusion serve solely as cotranslationally cleavable junctions between domains **A**, **B**, and **C**. They do not target the attached proteins for degradation (*8*). In addition, a lysine to arginine substitution at position 48 of these moieties (Ub^{R48}) prevents their conjugation to other Ub molecules at that position, so they cannot be involved in the formation of a major class of substrate-linked multi-Ub chains required for degradation by the 26S proteasome (*10, 11*).

UBP-mediated cleavage was previously used to examine the *in vivo* kinetics of protein translocation into the endoplasmic reticulum (ER) (*6*), where it was shown that cleavage was fast enough to be cotranslational. We directly verified this in the case of an **A**Ub-**B**Ub-**C**Ub fusion, using an *in vivo* radiolabeling regimen in which the labeling pulse was significantly shorter than the time required for the complete synthesis of **A**Ub-**B**Ub-**C**Ub, terminating the pulse by arresting translation. At the beginning of the pulse, the cell's ribosomes are at various stages in the synthesis of the fusion. As a result, the pulse produces a population of nascent **A**Ub-**B**Ub-**C**Ub chains that are labeled at various regions along the chain (Fig. 2B). The nascent chains that are just starting to be synthesized when the pulse begins will incorporate label into the N-terminal **A** domain, but they will not be elongated to full-length chains before the end of the pulse. Under these conditions free, *labeled*

AUb could be produced only if UBPs cleave the nascent polypeptide chains (Fig. 2B).

S. cerevisiae cells expressing a 190 kD **A**Ub-**B**Ub-**C**Ub of the form {DHFRhaUb}-{Me^{K}βgalUb}-{Me^{K}DHFRhaUb} (Fig. 2A) were labeled with ^{35}S-methionine/cysteine for 45 sec (*12*). In the eukaryotic cell types examined, the rate of translation was 2 to 10 residues/sec, while a rate of 12 residues/sec has been measured in *E. coli* (*13*). Assuming a rate of 5 residues/sec in *S. cerevisiae*, the above fusion would require ~350 sec for complete synthesis, more than 7 times the duration of the 45 sec pulse. The labeling was terminated by the addition of the translation inhibitor cycloheximide, and UBPs were simultaneously inactivated with N-ethylmaleimide (NEM) (*6*). Discrete bands corresponding to the labeled **A**Ub (DHFRhaUb) and **B**Ub (Me^{K}βgalUb) modules were observed upon immunoprecipitation, indicating that UBP-mediated cleavage at the **A**Ub-**B**Ub junction was cotranslational (Fig. 2C). Moreover, no full-length **A**Ub-**B**Ub-**C**Ub fusion was detected (Fig. 2C), indicating that the cotranslational cleavage by UBPs was highly efficient. High concentrations of NEM in these extracts increased nonspecific immunoprecipitation, yielding a crossreacting species that co-migrated with **C**Ub (Me^{K}DHFRhaUb). No such crossreacting band was present when NEM was omitted (Fig. 3A). Note that only the presence of free labeled **A**Ub was diagnostic of cotranslational cleavage; free **C**Ub was expected to be observed whether cleavage by UBPs was post- or cotranslational (Fig. 2B).

This result confirmed the key assumption of the Ub sandwich technique, validating its use to detect cotranslational degradation *in vivo*. Previous evidence bearing on cotranslational degradation was based on experiments with inhibitors or cell-free systems (*14*). Nascent polypeptide chains might be protected from degradation *in vivo*, either because they are sterically shielded by chaperones or because their translation time is short in comparison to the time required for targeting by the degradation machinery. To maximize the likelihood of detecting cotranslational degradation, a large, 118 kD, βgal-derived polypeptide carrying a strong N-terminal degradation signal was initially chosen as the **B** domain of the **A**Ub-**B**Ub-**C**Ub fusion (Fig. 2A). A protein of this size is unable to fit into the cavity of the cytosolic CCT chaperonin (*15*), ensuring that this type of shielding was unavailable to the test protein. In addition, the N-terminal location of the degradation signal made it potentially accessible from the beginning of translation.

The degradation signal used was an N-degron, which is targeted by Ubr1p, the E3 component of the N-end rule pathway (*5, 16*). The βgal-linked N-degron comprises the destabilizing N-terminal residue arginine (R) and a short, lysine-bearing extension, e^K (Fig. 2A), that is the site of multi-Ub chain attachment (*11, 17*). Re^K-βgal is rapidly degraded *in vivo* ($t_{1/2}$ ~2 min) (*5, 7*). Changing just the N-terminal residue of the protein to methionine (M) inactivates the degradation signal by precluding recognition by Ubr1p. The resulting Me^K-βgal is posttranslationally stable ($t_{1/2}$ > 20 hr) (*5, 7*). **A**Ub-**B**Ub-**C**Ub fusions in which domain **B** was either Re^K-βgal or its N-degron-lacking

counterpart Me^K-βgal (Fig. 2A) were expressed in *S. cerevisiae* strains containing different levels of Ubr1p (*18*). The extent of cotranslational degradation was assessed by radiolabeling for 30 min and immunoprecipitation (*19*) to determine the levels of **C**Ub relative to **A**Ub; this parameter is referred to below as the C/A ratio.

The results of a representative experiment are shown in Fig. 3A. The C/A ratio was significantly and reproducibly lower in cells expressing the N-degron-bearing domain **B**, but only in those strains that also expressed Ubr1p. To determine the percentage of nascent chains cotranslationally degraded by the N-end rule pathway, we averaged the C/A ratios over several trials, and compared the ratios for Re^K-βgal in wildtype (0.52) and Ubr1p-overexpressing strains (0.39) to the ratio found with the *ubr1Δ* strain (0.86) (Fig. 4A). This comparison indicated that ~40% of the nascent Re^K-βgal chains were cotranslationally degraded in the wildtype strain (Fig. 4B) (*20*). This fraction increased to ~55% when Ubr1p was overexpressed from the P_{GAL1} promoter.

A different way to measure the extent of N-degron-dependent cotranslational degradation was to compare the C/A ratios for Re^K-βgal and Me^K-βgal in the same strain. This comparison *independently* indicated ~40% cotranslational degradation of Re^K-βgal in the wildtype strain (R = 0.52; M = 0.82), and ~55% when Ubr1p was overexpressed (R = 0.39; M = 0.87). Note that the C/A ratios for Re^K-βgal in the *ubr1Δ* strain, and for Me^K-βgal in all the

strains used, were ~0.85 (Fig. 4A), significantly less than the 1.0 ratio expected in the absence of cotranslational degradation; this is discussed below.

The extent of cotranslational degradation was expected to depend on the length of the **B** domain (Fig. 1B), because a larger protein requires a longer time for synthesis, and possibly also because the nascent chain of a larger protein extends a greater distance from the ribosome. To address this issue, we determined the extent of cotranslational degradation of two other **B** domains. The mammalian Sindbis virus RNA polymerase, termed nsP4, is a 69 kD protein that naturally bears an N-degron (*21*). In virus-infected mammalian cells, nsP4 is synthesized as part of a viral polyprotein that is autocatalytically cleaved to expose the N-degron of nsP4 largely during translation (*22*). Xe^K-Ura3p (X=M or R) is a 34 kD enzyme of the *S. cerevisiae* uracil biosynthetic pathway that either carried (Re^K-Ura3p) or lacked (Me^K-Ura3p) an N-degron (*23*) (Fig. 2A). Initially, immunoblot analysis was used to estimate the steady-state C/A ratios for these **A**Ub-**B**Ub-**C**Ub fusions (Fig. 3B) (*24*). While the short-lived R-nsP4 (69 kD) and Re^K-βgal (118 kD) were cotranslationally degraded to similar extents, Re^K-Ura3p (34 kD), which was also short-lived posttranslationally (*23*), exhibited much less cotranslational degradation than the other two proteins (Fig. 3B). To measure C/A ratios more accurately, *in vivo* radiolabeling and immunoprecipitations were carried out (*19*). The results of several experiments, summarized in Figure 4, clearly showed that in the presence of a strong N-degron,

cotranslational degradation is significant for all three of the tested proteins, Re^K-βgal, R-nsP4, and Re^K-Ura3p.

Comparisons of the C/A ratios for R-nsP4 in the wildtype (*UBR1*) and *ubr1Δ* strains indicated that ~50% of the nascent chains were cotranslationally degraded by the N-end rule pathway in wildtype cells, and ~55% in cells overexpressing Ubr1p (Fig. 4B). These values were similar to, respectively, ~40% and ~55% cotranslational degradation of Re^K-βgal in these strains (Fig. 4B). In contrast, only ~20% cotranslational degradation was observed with the 34 kD Re^K-Ura3p in either wildtype or Ubr1p-overexpressing strains (Fig. 4B), suggesting that smaller proteins are less susceptible to cotranslational degradation. However, protein size was not directly proportional to the extent of cotranslational degradation observed, indicating that other factors must influence the accessibility or presentation of the degradation signal by the nascent chain.

These comparisons established the levels of cotranslational degradation by the Ubr1p-dependent N-end rule pathway. In addition, the C/A ratios for two of the N-degron-lacking **B** domains, Me^K-βgal and M-nsP4, were less than 1.0, suggesting that they might be cotranslationally degraded by a Ubr1p-independent pathway. To determine the baseline C/A ratio for negligible cotranslational degradation, we constructed an **A**Ub-**B**Ub-**C**Ub fusion in which domain **B** was an 18-residue sequence containing the FLAG epitope (Fig. 2A). This sequence bore no known degradation signals and would presumably be too short to be degraded cotranslationally. The C/A ratio observed was 0.97.

Thus Me^K-βgal exhibited a ~15% drop in C/A relative to the FLAG-bearing peptide, M-nsP4 a ~25% drop, whereas the C/A ratios obtained with Me^K-Ura3 and FLAG were indistinguishable (Fig. 4A). One interpretation of these differences is that, although Me^K-βgal and M-nsP4 are posttranslationally stable, they may be cotranslationally degraded to a significant extent. Premature termination of translation and/or transcription may also contribute to the drop in the C/A ratios for these two N-degron-lacking **B** domains. It is unlikely that premature termination of translation entirely accounts for the observed difference between the C/A ratios for FLAG and M-nsP4, because the codon adaptation index of the M-nsp4 open reading frame (0.1) is higher than the one for Me^K-βgal (0.07), but the drop in C/A ratio is greater with M-nsP4 (25%) than with Me^K-βgal (15%).

This work demonstrates that a nascent polypeptide carrying a degradation signal can be cotranslationally degraded *in vivo* (Fig. 4B). Cotranslational protein degradation, which cannot be detected by conventional pulse-chase assays, can now be studied using the Ub sandwich technique (Fig. 1). This method reveals that the extent of cotranslational degradation can be strikingly high: in the case of Re^K-βgal in Ubr1p-overexpressing cells, over 50% of nascent polypeptide chains never reach their full size before their destruction by processive proteolysis. Thus, if a nascent chain displays a degron of the Ub system, such a protein becomes a target of kinetic competition between cotranslational biogenesis and cotranslational degradation. Since the folding of a protein molecule begins

during its synthesis on the ribosome, a nascent polypeptide may cotranslationally expose degradation signals in the form of, for example, hydrophobic patches which become shielded through the folding of the newly formed protein (*1, 25*). Our findings with the posttranslationally long-lived Me^K-βgal and M-nsP4 (Fig. 4A) are consistent with this possibility.

If cotranslational protein degradation by the Ub system is found to be extensive *in vivo*, it could be accounted for as an evolutionary trade-off between the necessity of identifying and destroying degron-bearing mature proteins and the mechanistic difficulty of distinguishing between posttranslationally and cotranslationally presented degrons. Conditional degradation signals that require posttranslational phosphorylation for activation, such as those in IκBα and β-catenin (*26*), may have the additional feature of solving this mechanistic difficulty. Proteins might also be spared cotranslational degradation because their polypeptide chains are too short to be effectively targeted during synthesis, or because the machinery responsible for their degradation is physically segregated from the ribosomes. However, our experiments clearly demonstrate that a considerable fraction of degron-carrying polypeptides that are not subject to these constraints are degraded as nascent chains. Cotranslational protein degradation may represent a previously unrecognized form of protein quality control, which destroys nascent chains that fail to fold correctly. These and other questions about cotranslational protein degradation can now be addressed directly in living cells through the Ub sandwich technique.

References and Notes

1. M. Hochstrasser, *Annu. Rev. Genet.* **30**, 405-439 (1996); A. Varshavsky, *Trends Biochem. Sci.* **22**, 383-387 (1997); A. Hershko and A. Ciechanover, *Annu. Rev. Biochem.* **76**, 425-479 (1998); S. Sadis, C. Atienza, D. Finley, *Mol. Cell. Biol.* **15**, 4086-94 (1995); T. Gilon, O. Chomsky, R. G. Kulka, *EMBO J.* **17**, 2759-2766 (1998).
2. B. Alberts, et al., *Molecular Biology of the Cell,* p. 220. (Garland Publishing, New York, 1994).
3. J.-M. Peters, J. R. Harris, D. Finley, Eds., *Ubiquitin and the Biology of the Cell.* (Plenum Press, New York, NY, 1998).
4. K. Wilkinson and M. Hochstrasser, in *Ubiquitin and the Biology of the Cell.* J.-M. Peters, J. R. Harris, D. Finley, Eds. (Plenum Press, New York, NY, 1998).
5. A. Bachmair, D. Finley, A. Varshavsky, *Science* **234**, 179-186 (1986).
6. N. Johnsson and A. Varshavsky, *EMBO J.* **13**, 2686-2698 (1994).
7. A. Varshavsky, *Proc. Natl. Acad. Sci. USA* **93**, 12142-12149 (1996).
8. F. Lévy, N. Johnsson, T. Rumenapf, A. Varshavsky, *Proc. Natl. Acad. Sci. USA* **93**, 4907-4912 (1996).
9. W. Baumeister, J. Walz, F. Zühl, E. Seemüller, *Cell* **92**, 367-380 (1998); G. N. DeMartino and C. A. Slaughter *J. Biol. Chem.* **274,** 22123-22126 (1999).
10. C. M. Pickart, *FASEB J.* **11**, 1055-1066 (1997); M. Scheffner, S. Smith, S. Jentsch, in *Ubiquitin and the Biology of the Cell.* J.-M. Peters, J. R. Harris, D. Finley, Eds. (Plenum Press, New York, 1998) pp. 65-98.
11. V. Chau, et al., *Science* **243**, 1576-1583 (1989).

12. The expression of Ub sandwich proteins from the P_{GAL1} promoter was induced with 0.1 μM β-estradiol, using the Gal4.ER.VP16 chimeric protein, which consisted of the DNA-binding domain of Gal4p, the estrogen-binding domain of estrogen receptor, and the VP16 activation domain expressed from a low copy plasmid (*27*). Induction commenced when cultures were at OD_{600} ~0.2. Cells were harvested after ~10 hr of growth to OD_{600} 0.5-1. The harvested cells were washed in 0.8 ml SD medium (*28*) containing 0.1 μM β-estradiol and resuspended in 0.4 ml of the same medium. Cells were labeled for 30 sec with 0.28 mCi of ^{35}S-EXPRESS (New England Nuclear). Cycloheximide and NEM (final concentrations 0.5 mg/ml and 0.1 M respectively) were then added, and 0.2 ml of the resulting mixture transferred to a chilled tube containing 0.5 ml of 0.5-mm glass beads, 0.8 ml of ice-cold lysis buffer (1% Triton-X100, 0.15 M NaCl, 5 mM EDTA, 50 mM Na-HEPES, pH 7.5) and a mixture of protease inhibitors (final concentrations 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 0.5 μg/ml leupeptin, and 0.7 μg/ml pepstatin). Total handling time to the point of beginning the lysis was 45 sec. Extracts were prepared and immunoprecipitations carried out as described (*23*), using the following monoclonal antibodies, as appropriate: anti-ha 12CA5 (Boehringer), anti-FLAG M2 (Eastman Kodak), and anti-βgal (Promega). Immunoprecipitates were fractionated by SDS-13% PAGE. The bands were detected by autoradiography and quantitated by PhosphorImager (Molecular Dynamics).

13. A. S. Spirin, *Ribosome Structure and Protein Biosynthesis.* (Benjamin Cummings Publ. Co., Menlo Park, CA, USA, 1986); M. A. Sorensen, C. G. Kurland, S. Pedersen, *J. Mol. Biol.* **207**, 365-377 (1989).

14. W. Liao, S. C. J. Yeung, L. Chan, *J. Biol. Chem.* **273**, 27225-27230 (1998); L. Lin, G. N. DeMartino, W. C. Greene, *Cell* **92**, 819-828 (1998); S. Sato, C. L. Ward, R. R. Kopito, *J. Biol. Chem.* **273**, 7189-7192 (1998).

15. A. L. Horwich, K. B. Low, W. A. Fenton, I. N. Hirshfield, K. Furtak, *Cell* **74**, 909-917 (1993); J. Frydman and F. U. Hartl, *Science* **272**, 1497-1502 (1996); F. U. Hartl, *Nature* **381**, 571-580 (1996); J. L. Johnson and E. A. Craig, *Cell* **90**, 201-4 (1997); B. Bukau and A. L. Horwich, *Cell* **92**, 351-366 (1998); R. J. Ellis, *Curr. Biol.* **9**, 137-139 (1999).

16. B. Bartel, I. Wünning, A. Varshavsky, *EMBO J.* **9**, 3179-3189 (1990); K. Madura, R. J. Dohmen, A. Varshavsky, *J. Biol. Chem.* **268**, 12046-12054 (1993).

17. A. Bachmair and A. Varshavsky, *Cell* **56**, 1019-1032 (1989).

18. The *S. cerevisiae* strains JD52 (*MAT***a** *lys2-801 ura3-52 trp1-Δ63 his3-Δ200 leu2-3,112*), JD54 (P_{GAL1}*::UBR1*) and JD55 (*ubr1Δ*) were described previously (*23*).

19. As in (*12*), with the following modifications: cells were labeled for 30 min at 30°C with 0.11 mCi of ^{35}S-EXPRESS. Cells were pelleted, resuspended in 0.8 ml of ice-cold lysis buffer, then transferred to a chilled tube containing protease inhibitors, as described (*12*).

20. The C/A ratio was the amount of ^{35}S in the **C**Ub band divided by the amount of ^{35}S in the **A**Ub band. To determine the level of the Ubr1p-dependent cotranslational degradation, the C/A ratio observed for a particular

N-degron-bearing **B** domain in the wildtype or Ubr1p-overexpressing strain was divided by the C/A ratio for the same **B** domain in the *ubr1Δ* strain, and the resulting value was subtracted from 100%. For example, with Re^{K}-βgal, C/A was 0.52 in wildtype cells, and 0.86 in *ubr1Δ*, so the Ubr1p-dependent cotranslational degradation was $100\% \times (1 - 0.52/0.86) = 40\%$.

21. R. J. deGroot, T. Rümenapf, R. J. Kuhn, J. H. Strauss, *Proc. Natl. Acad. Sci. USA* **88**, 8967-8971 (1991).

22. W. F. Hardy and J. H. Strauss, *J. Virol.* **63**, 4653-4664 (1989).

23. M. Ghislain, R. J. Dohmen, F. Levy, A. Varshavsky, *EMBO J.* **15**, 4884-4899 (1996).

24. Yeast extracts were prepared for immunoblotting as described for immunoprecipitation (*19*), except that disruption of unlabeled cells was carried out in 0.2 ml of lysis buffer with 0.2 ml of glass beads. Immunoblotting was carried out with anti-ha at 1:1000 dilution, followed by a secondary horseradish peroxidase-conjugated goat anti-mouse antibody (BioRad) at 1:1000 dilution. The blots were developed using ECL (Amersham).

25. W. J. Netzer and F. U. Hartl, *Trends Biochem. Sci.* **23**, 68-73 (1998).

26. Z. J. Chen, et al., *Genes Dev.* **9**, 1586-1597 (1995), J. T. Winston, et al., *Genes Dev.* **13**, 270-283 (1999).

27. J. F. Louvion, B. Havauxcopf, D. Picard, *Gene* **131**, 129-134 (1993).

28. F. M. Ausubel, et al., Eds., *Current Protocols in Molecular Biology.* (Wiley-Interscience, New York, 1996).

29. D. Mumberg, R. Muller, M. Funk, *Nucl. Acids Res.* **22**, 5767-5768 (1994).

30. Plasmids encoding Ub sandwich fusions were constructed using standard PCR and recombinant DNA techniques (*28*), and the *E. coli* strain DH5α*F'(recA1)* to minimize recombination events. Construction details are available upon request. All Ub fusion proteins were expressed from the P_{GAL1} promoter on the high copy p426GAL1 vector (*29*).

31. Comparisons of means by the Mann-Whitney test were carried out using InStat 2.00 (Graphpad Software).

32. We thank F. Lévy and N. Johnsson for their advice at early stages of this work. We also thank R. Deshaies, T. Iverson, T-M Yi, and members of the Varshavsky laboratory for helpful discussions and comments on the manuscript. This study was supported by a grant to A. V. from the NIH. G. T. was supported in part by Amgen, Inc.

Fig. 1. The ubiquitin sandwich technique. (**A**) Organization of a Ub sandwich fusion. The polypeptide assayed for cotranslational degradation, **B**, is sandwiched between two stable reporter domains **A** and **C**. Red arrows indicate the locations of UBP cleavage sites. (**B**) The principle of the method. The reporter module **A**Ub is the first to be synthesized, and is cotranslationally released from **B**, thereby providing a measure of the number of nascent **B** chains that initially emerge from the ribosome. If the processive degradation of the emerging **B**, indicated by its insertion into the cylindrical proteasome, is strictly posttranslational, the UBP-mediated cleavage at the **B**Ub-**C** junction releases **C**Ub before **B** is degraded, so the molar yields of **C**Ub and **A**Ub are identical. However, if the degradation of **B** can be cotranslational, a significant fraction of **B**Ub-**C**Ub may be degraded as a unit. This will result in the molar yield of **C**Ub being lower than **A**Ub, the difference being a measure of cotranslational degradation.

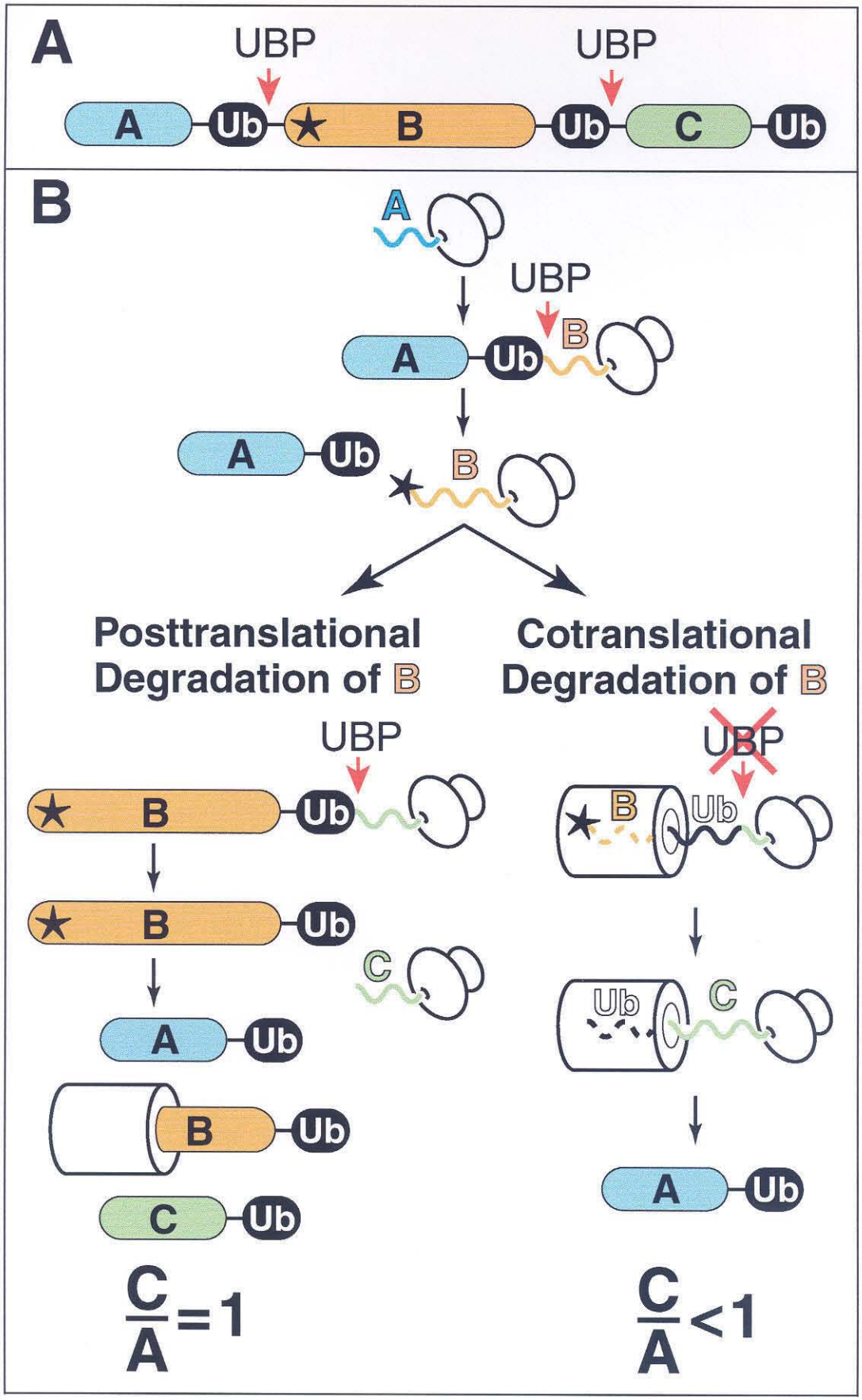

A
UBP
UBP
A
Ub
B
Ub
C
Ub
B
A
UBP
B
A
Ub
B
A
Ub
B
Posttranslational
Degradation of B
Cotranslational
Degradation of B
UBP
UBP
B
Ub
B
Ub
B
Ub
C
A
Ub
B
Ub
C
Ub
C/A=1
C/A<1

Fig. 2. UBP-mediated cleavage of ubiquitin sandwich fusions is cotranslational. (**A**) The protein fusions used (*30*). Domains **A** and **C** are mouse dihydrofolate reductase tagged with the influenza haemagglutinin-derived ha epitope (DHFRha). Domain **C** carries an N-terminal extension (e^K, see text) which makes it electrophoretically distinguishable from **A**. The different **B** domains are *E. coli* β-galactosidase (βgal), Sindbis virus RNA polymerase (nsP4) and *S. cerevisiae* Ura3. Unstable (N-degron-bearing) versions of these domains have an N-terminal arginine (R) residue; stable versions have methionine (M) (**B**) The population of nascent chains produced by a radiolabeling pulse significantly shorter than the time of translation of an **A**Ub-**B**Ub-**C**Ub fusion. Stretches of the polypeptide containing radiolabel are in red; unlabeled stretches are in black. If UBPs efficiently cleave the nascent chain, free radiolabeled **A**Ub, **B**Ub and **C**Ub should all be detected. If UBPs can cleave solely the full-length, mature protein, only the labeled **C**Ub module will be observed. (**C**) The UBP cleavage of Ub sandwich fusions is cotranslational. *S. cerevisiae* expressing an **A**Ub-**B**Ub-**C**Ub fusion of the form {DHFRhaUb}-{Me^KβgalUb}-{Me^KDHFRhaUb}, which is predicted to take ~350 sec to be synthesized, were subjected to a 45-sec ^{35}S-methionine pulse. Labeling was terminated by arresting translation with cycloheximide, and UBPs were simultaneously inactivated with NEM (*12*). The release of modules **A**Ub (DHFRhaUb), **B**Ub (Me^KβgalUb) and **C**Ub (Me^KDHFRhaUb) by UBP cleavage was assayed by immunoprecipitation. **C**Ub* denotes both the Me^KDHFRhaUb band and a crossreacting band present in these NEM-treated extracts but not in untreated ones (compare with Fig. 3A).

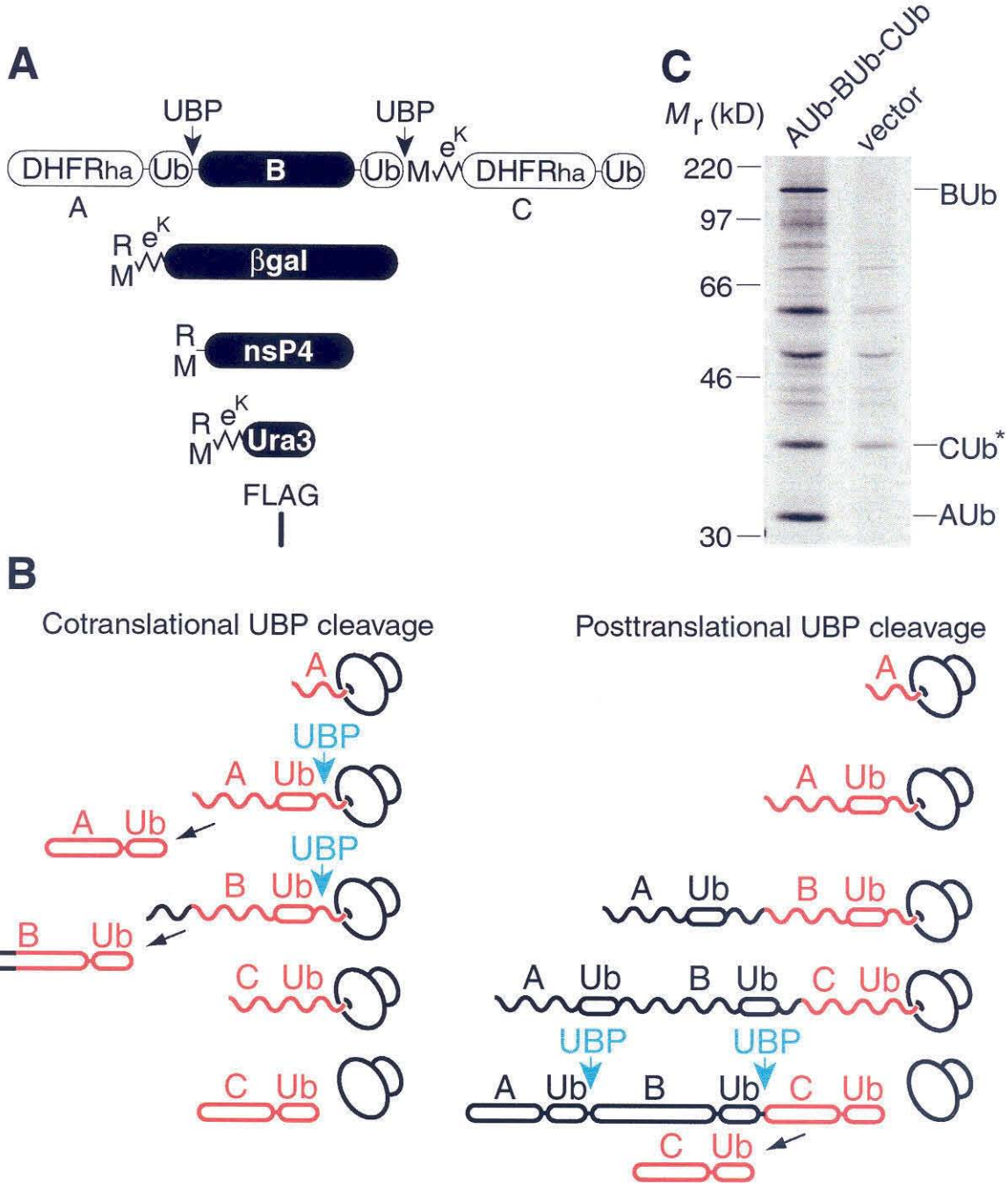

A
UBP
UBP
DHFRha
Ub
B
Ub
M
eK
DHFRha
Ub
A
C
R
M
eK
βgal
nsP4
Ura3
FLAG
C
Mr (kD)
AUb-BUb-CUb
vector
220
97
66
46
30
BUb
CUb*
AUb
B
Cotranslational UBP cleavage
Posttranslational UBP cleavage
A
UBP
A Ub
A Ub
UBP
B Ub
B Ub
C Ub
C Ub
A
A Ub
A Ub B Ub
A Ub B Ub C Ub
UBP
UBP
A Ub B Ub C Ub
C Ub

Figure 3. A fraction of nascent polypeptides bearing an N-terminal degradation signal is degraded cotranslationally. (**A**) Determination of C/A ratios through immunoprecipitation of *in vivo*-labeled **A**Ub-**B**Ub-**C**Ub fusion proteins (*19*). Two variants of βgal as domain **B** were used, one that carried an N-degron (Re^{K}βgal, $t_{1/2}$ ~2 min) and the otherwise identical βgal that differed by one residue and lacked this degradation signal (Me^{K}βgal, $t_{1/2}$ > 20 hr). The two βgal variants were expressed in *S. cerevisiae* strains containing different levels of Ubr1p, the rate-limiting recognition component of the N-end rule pathway. Each pair of lanes corresponds to two independent experiments. (**B**) Immunoblot analysis of the steady-state C/A levels for different **B** domains (described in Fig. 2A) (*24*). The identity of the N-terminal residue of each of the **B** domains, R (Arg) or M (Met), is indicated above each lane. F marks the lane corresponding to an **A**Ub-**B**Ub-**C**Ub fusion protein in which domain **B** was the 18-residue FLAG-containing moiety.

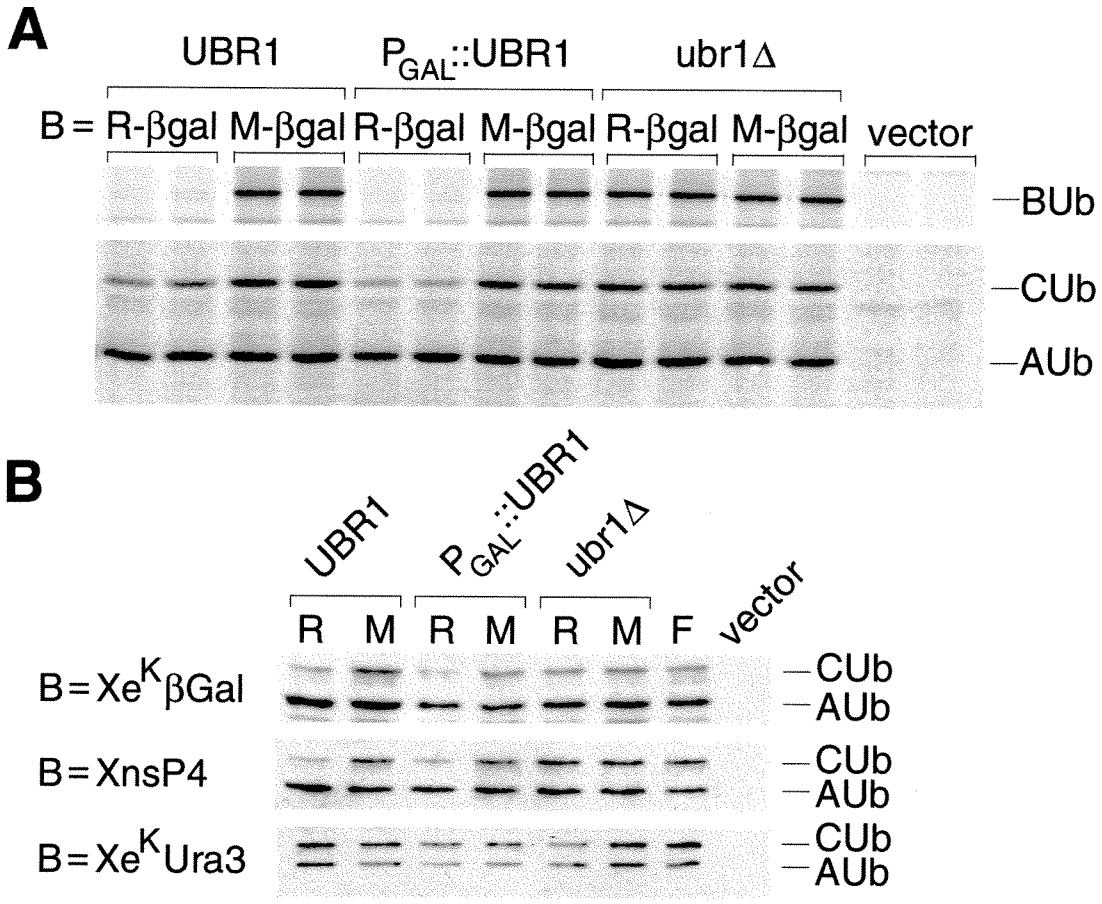
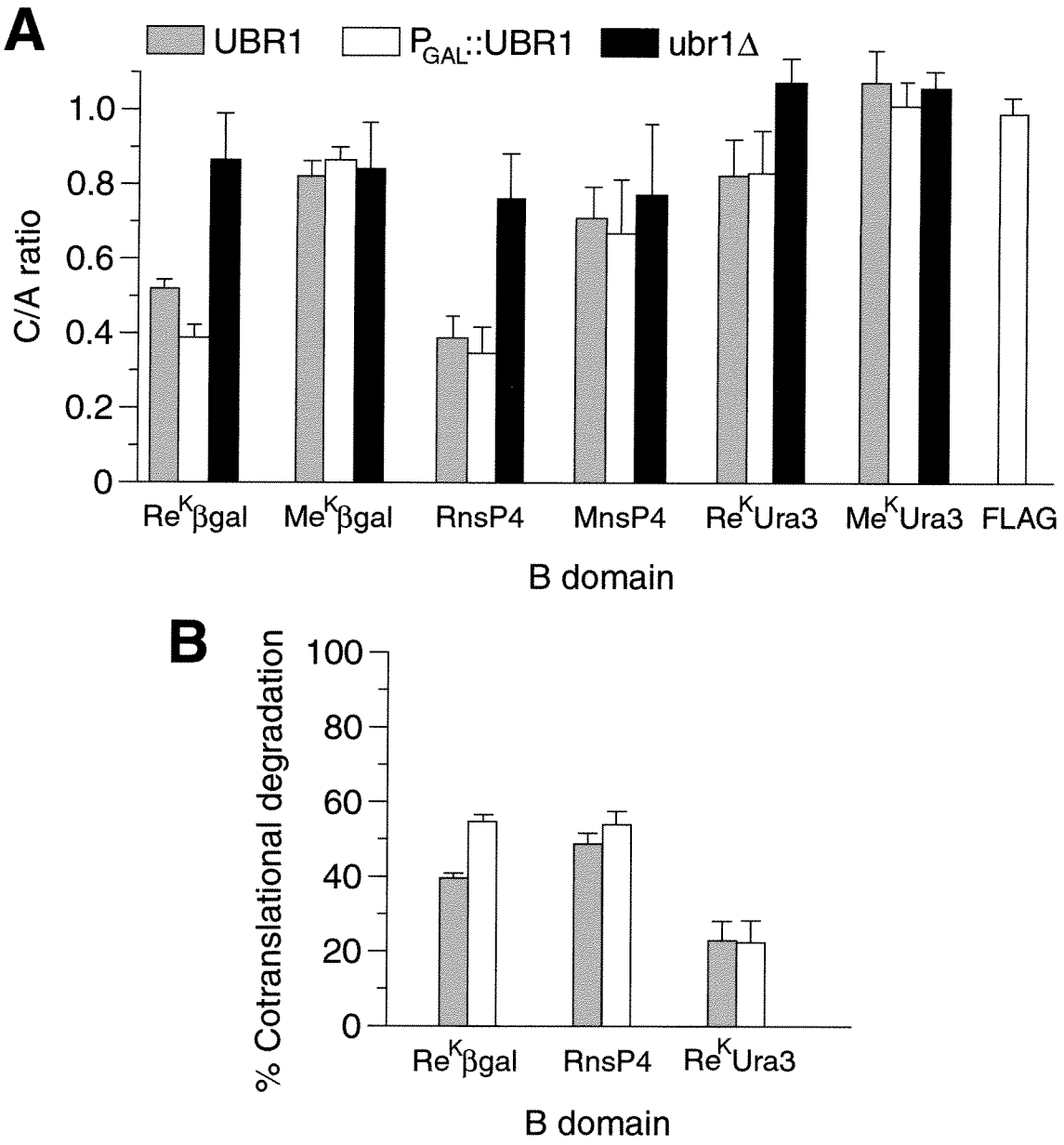

A
UBR1
P GAL ::UBR1
ubr1Δ
B = R-βgal M-βgal R-βgal M-βgal R-βgal M-βgal vector
—BUb
—CUb
—AUb
B
UBR1
P GAL ::UBR1
ubr1Δ
R M R M R M F vector
B=Xe K βGal
B=XnsP4
B=Xe K Ura3
—CUb
—AUb
—CUb
—AUb
—CUb
—AUb

Figure 4. The extent of cotranslational protein degradation depends on the presence of a degron, the activity of a degron-specific proteolytic pathway, and the nature and size of the protein. (**A**) C/A ratios obtained for the different **B** domains. (**B**) Ubr1p-dependent cotranslational degradation. These values were obtained by comparing C/A ratios observed for a particular N-degron-bearing **B** domain in wildtype or Ubr1p-overexpressing strains to the ratio determined in the *ubr1Δ* strain (*20*). Each bar represents a mean value derived from at least 4 independent experiments; standard errors are indicated. Differences between means were significant to at least $p < 0.05$ by the Mann-Whitney test (*31*).


A
UBR1
P$_{GAL}$::UBR1
ubr1Δ
C/A ratio
1.0
0.8
0.6
0.4
0.2
0
Re^{K}βgal
Me^{K}βgal
RnsP4
MnsP4
Re^{K}Ura3
Me^{K}Ura3
FLAG
B domain
B
% Cotranslational degradation
100
80
60
40
20
0
Re^{K}βgal
RnsP4
Re^{K}Ura3
B domain

Chapter 3

Peptides accelerate their uptake by activating a ubiquitin-dependent proteolytic pathway

Glenn C. Turner, Fangyong Du, and Alexander Varshavsky

(*Nature* in press)

Abstract

Protein degradation by the ubiquitin (Ub) system controls the intracellular concentrations of many regulatory proteins. The degradation signals presented by these proteins are recognized by the E3 components of the Ub system[1-3]. Here we describe the first example of physiological regulation of a Ub-dependent pathway through allosteric modulation of its E3 activity by small compounds. Ubr1p, the E3 of the N-end rule pathway in *Saccharomyces cerevisiae*, mediates the Ub/proteasome-dependent degradation of Cup9p, a transcriptional repressor of the peptide transporter Ptr2p[4]. Ubr1p also targets proteins that bear destabilizing N-terminal residues[5]. Here we show that the degradation of Cup9p is allosterically activated by dipeptides bearing destabilizing N-terminal residues. In the resulting positive feedback circuit, imported dipeptides bind to Ubr1p and accelerate the Ubr1p-dependent degradation of Cup9p, thereby derepressing the expression of Ptr2p and increasing the cell's capacity to import peptides. These findings identify the physiological rationale for the targeting of Cup9p by Ubr1p, and suggest that small compounds may regulate other Ub-dependent pathways as well.

Introduction, Results and Discussion

A protein target of the Ub system is conjugated to Ub through the action of three enzymes, E1, E2 and E3[2,3,6]. The resulting ubiquitylated protein bears a covalently linked multi-Ub chain, and is degraded by the 26S proteasome[7]. The selectivity of ubiquitylation is determined mainly by the E3, which recognizes a degradation signal (degron) carried by the target protein. The rate

of degradation of specific proteins is often regulated, in response either to signals from the environment or to signals produced intracellularly, for example, during cell cycle progression. In many cases, this is achieved by modulating the exposure or the structure of a degron in a target protein. For example, the degrons of the cyclin-dependent kinase inhibitors Sic1p and p27 are activated by phosphorylation, which is timed to bring about their destruction at key transition points in the cell cycle[8]. In other cases, phosphorylation regulates the activity of an E3 itself. For example, the anaphase-promoting complex (APC), a multisubunit E3, is activated only at mitosis[9].

One Ub-dependent proteolytic system, termed the N-end rule pathway, targets proteins carrying a degradation signal called the N-degron[10]. The essential determinants of an N-degron are a destabilizing N-terminal residue and a lysine residue of the substrate[11]. The N-end rule pathway is present in all organisms examined, from mammals and fungi to bacteria[12]. In *S. cerevisiae*, there are two classes of destabilizing residues, basic, or type 1 (Arg, Lys and His) and bulky hydrophobic, or type 2 (Phe, Leu, Tyr, Trp and Ile). Ubr1p, a 225K, RING-H2 finger-containing E3, directly recognizes these N-terminal residues[5,13]. The lysine determinant of an N-degron is the site of formation of a substrate-linked multi-Ub chain, which is produced by a complex of the E3 Ubr1p and the E2 enzyme Rad6p (Ubc2p)[14]. Dipeptides bearing a destabilizing N-terminal residue of either basic or hydrophobic type act as competitive inhibitors of the degradation of N-end rule substrates carrying the same type of destabilizing residue[15-17]. Thus Ubr1p contains two distinct N-terminal

residue-binding sites that are each capable of binding either a dipeptide or a protein, but not both at the same time.

The N-end rule pathway was discovered through the use of engineered reporter proteins[10]. Recent work identified the first physiological function of Ubr1p in *S. cerevisiae*, by demonstrating that Ubr1p is required for peptide import[18]. Ubr1p regulates the uptake of peptides by controlling degradation of the 35K homeodomain protein Cup9p, a transcriptional repressor of the di- and tripeptide transporter Ptr2p[4]. In contrast to the canonical N-end rule substrates, Ubr1p targets Cup9p through a degron located in the C-terminal half of Cup9p (F. Navarro-Garcia, G. Turner and A. Varshavsky, unpublished data). Despite this unexpected mode of recognition, we asked whether dipeptides bearing destabilizing N-terminal residues could affect the Ubr1p-mediated degradation of Cup9p, since dipeptides are able to inhibit degradation of canonical N-end rule substrates[17].

To address this question, it was necessary to produce a variant of the Cup9p repressor that could be moderately overexpressed for pulse-chase analyses without influencing the expression of Ptr2p and the uptake of dipeptides. An Asn (N) → Ser (S) substitution at position 265 of Cup9p, within the recognition helix of the homeodomain[19], was predicted to strongly reduce the affinity of Cup9p for DNA[20]. The N265S substitution did not alter the *in vivo* degradation of Cup9p (data not shown). This Cup9p derivative, tagged at the C-terminus with the FLAG epitope and denoted as $Cup9p_{NSF}$, was expressed as part of a fusion of the form $_{F}$DHFR-Ub-$Cup9p_{NSF}$, where $_{F}$DHFR was the N-terminally FLAG-tagged mouse dihydrofolate reductase. Ub-

specific proteases (UBPs) cotranslationally cleave this UPR (Ub/protein/reference) fusion at the Ub-Cup9p junction, yielding the test protein Cup9p$_{NSF}$ and the long-lived $_{F}$DHFR-Ub reference protein, which serves as an internal control for variations in expression levels and immunoprecipitation efficiency[21,22].

Cells expressing Cup9p$_{NSF}$ were grown in minimal medium containing allantoin as the nitrogen source to avoid the known effects of nitrogen catabolite repression on *PTR2* expression[23]. Leu-Ala and Arg-Ala, dipeptides bearing either type of destabilizing N-terminal residue (Leu, bulky hydrophobic; Arg, basic), were added to the medium prior to pulse-chase analysis, to a final concentration of 10 mM (see Methods). This dipeptide concentration results in maximal inhibition of degradation of N-end rule substrates[17]. Strikingly, the addition of either Leu-Ala or Arg-Ala exerted an *opposite* effect on Cup9p$_{NSF}$, strongly accelerating its degradation in wildtype (*UBR1*) cells. The half-life of Cup9p$_{NSF}$ decreased from ~5 min in the absence of dipeptides (Fig. 1c) to less than 1 min in their presence (Fig. 1b). This stimulatory effect was not observed in a *ubr1Δ* strain, indicating that the augmented degradation of Cup9p$_{NSF}$ was dependent on Ubr1p. The enhancement of degradation required dipeptides bearing destabilizing N-terminal residues: Ala-Leu and Ala-Arg, which bore a stabilizing N-terminal residue but had the same amino acid composition as, respectively, Leu-Ala and Arg-Ala, did not affect the degradation of Cup9p$_{NSF}$ ($t_{1/2}$ ~5 min) (Fig. 1b and data not shown). Similar results were obtained with cells expressing Cup9$_{NSF}$ that was not a part of a UPR fusion (data not shown).

To determine the concentration dependence of the stimulation, we measured the degradation of Cup9p at a range of concentrations of Trp-Ala, another peptide bearing a hydrophobic (type 2) destabilizing N-terminal residue. The enhancement of $Cup9p_{NSF}$ degradation was detectable at 1 μM Trp-Ala, the lowest concentration tested ($t_{1/2}$ ~1 min) (Fig. 1c). In contrast, the degradation of $Cup9p_{NSF}$ was not altered either by Ala-Trp or by Trp and Ala, the free amino acid components of Trp-Ala and Ala-Trp (Fig. 1c). These results indicate that the relevant signaling molecule in this process is a dipeptide bearing a destabilizing N-terminal residue. Experiments with other dipeptides carrying destabilizing N-terminal residues (Leu-Ala and Arg-Ala) yielded similar results (data not shown). Thus, the range of dipeptide concentrations that significantly stimulated Cup9p degradation was similar to physiologically active levels of many other nutrients. Interestingly, these concentrations (1-10 μM) were 1,000- to 10, 000-fold lower than the concentration (~10 mM) necessary for maximal inhibition of the Ubr1p-dependent degradation of protein substrates bearing N-degrons[17]. One possibility is that inhibiting N-degron-mediated proteolysis requires a dipeptide and a protein substrate to compete for the same binding site of Ubr1p, while no such competition exists when a dipeptide stimulates Cup9p degradation, since the dipeptide and Cup9p bind to two different sites of Ubr1p. It is also possible that only a small fraction of a cell's Ubr1p molecules have to be activated to enhance the degradation of Cup9p, while the Ubr1p pool may have to be almost saturated by dipeptides to suppress the targeting of a protein bearing an N-degron. Irrespective of the underlying mechanism,

the above results, together with the observation that a protein bearing a destabilizing N-terminal residue can be co-expressed with Cup9p without altering the kinetics of Cup9p degradation (data not shown), suggest that cells are capable of independently regulating the Ubr1p-dependent degradation of proteins bearing N-degrons and internal degrons.

Cup9p represses transcription of the transporter-encoding *PTR2* gene[4]. Thus, the dipeptide-induced acceleration of Cup9p degradation would be expected to increase the levels of *PTR2* mRNA, ultimately leading to an increase in dipeptide uptake. In this circuit, dipeptides bearing destabilizing N-terminal residues act as effectors of a Ubr1p-based positive feedback loop, enhancing their own uptake by accelerating the Ubr1p-mediated degradation of Cup9p. This conjecture was tested by examining the levels of *PTR2* mRNA in the presence or absence of dipeptides in the medium. At 25 μM, both Trp-Ala and Arg-Ala induced *PTR2* expression in the wildtype (*UBR1*) strain (Fig. 2a). The effect of Trp-Ala was particularly strong, resulting in levels of *PTR2* mRNA comparable to that of actin (*ACT1*) mRNA (Fig. 2a). Both Ubr1p and Cup9p were required for these effects, since the expression of *PTR2* was not altered by dipeptides in *ubr1Δ* and *cup9Δ* strains. Testing a range of concentrations of Trp-Ala showed that induction of *PTR2* mRNA could be observed at 1 μM Trp-Ala, increased substantially at 10 μM Trp-Ala, and increased more gradually at higher concentrations, in agreement with the observed changes in the half-life of Cup9p at different levels of Trp-Ala (Fig. 1c).

A plausible mechanism of the enhancement effect is that a dipeptide interacts with either the basic or hydrophobic N-terminal residue-binding sites of Ubr1p, while a distinct (third) substrate-binding site of Ubr1p recognizes the internal degron of Cup9p. In this model, the interaction of Ubr1p with dipeptides allosterically increases the ability of the Ubr1p-Rad6p (E3-E2) complex to ubiquitylate Cup9p. To test whether dipeptides act directly through Ubr1p, we examined the effect of dipeptides on Cup9p ubiquitylation in an *in vitro* system consisting of the following purified components: Ubr1p (E3), Rad6p (E2), Uba1p (E1), Ub, ATP, and radiolabeled Cup9p. In this system, Cup9p was significantly multi-ubiquitylated, in a Ubr1p/Rad6p-dependent reaction (data not shown), in the absence of added dipeptides (Fig. 3). This result was consistent with the relatively rapid *in vivo* degradation of Cup9p ($t_{1/2}$ ~5 min) in the absence of dipeptides (Fig. 1c).

The addition of dipeptides bearing either type of destabilizing N-terminal residue to the *in vitro* system substantially stimulated the Ubr1p-dependent multi-ubiquitylation of Cup9p (Fig. 3). Dipeptides of the same composition but bearing a stabilizing N-terminal residue did not stimulate multi-ubiquitylation, nor did the amino acid components of these dipeptides (Fig. 3). Given the composition of this *in vitro* system, our results demonstrate that dipeptides act directly through Ubr1p, without an intermediate signaling pathway. The underlying allosteric mechanism may involve increased affinity of Ubr1p for Cup9p, or enhanced ubiquitylation activity of the Ubr1p-Rad6p complex towards Cup9, or both.

The findings of this work show that the two binding sites of Ubr1p that interact with destabilizing N-terminal residues have a specific physiological function as allosteric effector binding sites. These sites enable Ubr1p to sense the presence of imported dipeptides, and to induce the expression of the Ptr2p transporter accordingly, by accelerating degradation of the Cup9p repressor. The resulting understanding of the regulation of peptide import is summarized in Figure 4a-d. This model predicts that a dipeptide bearing a destabilizing N-terminal residue, e. g., Leu-Ala, should stimulate its own uptake, in contrast to Ala-Leu, a dipeptide of the same composition bearing a stabilizing N-terminal residue. This prediction was borne out when we tested the ability of these two leucine-containing dipeptides to support the growth of *S. cerevisiae* auxotrophic for leucine. This Leu^- strain grew robustly on plates supplemented with 230 μM Leu-Ala, but it did not form visible colonies in the presence of 230 μM Ala-Leu (Fig. 4e).

Food sources that *S. cerevisiae* encounters outside the laboratory setting are likely to contain mixtures of short peptides, a subset of which would be capable of activating the Ubr1p-based positive feedback circuit, since 12 of the 20 amino acid residues are destabilizing in the yeast N-end rule[12]. We modeled this situation by providing cells with a mixture of Leu-Ala at a low concentration (2 μM) and Ala-Leu at a high concentration (230 μM). Although neither dipeptide supplement alone could satisfy the strain's requirement for leucine, a mixture of the two dipeptides supported robust growth (Fig. 4e). Moreover, a mixture of Ala-Leu (230 μM) and a dipeptide *lacking* leucine but bearing a destabilizing N-terminal residue (10 μM Arg-Ala or 1 μM Lys-Ala)

also rescued growth. In contrast, leucine-lacking dipeptides bearing stabilizing N-terminal residues, or the amino acid components of these dipeptides, could not rescue the growth of Leu$^-$ cells in the presence of 230 μM Ala-Leu (Fig. 4e). These results are predicted by the model described in Fig. 4a-d, in that a dipeptide bearing a destabilizing residue is expected to accelerate the import of all dipeptides, thereby enabling the uptake of sufficient Ala-Leu to satisfy the strain's leucine requirement.

This work establishes for the first time that the activity of an E3 can be directly linked to the presence of an environmental signal through an allosteric interaction with a small compound. Specifically, dipeptides bearing destabilizing N-terminal residues are shown to act as allosteric activators of Ubr1p, enhancing its ability to support the ubiquitylation and degradation of Cup9p. Physiologically, this results in a positive feedback circuit governing the uptake of peptides. By binding to Ubr1p, the imported dipeptides accelerate degradation of Cup9p, thereby derepressing the synthesis of the Ptr2p transporter and enhancing the cell's ability to import di- and tripeptides.

Most eukaryotic cells have the capacity to import peptides. The sequence conservation of both the basic and hydrophobic N-terminal residue-binding sites between the mouse and yeast Ubr1p proteins[24] suggests that the peptide-sensing physiological functions of these sites may be conserved among eukaryotes. In metazoans, many neurotransmitters and hormones bear destabilizing N-terminal residues, so it is possible that some of these signaling molecules act at least in part intracellularly, by modulating Ubr1p-mediated protein degradation. The ClpAP-dependent N-end rule

pathway of *E. coli* [25], whose physiological functions are unknown, may also play a role in regulating the import of peptides or related compounds, by analogy with the *S. cerevisiae* N-end rule pathway.

The discovery of peptide-mediated positive feedback (Fig. 4) identifies the physiological rationale for control of peptide import through the Ubr1p-dependent degradation of Cup9p: the N-terminal residue-binding sites of Ubr1p enable it to sense intracellular dipeptide concentrations and modulate the degradation of Cup9p accordingly. In addition, our results expand the set of mechanisms that regulate protein degradation from the previously known modulatory phosphorylation of degrons and E3's to allosteric regulation of an E3 by a small compound. The Ub system is either known or suspected to play major roles in the control of intermediary metabolism and the transport of small molecules across membranes[2,3]. Our findings suggest that these compounds, or their enzymatically produced derivatives, may modulate the functions of E3s in the Ub system similarly to the effects observed here with dipeptides and Ubr1p.

Methods

Yeast strains and plasmids. The *S. cerevisiae* strains used in pulse-chase experiments, JD52 (*MAT***a** *lys2-801 ura3-52 trp1-Δ63 his3-Δ200 leu2-3,112*), JD55 (*ubr1Δ*::HIS3), were described previously[26]. $_{F}$DHFR-Ub-Cup9p$_{NSF}$ was expressed from the P_{MET25} promoter on the centromeric vector p416MET25[27]. Construction details are available upon request. Strains used for Northern analyses were AVY30 (*MATα leu2-3,112 ubr1Δ::LEU2*), AVY 31 (*MATα leu2-3,112 cup9Δ::LEU2*), and AVY32 (*MATα LEU2*), constructed in the RJD350 background (*MATα leu2-3,112;* a gift from R. Deshaies) using restriction fragments obtained from plasmids pSOB30[5], pCB119[4] and pJJ252[28], respectively. The RJD350 strain was used for the colony formation assay (Fig. 4e). SHM plates[4] were supplemented with dipeptides or amino acids at the following concentrations: 230 μM Leu-Ala (or 2 μM where noted), 230 μM Ala-Leu, 230 μM each of Leu and Ala, 10 μM Arg-Ala, 10 μM Ala-Arg, 1 μM Lys-Ala, 1 μM Ala-Lys, 10 μM each of Arg and Ala, or 1 μM each of Lys and Ala.

Pulse-chase analysis. Cells were cultured in SHM[4] with auxotrophic supplements. Dipeptides were added to cultures at OD_{600} ~0.6 and incubation continued for 2.5 h in the experiments in Fig 1b, and for 30 min for Fig 1c. Cells were harvested, washed in 0.8 ml of SHM, resuspended in 0.4 ml of SHM, and labeled for 5 min at 30°C with 0.16 mCi of ^{35}S-EXPRESS (New England Nuclear). Cells were pelleted, and resuspended in fresh SHM containing 4 mM L-methionine and 2 mM L-cysteine. 0.1 ml samples were taken at the time points indicated and transferred to chilled tubes, each containing 0.5 ml of 0.5-

mm glass beads, 0.7 ml of ice-cold lysis buffer (1% Triton-X100, 0.15 M NaCl, 5 mM EDTA, 50 mM Na-HEPES, pH 7.5), and a mixture of protease inhibitors (final concentrations 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 0.5 μg/ml leupeptin, and 0.7 μg/ml pepstatin). Extracts were prepared and immunoprecipitations carried out as described[26], using anti-FLAG M2 resin (Sigma). Immunoprecipitates were fractionated by SDS-13% PAGE, and detected by autoradiography.

RNA preparation and Northern analysis. Cells were cultured in SHM to OD_{600} ~0.6. The indicated dipeptides were then added to a 25 μM final concentration, and the incubation was continued for an additional 30 min. Total RNA was prepared[29], and 25 μg samples were electrophoresed in 1% formaldehyde-agarose gels, followed by blotting for Northern analysis[30].

Ubr1p-dependent *in vitro* ubiquitylation system. The components of this system were purified as follows. N-terminally hexahistidine-tagged Uba1p was overexpressed in *S. cerevisiae* and purified by fractionation over Ni-NTA, ubiquitin affinity, and Superdex-200 columns. Rad6p was overexpressed in *E. coli*, and purified by fractionation over DEAE, Mono-Q and Superdex-75 columns. N-terminally FLAG-tagged Ubr1p was overexpressed in *S. cerevisiae*, and purified by fractionation over anti-FLAG M2, Ubc2p affinity, and Superdex-200 columns. N-terminally FLAG-tagged, C-terminally hexahistidine-tagged Cup9p was expressed and radiolabeled in *E. coli*, and purified by fractionation over Ni-NTA and anti-FLAG M2 columns. Details of these expression and purification protocols are available upon request.

The *in vitro* ubiquitylation reactions contained the following components: 7 μM Ub, 50 nM Uba1p, 50 nM Rad6p, 50 nM Ubr1p, 550 nM ^{35}S-labeled Cup9p, 25 mM HEPES/KOH (pH 7.5), 25 mM KCl, 5 mM $MgCl_2$, 2 mM ATP, 0.1 mM dithiothreitol, and 0.5 mg/ml ovalbumin as a carrier protein. Dipeptides or amino acids, as indicated, were added to a final concentration of 2 μM (top panel, Fig. 3) or 10 μM (bottom panel, Fig. 3). All components except Uba1p were mixed on ice for 10 minutes; Uba1p was then added and reactions shifted to 30°C. After the indicated times, the reactions were terminated by adding an equal volume of 2 x SDS-PAGE loading buffer and heating at 95°C for 5 min, followed by 8% SDS-PAGE.

Acknowledgments

We thank A. Webster for her valuable contributions to the establishment of the *in vitro* system, and R. Deshaies, J. Dohmen, L. Prakash, H. Rao and J. Sheng for their gifts of plasmids and strains. We also thank C. Byrd, H. Rao, and especially R. Deshaies for helpful discussions and comments on the manuscript. This work was supported by grants to A. V. from the NIH.
Correspondence and requests for materials should be addressed to A. V. (e-mail: avarsh@caltech.edu).

References:

1. Laney, J. D. & Hochstrasser, M. Substrate targeting in the ubiquitin system. *Cell* **97**, 427-430 (1999).
2. Hershko, A. & Ciechanover, A. The ubiquitin system. *Annu. Rev. Biochem.* **76**, 425-479 (1998).
3. Varshavsky, A. The ubiquitin system. *Trends Biochem. Sci.* **22**, 383-387 (1997).
4. Byrd, C., Turner, G. C. & Varshavsky, A. The N-end rule pathway controls the import of peptides through degradation of a transcriptional repressor. *EMBO J.* **17**, 269-277 (1998).
5. Bartel, B., Wünning, I. & Varshavsky, A. The recognition component of the N-end rule pathway. *EMBO J.* **9**, 3179-3189 (1990).
6. Scheffner, M., Smith, S. & Jentsch, S. in *Ubiquitin and the Biology of the Cell* (eds. Peters, J.-M., Harris, J. R. & Finley, D.) 65-98 (Plenum Press, New York, 1998).
7. Baumeister, W., Walz, J., Zühl, F. & Seemüller, E. The proteasome: paradigm of a self-compartmentalizing protease. *Cell* **92**, 367-380 (1998).
8. King, R. W., Deshaies, R. J., Peters, J. M. & Kirschner, M. W. How proteolysis drives the cell cycle. *Science* **274**, 1652-1659 (1996).
9. Kotani, S., Tanaka, H., Yasuda, H. & Todokoro, K. Regulation of APC activity by phosphorylation and regulatory factors. *J. Cell Biol.* **146**, 791-800 (1999).
10. Bachmair, A., Finley, D. & Varshavsky, A. *In vivo* half-life of a protein is a function of its amino-terminal residue. *Science* **234**, 179-186 (1986).

11. Bachmair, A. & Varshavsky, A. The degradation signal in a short-lived protein. *Cell* **56**, 1019-1032 (1989).

12. Varshavsky, A. The N-end rule: functions, mysteries, uses. *Proc. Natl. Acad. Sci. USA* **93**, 12142-12149 (1996).

13. Xie, Y. M. & Varshavsky, A. The E2-E3 interaction in the N-end rule pathway: the RING-H2 finger of E3 is required for the synthesis of multiubiquitin chain. *EMBO J.* **18**, 6832-6844 (1999).

14. Dohmen, R. J., Madura, K., Bartel, B. & Varshavsky, A. The N-end rule is mediated by the UBC2(RAD6) ubiquitin-conjugating enzyme. *Proc. Natl. Acad. Sci. USA* **88**, 7351-7355 (1991).

15. Reiss, Y., Kaim, D. & Hershko, A. Specificity of binding of N-terminal residues of proteins to ubiquitin-protein ligase. Use of amino acid derivatives to characterize specific binding sites. *J. Biol. Chem.* **263**, 2693-2269 (1988).

16. Gonda, D. K. *et al.* Universality and structure of the N-end rule. *J. Biol. Chem.* **264**, 16700-16712 (1989).

17. Baker, R. T. & Varshavsky, A. Inhibition of the N-end rule pathway in living cells. *Proc. Natl. Acad. Sci. USA* **87**, 2374-2378 (1991).

18. Alagramam, K., Naider, F. & Becker, J. M. A recognition component of the ubiquitin system is required for peptide transport in *Saccharomyces cerevisiae*. *Mol. Microbiol.* **15**, 225-234 (1995).

19. Knight, S. A. B., Tamai, K. T., Kosman, D. J. & Thiele, D. J. Identification and analysis of a *Saccharomyces cerevisiae* copper homeostasis gene encoding a homeodomain protein. *Mol. Cell. Biol.* **14**, 7792-7804 (1994).

20. Wolberger, C., Vershon, A. K., Liu, B. S., Johnson, A. D. & Pabo, C. O. Crystal-structure of a Matα-2 homeodomain-operator complex suggests a general model for homeodomain-DNA interactions. *Cell* **67**, 517-528 (1991).

21. Lévy, F., Johnsson, N., Rumenapf, T. & Varshavsky, A. Using ubiquitin to follow the metabolic fate of a protein. *Proc. Natl. Acad. Sci. USA* **93**, 4907-4912 (1996).

22. Suzuki, T. & Varshavsky, A. Degradation signals in the lysine-asparagine sequence space. *EMBO J.* **18**, 6017-6026 (1999).

23. Barnes, D., Lai, W., Breslav, M., Naider, F. & Becker, J. M. *PTR3*, a novel gene mediating amino acid-inducible regulation of peptide transport in *Saccharomyces cerevisiae*. *Mol. Microbiol.* **29**, 297-310 (1998).

24. Kwon, Y. T. *et al.* The mouse and human genes encoding the recognition component of the N-end rule pathway. *Proc. Natl. Acad. Sci. USA* **95**, 7898-7903 (1998).

25. Tobias, J. W., Shrader, T. E., Rocap, G. & Varshavsky, A. The N-end rule in bacteria. *Science* **254**, 1374-1377 (1991).

26. Ghislain, M., Dohmen, R. J., Levy, F. & Varshavsky, A. Cdc48p interacts with Ufd3p, a WD repeat protein required for ubiquitin-mediated proteolysis in *Saccharomyces cerevisiae*. *EMBO J.* **15**, 4884-4899 (1996).

27. Mumberg, D., Muller, R. & Funk, M. Regulatable promoters of *Saccharomyces cerevisiae* - comparison of transcriptional activity and their use for heterologous expression. *Nucl. Acids Res.* **22**, 5767-5768 (1994).

28. Jones, J. S. & Prakash, L. Yeast *Saccharomyces cerevisiae* selectable markers in pUC18 polylinkers. *Yeast* **6**, 363-366 (1990).

29. Schmitt, M. E., Brown, T. A. & Trumpower, B. L. A rapid and simple method for preparation of RNA from *Saccharomyces cerevisiae*. *Nucl. Acids Res.* **18**, 3091-3092 (1990).

30. Ausubel, F. M. *et al.* (eds.) *Current Protocols in Molecular Biology.* (Wiley-Interscience, New York, 1996).

Figure 1. Enhancement of Cup9p degradation by dipeptides bearing destabilizing N-terminal residues. **a**, The fusion protein used for pulse-chase analysis. The stable $_{F}$DHFR-Ub reference portion of the fusion is cotranslationally cleaved from Cup9$_{NSF}$ by UBPs. **b**, Pulse-chase analysis of $_{F}$DHFR-Ub-Cup9$_{NSF}$ in the presence of various dipeptides at 10 mM. Dipeptides bearing either basic (Arg-Ala) or bulky hydrophobic (Leu-Ala) destabilizing N-terminal residues strongly enhance Cup9$_{NSF}$ degradation, but only in strains expressing Ubr1p. Dipeptides bearing a stabilizing N-terminal residue (Ala-Arg and Ala-Leu) do not alter Cup9$_{NSF}$ degradation. **c**, The effects of different concentrations of Trp-Ala on the enhancement of Cup9$_{NSF}$ degradation in wildtype (*UBR1*) cells. Lanes marked by a dash indicate pulse-chase analysis performed in the absence of added dipeptides. Enhancement of Cup9$_{NSF}$ degradation was detectable at 1 μM Trp-Ala, and became substantially greater at 10 μM.

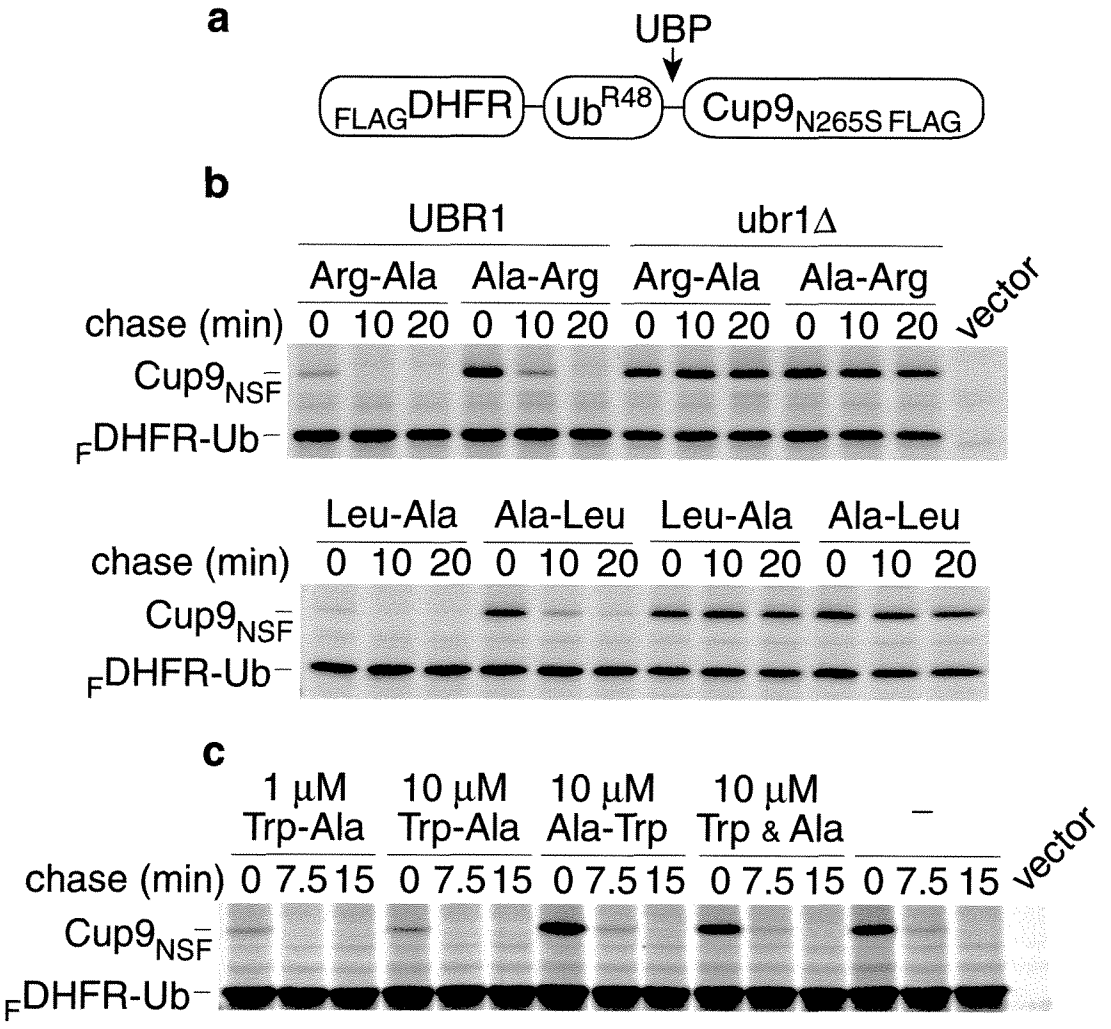

a
UBP
FLAGDHFR
Ub^R48
Cup9N265S FLAG
b
UBR1
ubr1Δ
Arg-Ala
Ala-Arg
Arg-Ala
Ala-Arg
vector
chase (min) 0 10 20 0 10 20 0 10 20 0 10 20
Cup9NSF
FDHFR-Ub
Leu-Ala
Ala-Leu
Leu-Ala
Ala-Leu
chase (min) 0 10 20 0 10 20 0 10 20 0 10 20
Cup9NSF
FDHFR-Ub
c
1 μM Trp-Ala
10 μM Trp-Ala
10 μM Ala-Trp
10 μM Trp & Ala
–
vector
chase (min) 0 7.5 15 0 7.5 15 0 7.5 15 0 7.5 15 0 7.5 15
Cup9NSF
FDHFR-Ub

Figure 2. Effects of dipeptides on expression of the dipeptide transporter gene *PTR2*. **a**, Induction of *PTR2* expression by dipeptides bearing destabilizing N-terminal residues (Trp-Ala and Arg-Ala) required both *UBR1* and *CUP9*. Dipeptides bearing a stabilizing N-terminal residue (Ala-Trp and Ala-Arg) had no effect on *PTR2* expression. *PTR2* mRNA and the *ACT1* mRNA loading control are indicated. **b**, Effect of different concentrations of Trp-Ala on the levels of *PTR2* mRNA.

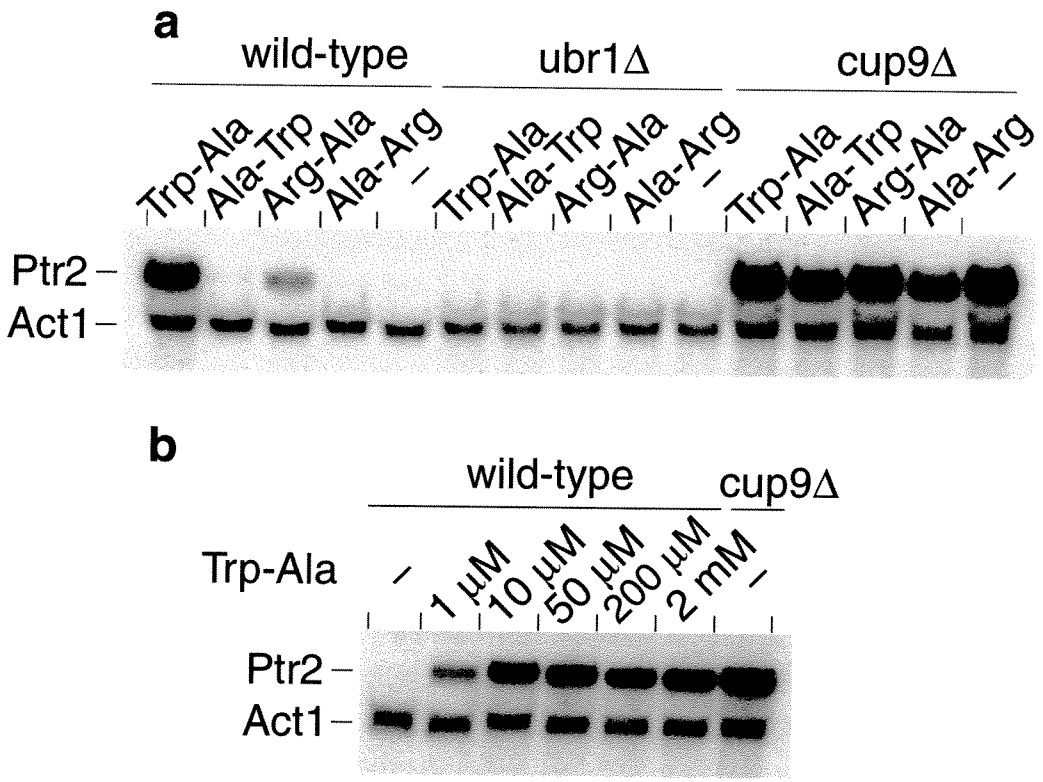

a
wild-type
ubr1Δ
cup9Δ
Trp-Ala
Ala-Trp
Arg-Ala
Ala-Arg
–
Ptr2
Act1
b
wild-type
cup9Δ
Trp-Ala
–
1 μM
10 μM
50 μM
200 μM
2 mM
–
Ptr2
Act1

Figure 3. *In vitro* ubiquitylation of Cup9p is enhanced by dipeptides bearing destabilizing N-terminal residues. Reactions consisting of purified Uba1p (E1), Rad6p (E2), Ubr1p (E3), Ub, ATP and radiolabeled Cup9p, were supplemented with the indicated dipeptides or amino acids (top panel, 2 μM, bottom panel, 10 μM), or left unsupplemented (denoted by a dash), and allowed to proceed at 30°C for the designated times. Radiolabeled input Cup9p, and its multi-ubiquitylated derivatives are indicated.

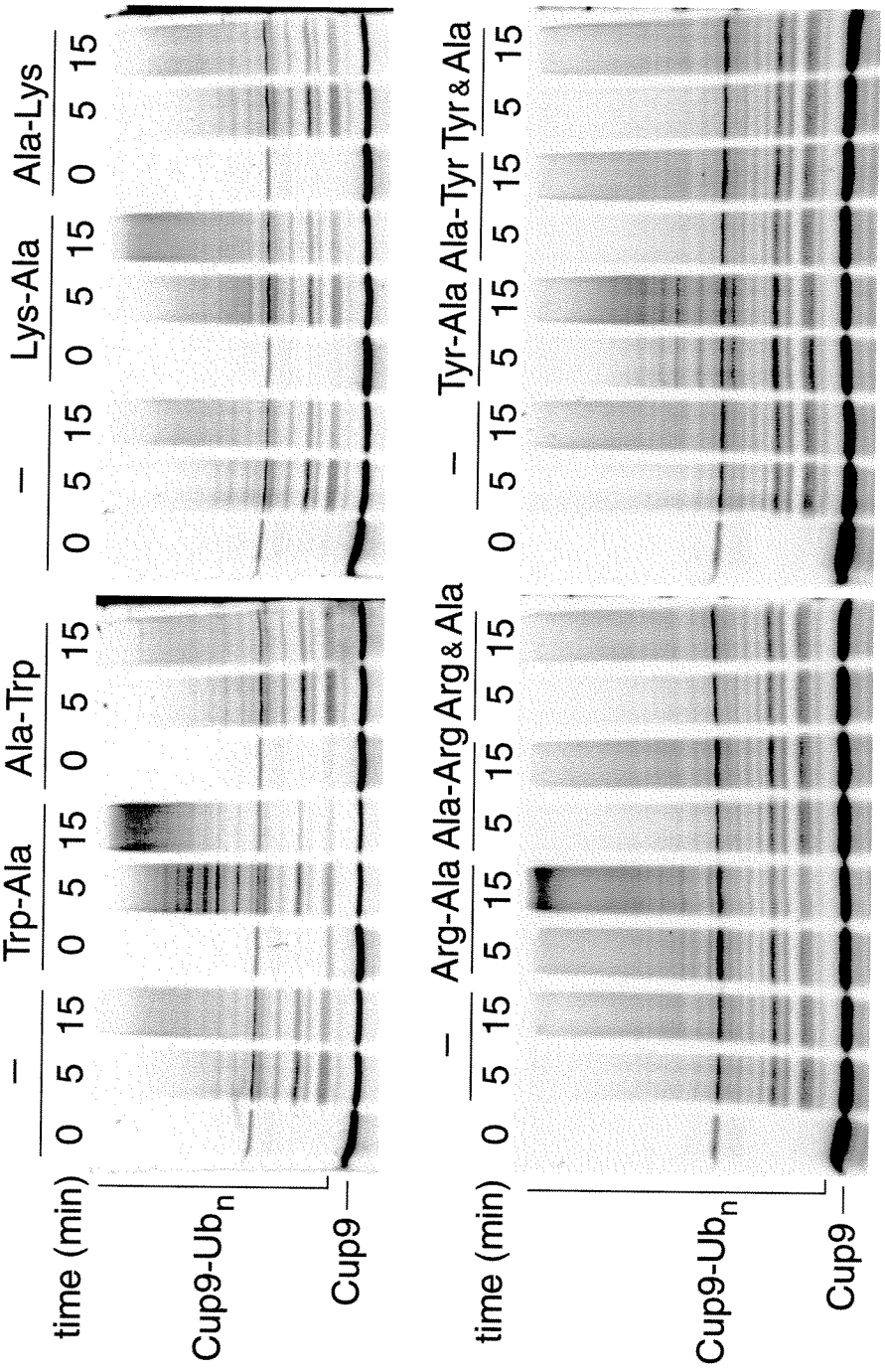
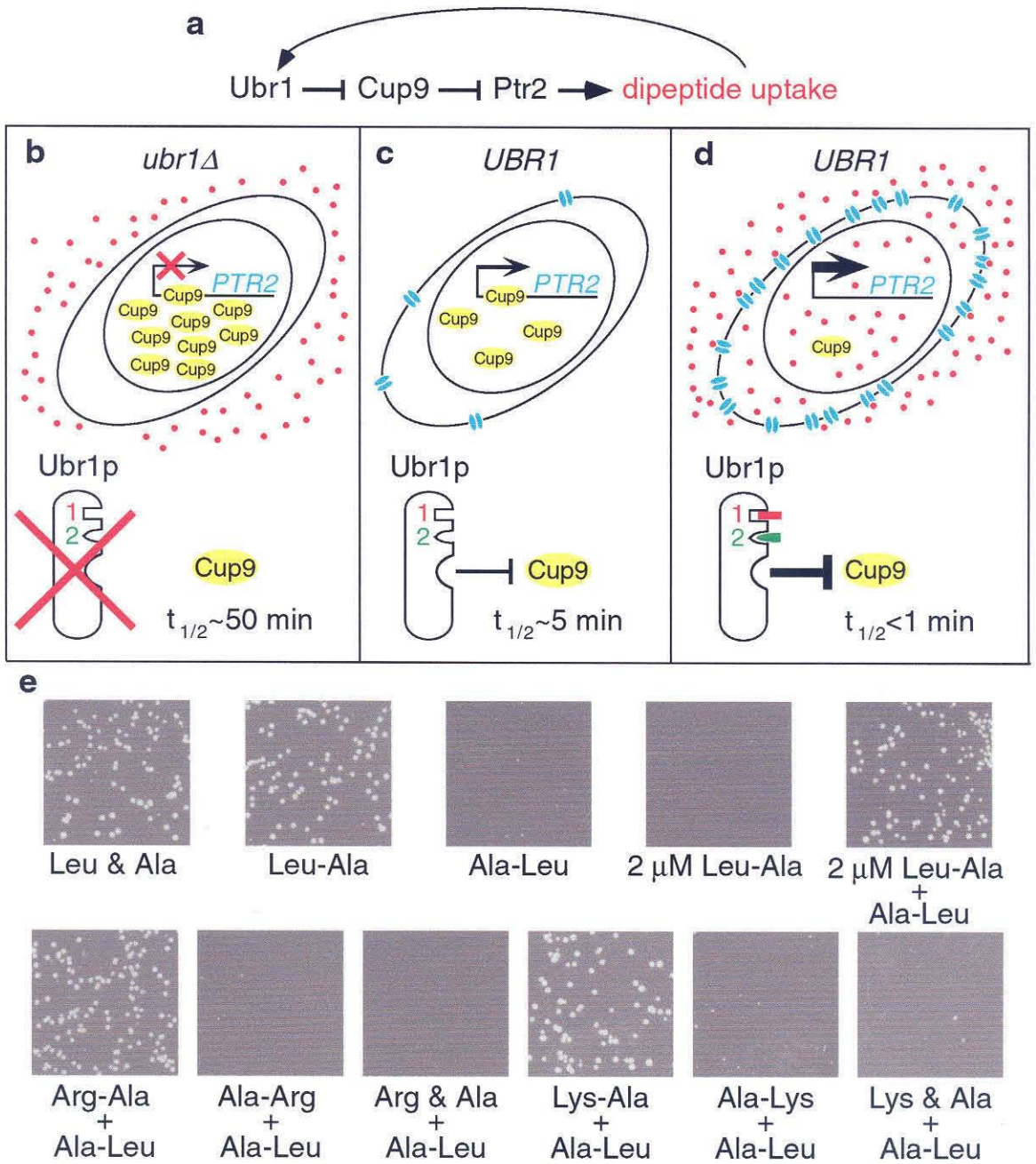

time (min)
–
0 5 15
Trp-Ala
0 5 15
Ala-Trp
0 5 15
–
0 5 15
Lys-Ala
0 5 15
Ala-Lys
0 5 15
Cup9-Ub$_n$
Cup9
time (min)
–
0 5 15
Arg-Ala
0 5 15
Ala-Arg
0 5 15
Arg&Ala
0 5 15
–
0 5 15
Tyr-Ala
0 5 15
Ala-Tyr
0 5 15
Tyr&Ala
0 5 15
Cup9-Ub$_n$
Cup9

Figure 4. Feedback regulation of peptide import in *S. cerevisiae*. **a**, Genetic diagram of the peptide transport circuit. **b**, Ubr1p is required for dipeptide uptake. In the absence of Ubr1p (*ubr1Δ*), the transcriptional repressor Cup9p is long-lived, accumulates to high levels, and extinguishes the expression of the *PTR2* gene. Thus, *ubr1Δ* cells cannot import dipeptides (red dots). **c,** In a wildtype (*UBR1*) cell growing in the absence of extracellular dipeptides, Ubr1p targets Cup9p for degradation ($t_{1/2}$ ~5 min), resulting in a moderate concentration of Cup9p and weak but significant expression of the Ptr2p transporter (blue double ovals). **d,** In wildtype (*UBR1*) cells growing in the presence of extracellular dipeptides some of which bear destabilizing N-terminal residues, the imported dipeptides bind to either the basic (type 1) or the hydrophobic (type 2) residue-binding site of Ubr1p. These peptides are denoted as a red block and a green wedge, respectively. Binding of either type of dipeptide to Ubr1p allosterically increases the rate of Ubr1p-mediated degradation of Cup9p. Peptides of both types are shown as bound to Ubr1p, but in fact the binding of either peptide accelerates Cup9p degradation. The resulting decrease of the half-life of Cup9p from ~5 min to less than 1 min results in a very low concentration of Cup9p, and consequently to a strong induction of the Ptr2p transporter. **e,** Colony formation assays. A *S. cerevisiae* strain requiring leucine for growth was incubated on plates supplemented with either dipeptides or their amino acid constituents at the following concentrations: Leu-Ala, 230 μM (or 2 μM, as indicated); Ala-Leu, 230 μM;

Arg-Ala, 10 μM; Ala-Arg, 10 μM; Lys-Ala, 1 μM; Ala-Lys, 1 μM; Arg and Ala, 10 μM each, Lys and Ala, 1 μM each.


a
Ubr1
Cup9
Ptr2
dipeptide uptake
b
ubr1Δ
PTR2
Cup9
Ubr1p
1
2
Cup9
t 1/2~50 min
c
UBR1
PTR2
Cup9
Ubr1p
1
2
Cup9
t 1/2~5 min
d
UBR1
PTR2
Cup9
Ubr1p
1
2
Cup9
t 1/2<1 min
e
Leu & Ala
Leu-Ala
Ala-Leu
2 μM Leu-Ala
2 μM Leu-Ala + Ala-Leu
Arg-Ala + Ala-Leu
Ala-Arg + Ala-Leu
Arg & Ala + Ala-Leu
Lys-Ala + Ala-Leu
Ala-Lys + Ala-Leu
Lys & Ala + Ala-Leu

Chapter 4

Amino acids induce dipeptide transport in *S. cerevisiae* by accelerating Ubr1-mediated degradation of the transcriptional repressor Cup9

Glenn C. Turner, Christopher C. Byrd, and Alexander Varshavsky

(prepared for submission)

ABSTRACT

The dipeptide transporter *PTR2* of *S. cerevisiae* is under multiple regulatory controls that link its expression to the quality of the available nitrogen source. Previous work has shown that free amino acids induce *PTR2* expression and that this induction requires Ssy1p and Ptr3p. Ssy1p is a homolog of amino acid transporters, and is proposed to function as an amino acid sensor (12, 17). Ptr3p is a novel protein downstream of Ssy1p in the amino acid signaling pathway (6, 21). Our results, described below, demonstrate that this signaling pathway regulates activity of a ubiquitin-dependent protein degradation pathway. Ubr1, the E3 of the N-end rule pathway (43), has been shown to regulate dipeptide uptake by controlling degradation of Cup9p, a homeodomain-containing repressor of *PTR2* (10). Our results demonstrate that activity of the Ssy1p-Ptr3p pathway stimulates Ubr1-dependent degradation of Cup9p, thereby inducing *PTR2* expression

INTRODUCTION

Protein degradation by the ubiquitin (Ub)-proteasome system controls the intracellular concentrations of many regulatory proteins (16, 24, 32, 44). Substrates of the Ub-proteasome system are ubiquitylated by a cascade of three enzymes, E1, E2 and E3. E1 utilizes ATP to form a high-energy thioester linkage with Ub. E1 transfers this Ub thioester to a cysteine residue of an E2 (Ub conjugating enzyme; Ubc). E2 enzymes function in concert with E3 ubiquitin-protein ligases to attach Ub to the substrate. E3s provide specificity to the ubiquitylation reaction by recognizing a degradation signal (degron) carried by the target. In some cases, E3s act by bringing E2 and substrate into proximity,

and then stimulating E2 to synthesize a multiUb chain linked to an accessible lysine residue of the substrate (35). Other classes of E3s directly participate in the transfer of Ub to the substrate (31). The resulting ubiquitylated protein bears a covalently linked multiUb chain, and is degraded by the 26S proteasome (reviewed in (8, 11)).

Recent work has shown that the uptake of peptides in *S. cerevisiae* is regulated by a component of the Ub-proteasome system (1, 10). Ubr1p, the E3 of the N-end rule pathway (43), regulates peptide uptake by controlling degradation of Cup9p, a homeodomain protein that represses transcription of the di- and tripeptide transporter Ptr2p (10).

Ubr1p was originally identified as the recognition component of the N-end rule pathway, which targets proteins carrying a degradation signal termed the N-degron (3, 7). An N-degron comprises a destabilizing N-terminal residue, and a lysine residue for multiUb chain attachment (4). In *S. cerevisiae*, Ubr1p directly recognizes two classes of destabilizing residues, basic, or type 1 (Arg, Lys and His) and bulky hydrophobic, or type 2 (Phe, Leu, Tyr, Trp and Ile) (43). Dipeptides bearing destabilizing N-terminal residue of either basic or hydrophobic type act as competitive inhibitors of the degradation of N-end rule substrates carrying the same type of destabilizing residue (5, 15, 30).

In contrast, the same dipeptides are allosteric activators of Ubr1p-dependent ubiquitylation of Cup9p (40). This interaction forms the basis of a positive feedback loop, where imported dipeptides accelerate Ubr1p-dependent degradation of Cup9p, thereby de-repressing the synthesis of the Ptr2p transporter and enhancing the cell's ability to import di- and tripeptides.

A variety of other regulatory inputs couple *PTR2* expression to the quality and availability of the nitrogen source(s) present. For example, *PTR2* expression is repressed by certain nitrogen sources, including ammonia, but not by others, such as urea and allantoin (9, 28). This ensures that a cell does not expend energy synthesizing proteins required for dipeptide uptake when dipeptides are absent and a nitrogen source that supports optimal growth, such as ammonia, is present.

A separate pathway induces *PTR2* expression in the presence of any of a number of different amino acids (18). Like all fungi, yeast are scavenging heterotrophs, so the amino acids they encounter outside a laboratory environment are usually the breakdown products of peptides. Thus, the presence of amino acids may signal the availability of di- and tripeptides in the environment. Also, the possibility that Ptr2p itself transports amino acids, although unlikely, has not been excluded. For example, AtPtr2 and AtCHL1, members of the Ptr2 family from *Arabidopsis thaliana*, transport histidine and nitrate respectively (13, 39). Either of these scenarios would explain why amino acids are important regulators of *PTR2* expression.

Two components of the amino acid induction pathway have been identified. Ssy1p, a plasma membrane protein with similarity to amino acid transporters, has been proposed to act as a sensor of extracellular amino acid concentrations (12, 17). Ptr3p, a novel protein of unknown function, is presumed to be a component of the signal transduction apparatus (1, 6, 21). Both Ssy1p and Ptr3p are required for the induction of the dipeptide transporter Ptr2p in response to amino acid concentrations as low as 1 μM (18). A variety of

amino acid transporters are also subject to amino acid induction via the Ssy1p-Ptr3p pathway (12, 17, 21).

Here we show that this amino acid induction pathway controls dipeptide uptake by regulating Ubr1p-dependent degradation of the Cup9p repressor. Genetic and biochemical interaction assays indicate that Cup9p acts with the corepressors Tup1p and Ssn6p to repress *PTR2*. Ssy1p and Ptr3p, components of the amino acid sensing pathway, are shown to induce *PTR2* expression by accelerating Ubr1-dependent degradation of Cup9p, thereby alleviating Cup9p-Tup1p-Ssn6p repression. These results establish a link between a signaling pathway responsible for the induction of dipeptide transport by amino acids, and the activity of a specific Ub-dependent proteolytic pathway.

MATERIALS AND METHODS

Yeast strains, media, and genetic techniques.

Yeast strain descriptions are shown in Table 1. Strains AVY24 and AVY25 were constructed in the RJD347 background (*MATα ura3-52*; a gift from R. J. Deshaies, Caltech) by a PCR-based knock-out strategy (34) utilizing DNA fragments containing the MYC_3-URA3-MYC_3 cassette (MYC_3 is three repeats of the MYC epitope) flanked on either side by 50 bp of homology to the *SSY1* or *PTR3* locus, respectively. This strategy was used to create precise deletions of the *SSY1* and *PTR3* open reading frames. Strains containing *ssn6Δ::HisG* or *tup1Δ::HisG* deletions were constructed using a NotI-EcoRI restriction fragment carrying *ssn6Δ::HisG-URA3-HisG* from pSsn6Δ and a SpeI-XhoI fragment carrying *tup1Δ::HisG-URA3-HisG* from pTup1Δ, respectively. To select for

recombination within the *HisG-URA3-HisG* cassette, which generates *ssn6Δ::HisG* and *tup1Δ::HisG*, 5-fluoroorotic acid-resistant colonies were isolated, and strain identity confirmed by Southern blotting (data not shown). This procedure was performed in the AVY1 background (19) to generate strains AVY60 and AVY61, in the JD55 background (*MAT**a** ura3-52 his3-Δ200 leu2-3,112 trp1-Δ63 lys2-801 ubr1Δ::HIS3*) (26) to generate AVY62 and AVY63, in the AVY50 background (10) to generate AVY64 and AVY65, and in the AVY51 background (10) to generate AVY66 and AVY67.

In Figure 1, yeast strains AVY1, AVY107, AVY51, AVY62 and AYV63 were cultured on synthetic medium (SD) containing 2% glucose, 0.67% yeast nitrogen base without amino acids (containing 5 g/L ammonium sulfate), supplemented with 20 mg/L each of histidine, leucine, tryptophan and uracil, and 20 mg/L of lysine, or lysyl-alanine dipeptide (Lys-Ala), as indicated. For the experiments in Figure 2A, yeast strains AVY1, AVY107, AVY50, AVY51, and AVY60 through AVY67 were cultured in SD supplemented with 20 mg/L each of histidine, leucine, lysine, tryptophan and uracil. For the experiments in Figure 3, yeast were cultured in SHM medium, either containing or lacking 20 mg/L tryptophan. SHM consists of 2% glucose, 0.67% yeast nitrogen base without amino acids and without ammonium sulfate, and allantoin (1 g/L).

Plasmids.

$_{F}$DHFR-Ub-Cup9p$_{NSF}$ was expressed from the P_{MET25} promoter on the centromeric vector p416MET25 (27, 40). Ssn6p tagged at the C-terminus with two copies of the MYC epitope (Ssn6$_{MYCx2}$) expressed from its own promoter on a high copy (2μ) plasmid was a generous gift from R. Zitomer (SUNY Albany).

For plasmid-based complementation assays, plasmids containing *SSN6* (pSSN6) and *TUP1* (pTUP1) were constructed by standard subcloning and PCR-amplification techniques (2). PCR-amplification of genomic DNA was used to generate a fragment containing*SSN6* flanked by 901 bp of upstream sequence and 400 bp of downstream sequence that was inserted into the NotI and EcoRI sites of plasmid pRS314 (36). The same procedure was used to generate a fragment containing *TUP1* flanked by 459 bp of upstream sequence and 400 bp of downstream sequence that was inserted into the SpeI and ClaI sites of plasmid pRS416 (36).

pSsn6Δ, which carries *ssn6Δ::HisG-URA3-HisG*, was constructed by first subcloning the NotI-EcoRI fragment containing *SSN6* from pSSN6 into the NotI and EcoRI sites of pBluescript (Stratagene), generating pBlueSSN6. PCR was then used to introduce a deletion into the plasmid from 50 bp upstream of the *SSN6* start codon to position 2585 of the open reading frame, while simultaneously introducing a EclXI site at the upstream breakpoint of the deletion. This enabled the insertion of a EclXI-PstI fragment carrying the *HisG-URA3-HisG* cassette from pAS135, a gift of A. Sil (UCSF). Integration of the NotI-EcoRI fragment of this plasmid, carrying *ssn6Δ::HisG-URA3-HisG* , creates a deletion of the *SSN6* open reading frame spanning positions -50 to +2585 (relative to the start codon). pTup1Δ, which carries *tup1Δ::HisG-URA3-HisG*, was constructed using a similar strategy. The SpeI-ClaI fragment containing *TUP1* from pTUP1 was subcloned into the corresponding sites of pBluescript, generating pBlueTUP1. PCR was used to introduce a deletion from 7 bp upstream of the *TUP1* start codon to position 2016 of the open reading frame,

while simultaneously introducing a BamHI site at the deletion breakpoint. A BamHI-EcoRI fragment carrying the *HisG-URA3-HisG* cassette from pAS135 was then inserted. Integration of the SpeI-XhoI fragment of this plasmid carrying*tup1Δ::HisG-URA3-HisG* creates a deletion of *TUP1* spanning positions -7 to +2016 (relative to the start codon).

RNA preparation and Northern analysis.

Strains and growth medium for each experiment are described above. Yeast were cultured to OD_{600} ~0.6. Total RNA was prepared (33), and 25 μg samples were electrophoresed in 1% formaldehyde-agarose gels, followed by blotting for Northern analysis (2).

Pulse-chase analysis and immunoprecipitations.

For coimmunoprecipitation experiments, cells bearing the indicated plasmids were cultured to OD_{600} ~1.0 in SD containing minimal auxotrophic supplements. Cells were harvested, washed in 1.0 mL of water and resuspended in 0.8 mL of ice-cold buffer C (50 mM Na-HEPES pH 7.5, 1 mM EDTA, 250 mM NaCl, 1 mg/mL ovalbumin) and transferred to chilled tubes, each containing 0.5 mL of 0.5-mm glass beads and a mixture of protease inhibitors (final concentrations 1 mM phenylmethylsulfonyl fluoride, 2 μg/mL aprotinin, 0.5 μg/mL leupeptin, and 0.7 μg/mL pepstatin). Extracts were prepared and immunoprecipitations carried out as described (14), using anti-FLAG M2 resin (Sigma), with two modifications. First, following incubation of the extract and anti-FLAG resin for 45 min at 4°C, the immunoprecipitate was washed once with 1 mL of buffer C, and then twice more with 1 mL buffer C lacking ovalbumin. Second, immunoprecipitates were eluted from the anti-

FLAG resin for 15 min at room temperature with 0.5 mg/mL FLAG peptide in buffer C lacking ovalbumin. This eluate was then combined with an equal volume of 2x SDS-PAGE loading buffer, and then the immunoprecipitate was fractionated by 13% SDS-PAGE, and immunoblotted as described (14). Immunoblotting was carried out with anti-MYC at 1:1000 dilution (Babco), followed by a secondary horseradish peroxidase-conjugated goat anti-mouse antibody (BioRad) at 1:1000 dilution. The blots were developed using ECL (Amersham).

For pulse-chase analysis, cells were cultured to OD_{600} ~0.8 in SHM either containing or lacking 20 mg/L tryptophan, an inducing amino acid (18). Cells were harvested, washed in 0.8 mL of SHM ± tryptophan, resuspended in 0.4 mL of the same medium, and radiolabeled for 5 min at 30°C with 0.16 mCi of ^{35}S-EXPRESS (New England Nuclear). Cells were pelleted, and resuspended in fresh SHM ± tryptophan containing 4 mM L-methionine and 2 mM L-cysteine. 0.1 mL samples were taken at the time points indicated and transferred to chilled tubes, each containing 0.5 mL of 0.5-mm glass beads, 0.7 mL of ice-cold lysis buffer (1% Triton-X100, 0.15 M NaCl, 5 mM EDTA, 50 mM Na-HEPES, pH 7.5), and the mixture of protease inhibitors described above. Extracts were prepared and immunoprecipitations carried out as described (14), using anti-FLAG M2 resin (Sigma). Immunoprecipitates were fractionated by 13% SDS-PAGE, and detected by autoradiography.

RESULTS

Cup9p interacts with the corepressor complex Tup1p-Ssn6p to repress *PTR2* expression.

Ubr1p regulates dipeptide uptake by controlling expression of the dipeptide transporter Ptr2p. Levels of *PTR2* mRNA are dramatically reduced in *ubr1Δ* strains (1). This suggests that a short-lived repressor of *PTR2* accumulates in *ubr1Δ* cells, shutting down *PTR2* transcription. A bypass screen for mutations that restore dipeptide uptake and *PTR2* expression in *ubr1Δ* cells identified the homeodomain protein Cup9p as a repressor of *PTR2* (10). Further analysis of this collection of bypass mutants identified additional isolates that did not fall into the Cup9 complementation group. To understand more clearly how Ubr1p regulates dipeptide uptake, we sought to identify these other bypass mutants.

Many of these mutants exhibit clumpy growth in liquid culture (flocculation), and reduced mating ability, phenotypes associated with a defect in either Tup1p or Ssn6p, two corepressor proteins. Tup1p and Ssn6p form a complex that is able to directly antagonize the basal transcription machinery; however, they must be recruited to individual promoters through interaction with specific DNA binding proteins (20, 29, 42, 46). One such DNA binding protein is the mating type regulator α2 (23, 37). In the absence of either Tup1p or Ssn6p, α2 repression of *MATa*-specific genes is defective, resulting in a mating-deficient, pseudo-diploid state (45).

To test the possibility that these isolates represent *tup1* or *ssn6* mutants, they were transformed with low copy plasmids containing either the *TUP1* or *SSN6* open reading frame expressed from their natural/endogenous promoters

(see Methods). These plasmids could rescue the clumpy growth and peptide uptake defects of all the flocculent bypass mutants. We classified the entire collection of 201 bypass suppressors into complementation groups using a combination of this plasmid-based complementation and mating-based complementation assays (which could not be comprehensive due to the severity of the sterility of certain isolates). The 201 isolates fall into three complementation groups defined by the *CUP9, TUP1* and *SSN6* genes (Table 2). There is a high likelihood that this screen is saturated, based on the number of isolates of each complementation group (Table 2; 30 isolates in the smallest (*tup1*) complementation group). However, as these bypass suppressors were not isolated in a conditional (e.g., temperature sensitive) screen, genes that are required for repression of *PTR2* in *ubr1Δ* cells but that are also essential for viability would not have been identified.

To verify the role of Tup1p and Ssn6p in regulating peptide uptake, we tested the effects of disrupting *TUP1* or *SSN6* on the ability of a *ubr1Δ* strain to import dipeptides. Congenic *tup1Δ ubr1Δ* and *ssn6Δ ubr1Δ* double mutants were constructed in a lysine auxotrophic (*lys2*) strain background by homologous recombination, and their identity verified by Southern blotting (data not shown). The ability of these double mutants to import dipeptides was then assayed by following their growth on medium containing the dipeptide lysyl-alanine (Lys-Ala) as the sole source of lysine (Figure 1). A *UBR1 lys2* strain is able to import sufficient Lys-Ala to support its growth on this medium, while a congenic *ubr1Δ lys2* strain cannot satisfy its lysine requirement with Lys-Ala, and fails to form visible colonies under these conditions. Both *tup1Δ ubr1Δ lys2*

and *ssn6Δ ubr1Δ lys2* strains formed colonies on Lys-Ala plates, as did a *cup9Δ ubr1Δ lys2* strain. Thus, deletions of *CUP9, TUP1* or *SSN6* are each capable of restoring dipeptide uptake to a *ubr1Δ* strain. The slightly smaller colonies formed by the *ssn6Δ ubr1Δ* strain on Lys-Ala supplemented plates can be attributed to a well-documented growth defect of *ssn6Δ* strains (45), which we also observe in the growth of this strain on a control lysine-supplemented plate.

The observation that *cup9, tup1*and *ssn6* mutants all bypass the block to dipeptide uptake in a *ubr1Δ* strain immediately suggests that Cup9p represses Ptr2p expression by recruiting the Tup1p-Ssn6p complex to the *PTR2* promoter. To test this model, we examined the effects of *tup1Δ* and *ssn6Δ* mutants on levels of Ptr2 mRNA (Figure 2A). Northern analysis shows that *PTR2* mRNA levels are strongly elevated in *cup9Δ ubr1Δ, tup1Δ ubr1Δ* and *ssn6Δ ubr1Δ* strains, relative to the *ubr1Δ* strain. Thus, *cup9Δ, tup1Δ* and *ssn6Δ* mutations all bypass the block to dipeptide uptake in *ubr1Δ* cells by relieving transcriptional repression of the dipeptide transporter *PTR2*. Moreover, *PTR2* mRNA levels are induced to a similar level in *cup9Δ, tup1Δ* and *ssn6Δ* single mutants, indicating that these genes also repress *PTR2* expression in *UBR1* cells. Fine comparisons of *PTR2* mRNA levels in these strains were not feasible due to lane to lane variability in the total amount of RNA loaded, as indicated by the uneven levels of *ACT1* mRNA in the different lanes. We attribute this to two factors: *tup1Δ* and *ssn6Δ* mutations impair the growth of yeast, leading to a reduced yield of RNA from these strains relative to strains without a significant growth defect, even when cultures are harvested at a comparable stage in the growth cycle. Moreover, *tup1*and *ssn6* mutations influence expression of many genes in *S.*

cerevisiae. These two factors complicate efforts to normalize levels of RNA between samples.

To verify that Cup9p acts in concert with the Tup1p-Ssn6p complex to repress *PTR2*, we examined the ability of Cup9p to interact with Ssn6p in a coimmunoprecipitation assay. Over-expression of wildtype Cup9p impedes cell growth (data not shown), which could interfere with the interpretation of these experiments. To avoid any such complications, we utilized a nontoxic derivative of Cup9p (40). Cup9p interacts with DNA through its homeodomain motif (22). Based on the previously studied interactions of other homeodomain proteins with DNA, an Asn (N) → Ser (S) substitution at position 265, within the recognition helix of the Cup9p homeodomain, is predicted to strongly reduce the affinity of Cup9p for DNA without causing a significant structural perturbation (47). This Cup9p derivative, tagged at the C-terminus with the FLAG epitope, is denoted $Cup9p_{NSF}$.

Extracts were prepared from cells co-expressing $Cup9p_{NSF}$ and C-terminally MYC-tagged Ssn6p. Anti-FLAG immunoprecipitates from these extracts were separated by SDS-PAGE and subjected to immunoblotting with anti-MYC antibodies. $Ssn6_{MYC}$ was specifically coprecipitated with $Cup9p_{NSF}$ in this assay; no $Ssn6_{MYC}$ was detected in immunoprecipitates of extracts from cells that did not also express by $Cup9p_{NSF}$ (Figure 2B). Although we did not examine the $Cup9p_{NSF}$ complexes we isolated for the presence of Tup1p, previous work has established that Ssn6p is found in a complex with Tup1p (42, 46), strongly suggesting that it is in fact a complex of Ssn6p and Tup1p that interacts with Cup9p.

Cup9 and Ubr1 are required for amino acid induction of Ptr2 mRNA levels.

PTR2 expression is induced when strains are grown in a medium containing certain amino acids (Leu, Trp, Lys, Ile, Thr, Phe, Val, His, Ala, Tyr, Gly) (18). Hydrophobic amino acids are amongst the strongest inducers, with effective concentrations as low as 1 μM (18). Increased Ubr1p-dependent degradation of Cup9p strongly enhances Ptr2p expression in response to imported dipeptides (40), so it was plausible that other signals regulating Ptr2 expression, such as inducing amino acids, may also act via Ubr1p and Cup9p.

To test this possibility we examined the effects of tryptophan, a strongly inducing amino acid, on Ptr2p mRNA levels in *ubr1Δ* and *cup9Δ* strains. These strains were grown in allantoin-based media (SHM; see Methods) containing or lacking 20 mg/L tryptophan. Allantoin is a non-repressing nitrogen source, so *PTR2* expression is not influenced by nitrogen catabolite repression under these conditions. *PTR2* mRNA levels were strongly induced by tryptophan in a *UBR1 CUP9* (wildtype) strain. *PTR2* levels were extremely low in cells grown in medium lacking amino acids; however, expression increased to a level comparable to that of *ACT1* in the presence of 20 mg/L tryptophan (Figure 3). In contrast, *PTR2* expression was not altered by the presence of tryptophan in a *cup9Δ* strain; *PTR2* was highly expressed in both growth conditions. *PTR2* mRNA was undetectable in a *ubr1Δ* strain grown in the presence or absence of tryptophan. Thus, both *UBR1* and *CUP9* are required for amino acid induction of *PTR2* expression by amino acids.

Two genes, *SSY1* and *PTR3*, are proposed to be part of an amino acid-sensing pathway in *S. cerevisiae* (21). Ssy1p is a transmembrane protein with homology to amino acid transporters (12, 17). Ptr3p is a novel protein, which interacts with Ssy1p (6, 21). Both *SSY1* and *PTR3* are required for amino acid induction of *PTR2* and several amino acid transporter genes. We examined the effects of 20 mg/L tryptophan on levels of *PTR2* mRNA in *ssy1Δ* and *ptr3Δ* strains, to compare with the effects of *ubr1Δ* and *cup9Δ* mutants. *PTR2* induction was dramatically reduced in both *ssy1Δ* and *ptr3Δ* strains; however, a small activation was still observed (Figure 3). These results indicate that the requirement for *UBR1* and *CUP9* for amino acid induction of *PTR2* is at least as great, if not greater than the requirement for *SSY1* and *PTR3*.

The components of an amino acid-sensing pathway, Ssy1p and Ptr3p, induce *PTR2* expression by enhancing Ubr1p-dependent degradation of Cup9p

The requirement for both *UBR1* and *CUP9* in amino acid induction of *PTR2* suggests that induction occurs by increasing Ubr1p-dependent degradation of Cup9p. We tested this possibility by measuring Cup9p degradation in response to amino acids. The DNA binding defective $Cup9p_{NSF}$, (described above; see also Methods) was expressed as part of a fusion of the form $_{F}$DHFR-Ub-$Cup9p_{NSF}$, where $_{F}$DHFR was the N-terminally FLAG-tagged mouse dihydrofolate reductase. Ub specific proteases (UBPs) cotranslationally cleave this fusion at the Ub-Cup9p junction, yielding the long-lived $_{F}$DHFR-Ub reference protein and the test protein $Cup9p_{NSF}$. The reference protein serves as an internal control for variations in expression levels and immunoprecipitation

efficiency, thereby increasing the accuracy of pulse-chase assays. This generally applicable method for producing a reference protein is called the UPR (Ub/protein/reference) technique (25, 38). The *in vivo* degradation of Cup9p was not altered by either the N265S substitution in $Cup9p_{NSF}$ or the incorporation of this protein into a UPR fusion (40).

Pulse-chase analysis (see Methods) shows that in wild type (*UBR1*) cells grown in SHM lacking amino acids, $Cup9p_{NSF}$ is degraded with a half-life of ~10 min (Figure 4). The presence of 20 mg/L tryptophan in the medium produced a significant, reproducible decrease in $Cup9p_{NSF}$ half-life to ~2.5 min (Figure 4 and data not shown). In contrast, $Cup9p_{NSF}$ half-life was not altered by tryptophan in a *ubr1Δ* strain ($t_{1/2}$ ~45 min; data not shown), indicating that Ubr1p was responsible for the enhancement of degradation by tryptophan. Additionally, both Ssy1p and Ptr3p were partially required for the tryptophan-accelerated degradation of $Cup9p_{NSF}$ (Figure 4). Consistent with the incomplete block to amino acid induction of Ptr2 observed in *ssy1Δ* and *ptr3Δ* mutants, $Cup9p_{NSF}$ degradation was still detectably induced by amino acids in these strains ($t_{1/2}$ ~5 min). These results demonstrate that Ubr1-dependent degradation of Cup9 degradation is induced by amino acids, and that Ssy1 and Ptr3 are partly required for this induction.

DISCUSSION

The uptake of dipeptides is controlled by multiple regulatory systems in *S. cerevisiae*. Nitrogen catabolite repression inhibits dipeptide transport in the

presence of a favorable nitrogen source, such as ammonia (9, 28). A separate regulatory pathway induces dipeptide transport when specific amino acids are present in the growth medium (18). Presumably, since yeast, like all fungi, are decomposers, the presence of amino acids in their environment indicates that dipeptides are also present. Previous work has shown that two gene products, Ssy1p and Ptr3p, are required for the amino acid-mediated transcriptional activation of the dipeptide transporter *PTR2*, and several amino acid permeases (21). Ssy1p encodes a protein with similarity to amino acid transporters (12, 17). Ptr3p is a novel protein, which has recently been shown to physically interact with Ssy1p (6, 41). Elevated intracellular levels of tryptophan do not induce amino acid permeases, suggesting that Ssy1p senses extracellular concentrations of amino acids (17). Ptr3p has a small motif found in many other proteins involved in amino acid metabolism (21). If this motif were shown to be an amino acid-binding domain, this would suggest that Ssy1p may transmit information to Ptr3p by modulating its interaction with amino acids, perhaps by loading amino acids directly onto Ptr3p.

We show here that amino acids induce dipeptide transport by activating Ubr1p-dependent degradation of Cup9p, thereby de-repressing *PTR2* expression. The results of this paper indicate that Cup9p exerts this repression via the corepressors Tup1p and Ssn6p. Tup1p and Ssn6p form a complex that directly interferes with the basal transcription machinery (29, 42, 46). The Tup1p-Ssn6p complex cannot bind to DNA; rather it is recruited to individual promoters by interaction with specific DNA binding proteins (20). Cup9p has previously been shown to bind directly to the *PTR2* promoter (10). Here we show that *CUP9*, *TUP1* and *SSN6* are all required for repression of *PTR2*.

Additionally, Cup9p is found in a complex with Ssn6p in immunoprecipitates from yeast extracts. Together, these results indicate that Cup9p represses *PTR2* expression by directing the Tup1p-Ssn6p complex to the *PTR2* promoter.

The induction of *PTR2* mRNA levels by amino acids requires both *UBR1* and *CUP9*. Moreover, this induction is accompanied by an increase in the rate of Ubr1p-mediated degradation of Cup9p. Thus amino acids elevate *PTR2* expression by inducing degradation of the Cup9p repressor. Both Ssy1p and Ptr3p, previously found to be components of the amino acid signaling pathway, are required for the acceleration of Cup9p degradation.

These results suggest a model in which Ssy1p senses extracellular concentrations of amino acids and initiates a signal transduction cascade, of which Ptr3p is a component. This cascade activates Ubr1p, increasing the rate of Cup9p degradation, thereby de-repressing *PTR2* expression (Figure 5). The precise mechanism by which this signal transduction cascade activates Ubr1p remains a topic for future investigation.

REFERENCES

1. **Alagramam, K., F. Naider, and J. M. Becker.** 1995. A recognition component of the ubiquitin system is required for peptide transport in *Saccharomyces cerevisiae*. Mol. Microbiol. **15:**225-234.

2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. A. Smith, J. G. Seidman, and K. Struhl (ed.).** 1996. Current Protocols in Molecular Biology. Wiley-Interscience, New York.

3. **Bachmair, A., D. Finley, and A. Varshavsky.** 1986. *In vivo* half-life of a protein is a function of its amino-terminal residue. Science **234:**179-186.

4. **Bachmair, A., and A. Varshavsky.** 1989. The degradation signal in a short-lived protein. Cell **56:**1019-1032.

5. **Baker, R. T., and A. Varshavsky.** 1991. Inhibition of the N-end rule pathway in living cells. Proc. Natl. Acad. Sci. USA **87:**2374-2378.

6. **Barnes, D., W. Lai, M. Breslav, F. Naider, and J. M. Becker.** 1998. *PTR3*, a novel gene mediating amino acid-inducible regulation of peptide transport in *Saccharomyces cerevisiae*. Mol. Microbiol. **29:**297-310.

7. **Bartel, B., I. Wünning, and A. Varshavsky.** 1990. The recognition component of the N-end rule pathway. EMBO J. **9:**3179-3189.

8. **Baumeister, W., J. Walz, F. Zühl, and E. Seemüller.** 1998. The proteasome: paradigm of a self-compartmentalizing protease. Cell **92:**367-380.

9. **Becker, J. M., and F. Naider.** 1980. Peptide transport in yeast: uptake of radioactive trimethionine in *S. cerevisiae*. Arch. Biochem. Biophys. **178:**245-255.

10. **Byrd, C., G. C. Turner, and A. Varshavsky.** 1998. The N-end rule pathway controls the import of peptides through degradation of a transcriptional repressor. EMBO J. **17:**269-277.

11. **DeMartino, G. N., and C. A. Slaughter.** 1999. The proteasome, a novel protease regulated by multiple mechanisms. J. Biol. Chem. **274:**22123-22126.

12. **Didion, T., B. Regenberg, M. U. Jørgesen, M. C. Kielland-Brandt, and H. A. Andersen.** 1998. The permease homologue Ssy1p controls the expression of amino acid and peptide transporter genes in *Saccharomyces cerevisiae*. Mol. Microbiol. **27:**643-650.

13. **Frommer, W. B., S. Hummel, and D. Rentsch.** 1994. Cloning of an *Arabidopsis* histidine transporting protein related to nitrate and peptide transporters. FEBS Letters **347:**185-189.

14. **Ghislain, M., R. J. Dohmen, F. Levy, and A. Varshavsky.** 1996. Cdc48p interacts with Ufd3p, a WD repeat protein required for ubiquitin-mediated proteolysis in *Saccharomyces cerevisiae*. EMBO J. **15:**4884-4899.

15. **Gonda, D. K., A. Bachmair, I. Wünning, J. W. Tobias, W. S. Lane, and A. Varshavsky.** 1989. Universality and structure of the N-end rule. J. Biol. Chem. **264:**16700-16712.

16. **Hershko, A., and A. Ciechanover.** 1998. The ubiquitin system. Annu. Rev. Biochem. **76:**425-479.

17. **Iraqui, I., S. Vissers, F. Bernard, J. O. De Craene, E. Boles, A. Urrestarazu, and B. Andre.** 1999. Amino acid signaling in *Saccharomyces cerevisiae:* a permease-like sensor of external amino acids and F-box protein Grr1p are required for transcriptional induction of the *AGP1* gene, which encodes a broad-specificity amino acid permease. Mol. Cell. Biol. **19:**989-1001.

18. **Island, M. D., F. Naider, and J. M. Becker.** 1987. Regulation of dipeptide transport in *S. cerevisiae* by micromolar amino acid concentrations. J. Bact. **169:**2132-2136.

19. **Johnson, E. S., P. C. Ma, I. M. Ota, and A. Varshavsky.** 1995. A proteolytic pathway that recognizes ubiquitin as a degradation signal. J. Biol. Chem. **270:**17442-17456.

20. **Keleher, C. A., M. J. Redd, J. Schultz, M. Carlson, and A. D. Johnson.** 1992. Ssn6-Tup1 is a general repressor of transcription in yeast. Cell **68:**709-719.

21. **Klasson, H., G. R. Fink, and P. O. Ljungdahl.** 1999. Ssy1p and Ptr3p are plasma membrane components of a yeast system that senses extracellular amino acids. Mol. Cell. Biol. **19:**5405-5416.

22. **Knight, S. A. B., K. T. Tamai, D. J. Kosman, and D. J. Thiele.** 1994. Identification and analysis of a *Saccharomyces cerevisiae* copper homeostasis gene encoding a homeodomain protein. Mol. Cell. Biol. **14:**7792-7804.

23. **Komachi, K., M. J. Redd, and A. D. Johnson.** 1994. The WD repeats of Tup1 interact with the homeodomain protein α2. Genes Dev. **8:**2857-2867.

24. **Laney, J. D., and M. Hochstrasser.** 1999. Substrate targeting in the ubiquitin system. Cell **97:**427-30.

25. **Lévy, F., N. Johnsson, T. Rumenapf, and A. Varshavsky.** 1996. Using ubiquitin to follow the metabolic fate of a protein. Proc. Natl. Acad. Sci. USA **93:**4907-4912.

26. **Madura, K., and A. Varshavsky.** 1994. Degradation of Gα by the N-end rule pathway. Science **265:**1454-1458.

27. **Mumberg, D., R. Muller, and M. Funk.** 1994. Regulatable promoters of *Saccharomyces cerevisiae* - comparison of transcriptional activity and their use for heterologous expression. Nucl. Acids Res. **22:**5767-5768.

28. **Nisbet, T. M., and J. W. Payne.** 1979. Peptide uptake in *Saccharomyces cerevisiae*: characteristics of a transport system shared by di and tripeptides. J. Gen. Microbiol. **115:**127-133.

29. **Redd, M. J., M. B. Arnaud, and A. D. Johnson.** 1997. A complex composed of Tup1 and Ssn6 represses transcription *in vitro*. J. Biol. Chem. **272:**11193-11197.

30. **Reiss, Y., D. Kaim, and A. Hershko.** 1988. Specificity of binding of N-terminal residues of proteins to ubiquitin-protein ligase. Use of amino acid derivatives to characterize specific binding sites. J. Biol. Chem. **263:**2693-269.

31. **Scheffner, M., U. Nuber, and J. M. Huibregtse.** 1995. Protein ubiquitination involving an E1-E2-E3 enzyme ubiquitin thioester cascade. Nature **373:**81-83.

32. **Scheffner, M., S. Smith, and S. Jentsch.** 1998. The ubiquitin conjugation system., p. 65-98. *In* J.-M. Peters, J. R. Harris, and D. Finley (ed.), Ubiquitin and the Biology of the Cell. Plenum Press, New York.

33. **Schmitt, M. E., T. A. Brown, and B. L. Trumpower.** 1990. A rapid and simple method for preparation of RNA from *Saccharomyces cerevisiae*. Nucl. Acids Res. **18:**3091-3092.

34. **Schneider, B. L., W. Seufert, B. Steiner, Q. H. Yang, and A. B. Futcher.** 1995. Use of polymerase chain-reaction epitope tagging for protein tagging in *Saccharomyces cerevisiae*. Yeast **11:**1265-1274.

35. **Seol, J. H., R. M. Feldman, W. Zachariae, A. Shevchenko, C. C. Correll, S. Lyapina, Y. Chi, M. Galova, J. Glaypool, S. Sanmeyer, K. Nasmyth, and R. J. Deshaies.** 1999. Cdc53/cullin and the essential Hrt1 RING-H2 subunit of SCF define a ubiquitin ligase module that activates the E2 enzyme Cdc34. Genes Dev. **13:**1614-1626.

36. **Sikorski, R. S., and P. Hieter.** 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *S. cerevisiae*. Genetics **122:**19-27.

37. **Smith, R. L., M. J. Redd, and A. D. Johnson.** 1995. The tetratricopeptide repeats of Ssn6 interact with the homeodomain of α2. Genes Dev. **9:**2903-2910.

38. **Suzuki, T., and A. Varshavsky.** 1999. Degradation signals in the lysine-asparagine sequence space. EMBO J. **18:**6017-6026.

39. **Tsay, Y. F., J. I. Schroeder, K. A. Feldmann, and N. M. Crawford.** 1993. The herbicide sensitivity gene *Chl1* of *Arabidopsis* encodes a nitrate-inducible nitrate transporter. Cell **72:**705-713.

40. **Turner, G. C., F. Du, and A. Varshavsky.** in press. Peptides accelerate their uptake by activating a ubiquitin-dependent proteolytic pathway. Nature.

41. **Uetz, P., L. Giot, G. Cagney, T. A. Mansfield, R. S. Judson, J. R. Knight, D. Lockshon, V. Narayan, M. Srinivasan, P. Pochart, A. Qureshi-Emili, Y. Li, B. Godwin, D. Conover, T. Kalbfleisch, G. Vijayadamodar, M. J. Yang, M. Johnston, S. Fields, and J. M. Rothberg.** 2000. A comprehensive analysis of protein-protein interactions in *Saccharomyces cerevisiae*. Nature **403:**623-627.

42. **Varanasi, U. S., M. Klis, P. B. Mikesell, and R. J. Trumbly.** 1996. The Cyc8 (Ssn6)-Tup1 corepressor complex is composed of one Cyc8 and four Tup1 subunits. Mol. Cell. Biol. **16:**6707-6714.

43. **Varshavsky, A.** 1997. The N-end rule pathway of protein degradation. Genes Cells **2:**13-28.

44. **Varshavsky, A.** 1997. The ubiquitin system. Trends Biochem. Sci. **22:**383-387.

45. **Wahi, M., and A. D. Johnson.** 1995. Identification of genes required for α2 repression in *Saccharomyces cerevisiae*. Genetics **140:**79-90.

46. **Williams, F. E., U. Varanasi, and R. J. Trumbly.** 1991. The Cyc8 and Tup1 Proteins involved in glucose repression in *Saccharomyces cerevisiae* are associated in a protein complex. Mol. Cell. Biol. **11:**3307-3316.

47. **Wolberger, C., A. K. Vershon, B. S. Liu, A. D. Johnson, and C. O. Pabo.** 1991. Crystal-structure of a Mat α2 homeodomain-operator complex suggests a general model for homeodomain-DNA interactions. Cell **67:**517-528.

Strain	Genotype	Reference
AVY1	*MAT**a** ura3-52 his3-Δ200 leu2-3,112 trp1-Δ63 lys2-801*	JD52 in (19)
AVY24	*MATα ura3-52 ssy1Δ::* MYC_3*-URA3-*MYC_3	This study
AVY25	*MATα ura3-52 ptr3Δ::*MYC_3*-URA3-*MYC_3	This study
AVY30	*MATα leu2-3,112 ubr1Δ::LEU2*	(40)
AVY31	*MATα leu2-3,112 cup9Δ::LEU2*	(40)
AVY32	*MATα LEU2*	(40)
AVY50	*MAT**a** ura3-52 his3-Δ200 leu2-3,112 trp1-Δ63 lys2-801 cup9Δ::LEU2*	CBY19 in(10)
AVY51	*MAT**a** ura3-52 his3-Δ200 leu2-3,112 trp1-Δ63 lys2-801 ubr1Δ::HIS3 cup9Δ::LEU2*	CBY17 in(10)
AVY60	*MAT**a** ura3-52 his3-Δ200 leu2-3,112 trp1-Δ63 lys2-801 ssn6Δ::HisG*	This study
AVY61	*MAT**a** ura3-52 his3-Δ200 leu2-3,112 trp1-Δ63 lys2-801 tup1Δ::HisG*	This study
AVY62	*MAT**a** ura3-52 his3-Δ200 leu2-3,112 trp1-Δ63 lys2-801 ubr1Δ::HIS3 ssn6Δ::HisG*	This study
AVY63	*MAT**a** ura3-52 his3-Δ200 leu2-3,112 trp1-Δ63 lys2-801 ubr1Δ::HIS3 tup1Δ::HisG*	This study
AVY64	*MAT**a** ura3-52 his3-Δ200 leu2-3,112 trp1-Δ63 lys2-801 cup9Δ::LEU2 ssn6Δ::HisG*	This study
AVY65	*MAT**a** ura3-52 his3-Δ200 leu2-3,112 trp1-Δ63 lys2-801 cup9Δ::LEU2 tup1Δ::HisG*	This study
AVY66	*MAT**a** ura3-52 his3-Δ200 leu2-3,112 trp1-Δ63 lys2-801 ubr1Δ::HIS3 cup9Δ::LEU2 ssn6Δ::HisG*	This study
AVY67	*MAT**a** ura3-52 his3-Δ200 leu2-3,112 trp1-Δ63 lys2-801 ubr1Δ::HIS3 cup9Δ::LEU2 tup1Δ::HisG*	This study
AVY107	*MAT**a** ura3-52 his3-Δ200 leu2-3,112 trp1-Δ63 lys2-801 ubr1Δ:::*MYC_3	(40)

Table 1. Yeast strains used in this study.

Gene	# representatives
cup9	104
ssn6	67
tup1	30

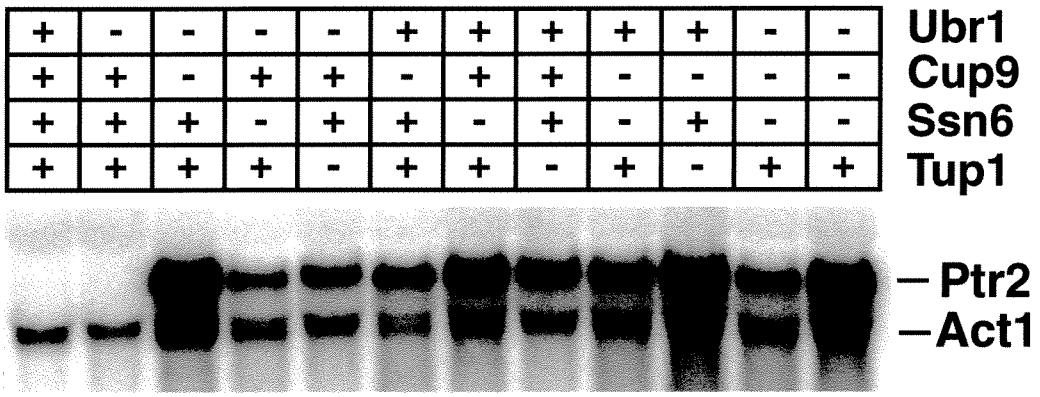
Table 2. Suppressors of the block to dipeptide uptake in *ubr1Δ*. A collection of 201 mutants that restored the ability of a *ubr1Δ* strain to import dipeptides were classed into complementation groups using plasmid-based or mating-based complementation tests. The number of representatives in each complementation group, *cup9*, *tup1*, and *ssn6*, is shown.

Figure 1. *ssn6Δ* and *tup1Δ* restore dipeptide uptake to a *ubr1Δ* strain. Strains with genotypes indicated on the schematic plate were tested for the ability to use dipeptides as a nutrient source. All strains were auxotropic for lysine (*lys2*). Strains were tested for growth on solidified SD medium lacking lysine (left), supplemented with lysine (right) or supplemented with lysyl-alanine dipeptide (Lys-Ala; center). Only strains capable of importing dipeptides could form colonies on the Lys-Ala plate. Deletion of either *SSN6*, *TUP1* or *CUP9* restored dipeptide uptake to a *ubr1Δ* strain.

-lys
-lys + Lys-Ala
+lys
UBR1
ubr1Δ
ubr1Δ tup1Δ
ubr1Δ ssn6Δ
ubr1Δ cup9Δ

Figure 2. *CUP9* represses *PTR2* transcription via *SSN6* and *TUP1*

(A) *SSN6*, *TUP1* and *CUP9* are required for repression of *PTR2*. RNA blot analysis of *PTR2* expression in *ssn6Δ*, *tup1Δ*, *cup9Δ* and *ubr1Δ* mutants and various combinations thereof is shown. The status of each gene (- = deleted; + = wildtype) is denoted above. *PTR2* and *ACT1* mRNA are indicated. (B) Coimmunoprecipitation of MYC-tagged Ssn6p ($Ssn6_{MYCx2}$) with a FLAG-tagged Cup9p variant ($Cup9p_{NSF}$; see Results). Extracts from cells expressing the indicated combinations of $Ssn6_{MYCx2}$ and $Cup9p_{NSF}$ were immunoprecipitated with anti-FLAG resin, separated by SDS-PAGE, and immunoblotted with anti-MYC antibody.


+ - - - - + + + + + - -
+ + - + + - + + - - - -
+ + + - + + - + - + - -
+ + + + - + + - + - + +
Ubr1
Cup9
Ssn6
Tup1
— Ptr2
—Act1

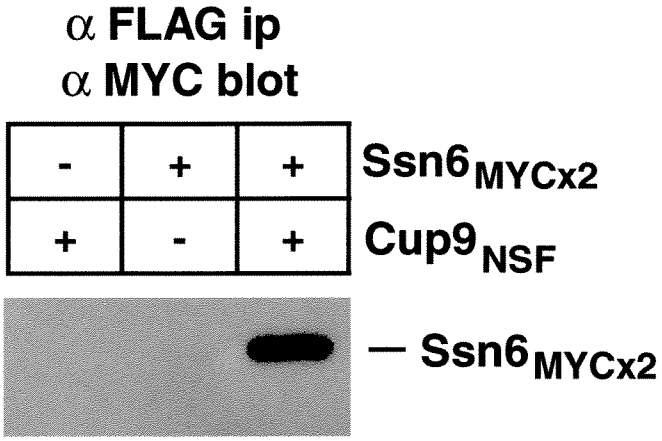

α FLAG ip
α MYC blot
- + +
+ - +
Ssn6MYCx2
Cup9NSF
— Ssn6MYCx2

Figure 3. Amino acid induction of *PTR2* requires *UBR1* and *CUP9*. Strains of the indicated genotype were grown in SHM containing or lacking 20 mg/L tryptophan. RNA was harvested, and blotted for levels of *PTR2* and *ACT1*, as indicated.

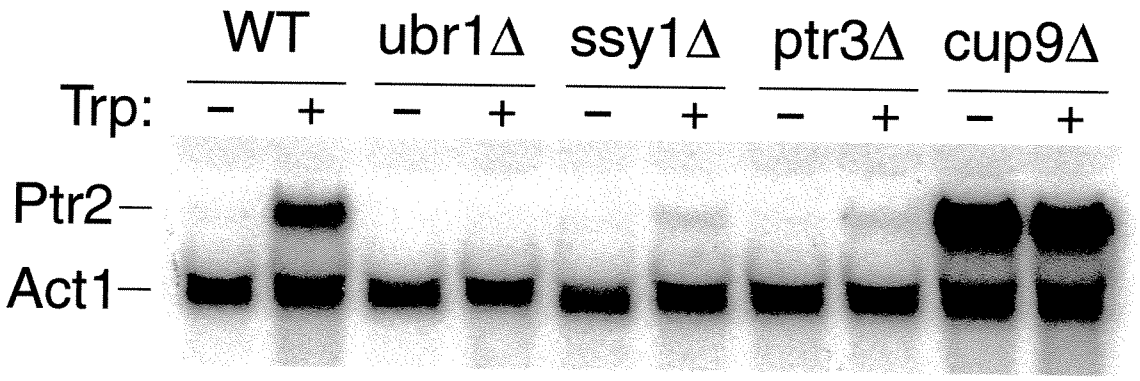
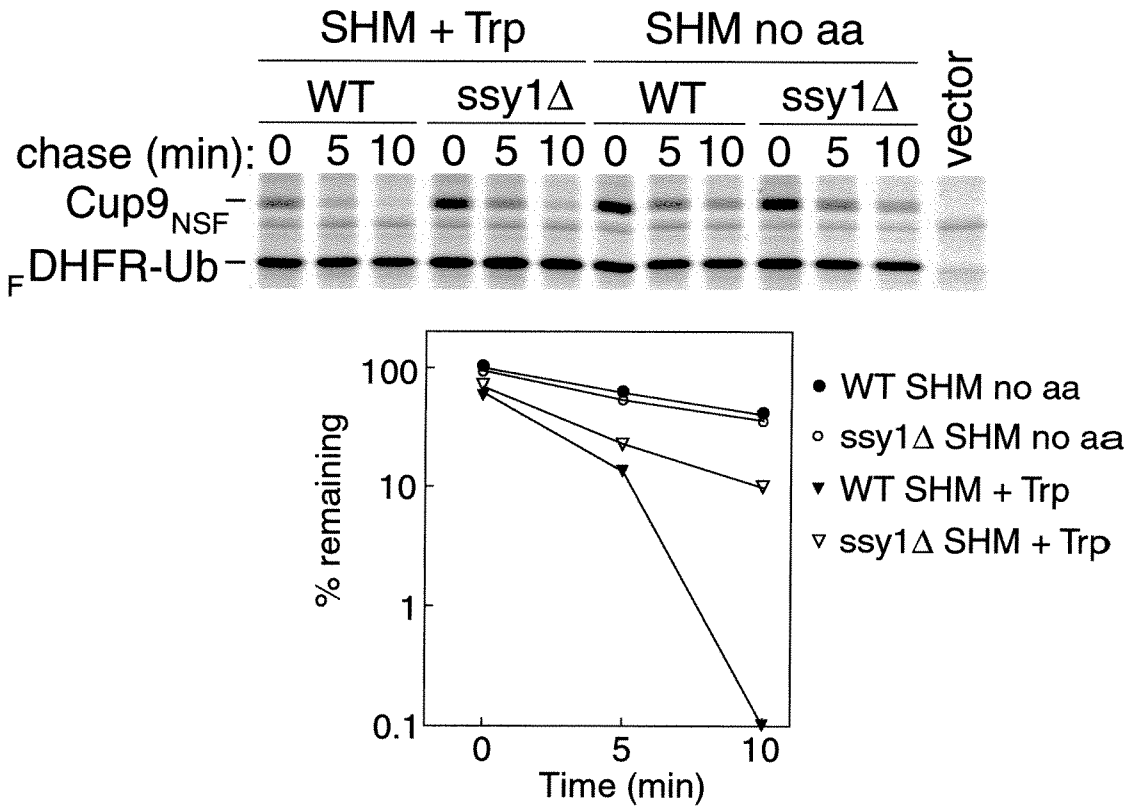

WT
ubr1Δ
ssy1Δ
ptr3Δ
cup9Δ
Trp:
– + – + – + – + – +
Ptr2–
Act1–

Figure 4. *SSY1* and *PTR3* enhance $Cup9_{NSF}$ degradation in the presence of amino acids. Pulse chase analysis of the $_{F}$DHFR-Ub-$Cup9p_{NSF}$ fusion protein is shown (see Results). Strains of the indicated genotype were grown in SHM containing or lacking 20 mg/L tryptophan. Cells were subjected to a 5 min radiolabel pulse, followed by a chase with 0, 5 and 10 min time points. Both the $_{F}$DHFR-Ub reference protein and $Cup9p_{NSF}$ were detected by immunoprecipitation with anti-FLAG. Decay curves of $Cup9p_{NSF}$ are plotted to the right, showing both the acceleration of $Cup9p_{NSF}$ degradation by the presence of tryptophan, and the partial dependence of this acceleration on *SSY1* and *PTR3*.


SHM + Trp
SHM no aa
WT
ssy1Δ
WT
ssy1Δ
vector
chase (min): 0 5 10 0 5 10 0 5 10 0 5 10
Cup9NSF–
FDHFR-Ub–
100
10
1
0.1
% remaining
0
5
10
Time (min)
WT SHM no aa
ssy1Δ SHM no aa
WT SHM + Trp
ssy1Δ SHM + Trp

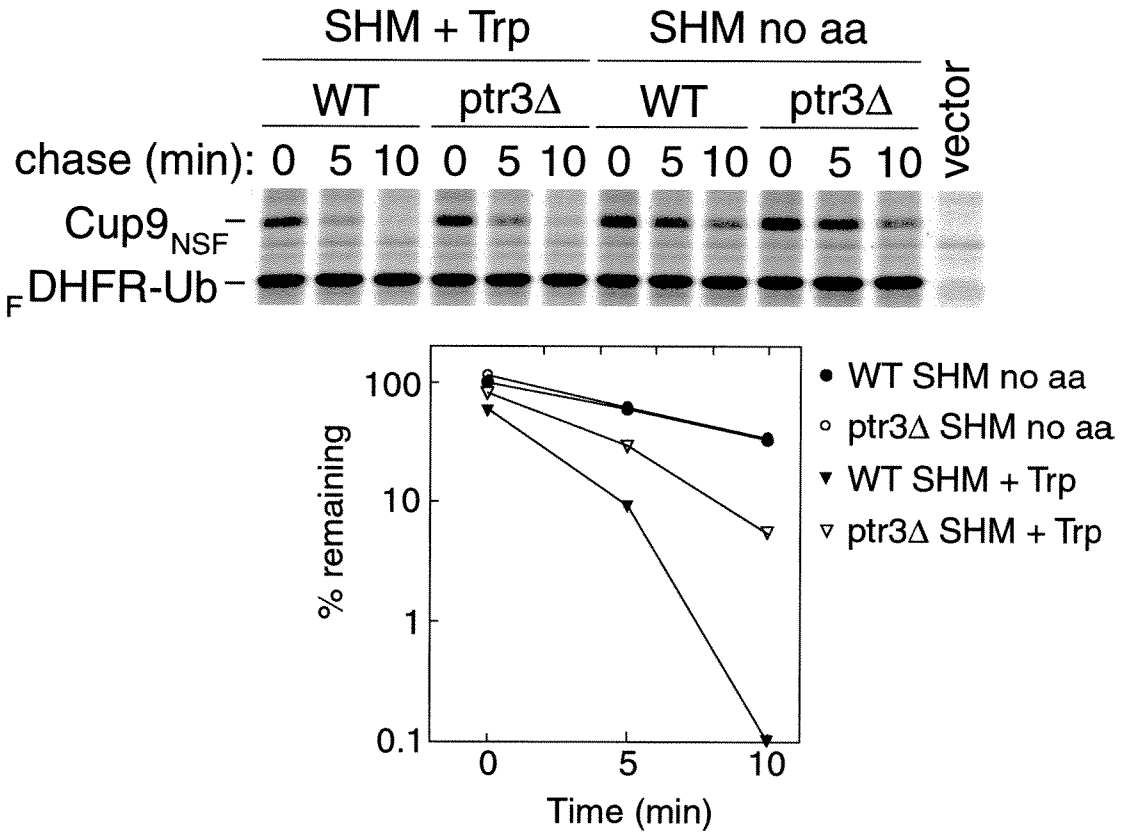
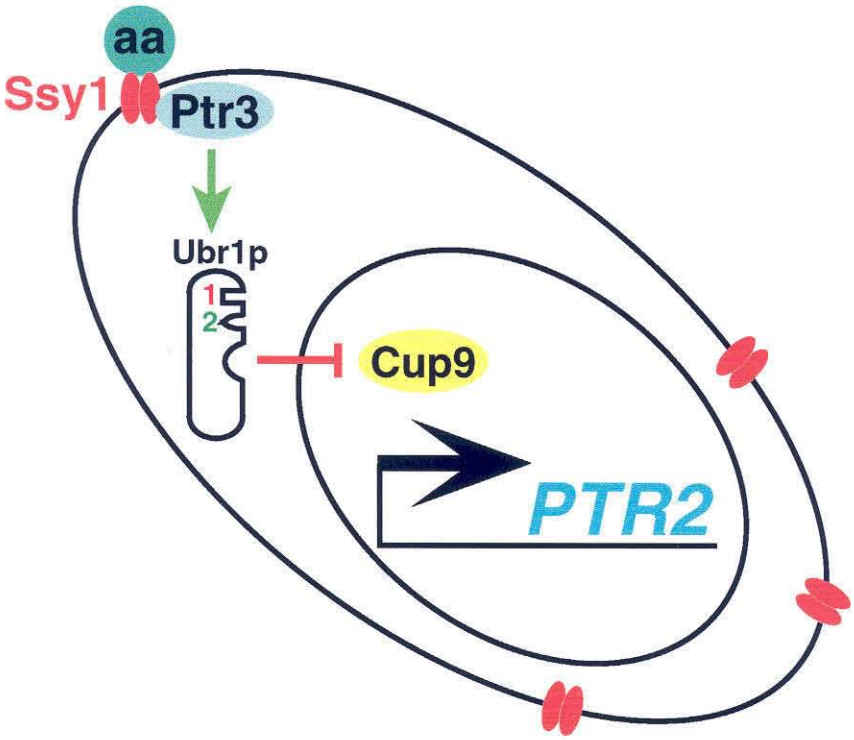

SHM + Trp
SHM no aa
WT
ptr3Δ
WT
ptr3Δ
vector
chase (min): 0 5 10 0 5 10 0 5 10 0 5 10
Cup9NSF–
FDHFR-Ub–
100
10
1
0.1
% remaining
0
5
10
Time (min)
WT SHM no aa
ptr3Δ SHM no aa
WT SHM + Trp
ptr3Δ SHM + Trp

Figure 5. Amino acid regulation of *PTR2*. Ssy1p, a homolog of amino acid transporters, is proposed to be a sensor of extracellular amino acids (12, 17). Ptr3p, a is a novel protein of unknown function that is downstream of Ssy1p in the amino acid-sensing pathway (1, 6, 21). In response to amino acids, the Ssy1p-Ptr3p signaling pathway activates Ubr1p, stimulating Cup9p degradation, thereby de-repressing *PTR2* expression.


aa
Ssy1
Ptr3
Ubr1p
1
2
Cup9
PTR2

Chapter 5

Proposed studies of cotranslational protein degradation and regulation of the Ubr1 pathway

Outline

Protein degradation serves two functions: protein quality control, and concentration regulation of components of biochemical switches and oscillators. In this chapter I will briefly discuss how the work in this thesis has contributed to the understanding of these functions. I will then suggest how these investigations might be carried on, describe some preliminary experiments I have done, and some additional experiments that might yield some interesting information.

Cotranslational protein degradation

Misfolded proteins are subject to two possible quality control efforts: refolding by the chaperone machinery, and degradation by the Ub-proteasome system. In both cases, the underlying recognition signal is probably an exposed patch of hydrophobic residues. Thus, the fate of a misfolded protein is determined by a kinetic competition between the chaperones, and the Ub-proteasome system for recognition of that signal (7).

Nascent polypeptides emerging from the ribosome may, in the process of folding, present hydrophobic degradation signals similar to those recognized in mature misfolded proteins. Using a novel method termed the Ub sandwich technique, I have shown that a nascent protein carrying a degradation signal is also subject to kinetic partitioning between folding and degradation (see Chapter 2). Up to 50% of these polypeptides can be degraded cotranslationally, as the nascent chain is in the process of being synthesized. These observations can be expanded in a number of interesting ways:

1- What role do the chaperones play in cotranslational degradation?

One would predict that inactivating the chaperone machinery would bias the fate of nascent chains away from proper folding and towards degradation. In fact, the cylindrical GroEL chaperonin has been shown to shield proteins from degradation within its central cavity (5). Do other chaperones play a similar role in the kinetic competition between folding and degradation?

i) The *SSB*s – ribosome-associated members of the Hsp70 family (17, 20).

Surprisingly, preliminary experiments suggest that the chaperone machinery is required for cotranslational degradation. Mutational inactivation of the *SSB* family of ribosome-associated hsp70 chaperones actually impairs degradation of an N-end rule substrate:

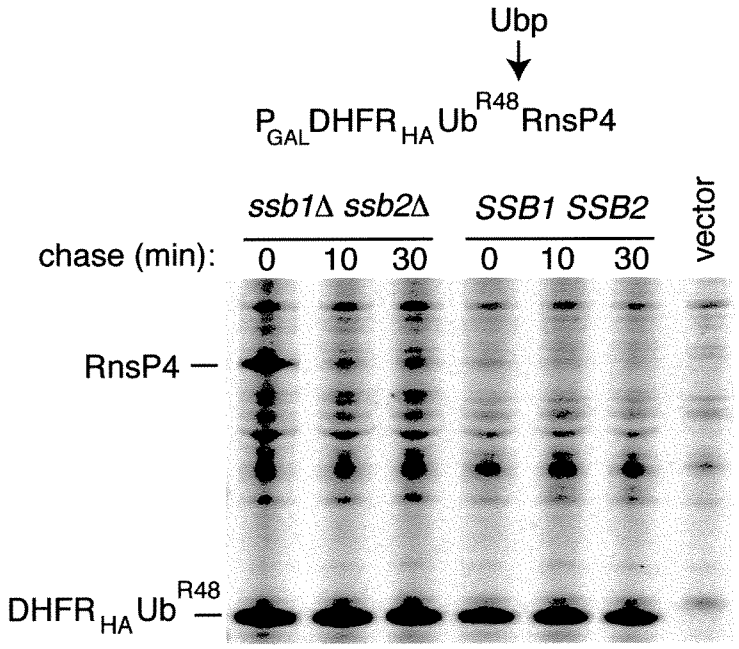


Figure 5.1) Pulse chase analysis of UPR R-nsP4 in *ssb1Δ ssb2Δ* and *SSB1 SSB2* strains (5 min radiolabeling, immunoprecipitation with anti-HA and anti-nsP4) (17). RnsP4 is abundant at t=0 in the *ssb1Δ ssb2Δ* mutant, but barely detectable in wildtype (*SSB1 SSB2*).

Obviously, this effect may be very indirect; the Ssbs may be required for the folding of any of the components of the N-end rule pathway, or the proteasome, rather than for acting directly on the RnsP4 substrate. However, these less interesting possibilities can be excluded with some additional experimental work. The Ssbs are ribosome-associated chaperones, suggesting that they act during cotranslational folding of nascent chains, but not on mature properly folded proteins (17, 20). Thus, the effects of Ssb inactivation on the degradation of the N-end rule substrate may be confined to the cotranslational time window. The data in Figure 5.1 support this possibility, since the main difference in the decay curves is that there is more RnsP4 at the early time points of the chase in the *ssb1Δ ssb2Δ* strain. If the posttranslational degradation of the substrate is intact (measured at the 10 through 60 min time points of a traditional pulse chase), but the cotranslational degradation of the same substrate is diminished (measured using the Ub sandwich technique), then the components of the N-end rule pathway and the proteasome must be properly functional, but the cotranslational presentation of the substrate to the degradation machinery must be defective.

Other evidence also suggests that chaperones may assist the degradation of N-end rule substrates. The N-end rule substrate Tyr-e^K β-gal becomes resistant to degradation over time, suggesting that it may slowly evolve into an undegradable conformation (24). Additionally, ubiquitinated β-gal-based substrates are not efficiently degraded by the proteasome *in vitro* (26), although they are rapidly degraded *in vivo* (11), suggesting that chaperones may assist in maintaining these substrates in a degradation-competent conformation.

ii) Nascent Chain Associated Complex (NAC)

Other chaperones may also influence the competition between folding and degradation. NAC influences kinetic partitioning of protein localization between transport to different organelles (endoplasmic reticulum and mitochondria) versus remaining localized to the cytoplasm (6, 27). Proteins normally targeted to the mitochondria may be degraded in strains carrying temperature sensitive alleles of the NAC subunits. This suggests that NAC may be required to shield these proteins from cotranslational degradation, rather than assisting their degradation, as proposed above for the Ssb family of Hsp70 chaperones. This could be easily tested using the Ub sandwich technique.

iii) Hsp90

The possibility that the Hsp90 chaperone facilitates degradation of certain proteins should also be tested. The ansamycins, small molecule Hsp90 inhibitors, stall misfolded proteins in complex with Hsp90 (23). These stalled substrates are rapidly degraded, suggesting that Hsp90 presents these proteins to the degradation machinery (23). One could test the possibility that Hsp90 cotranslationally delivers proteins to the degradation machinery in ansamycin-treated cells. Src kinases and Gα subunits would be important substrates to test. Nascent Src kinase is known to interact with Hsp90, making it a good candidate for this experiment (28). Ansamycins induce $G\alpha_o$ degradation in mammalian cells (2), and yeast Gpa1 undergoes rapid Ubr1-dependent degradation under some circumstances, making it an appealing candidate (15).

2- Are folding-defective proteins cotranslationally degraded?

Cotranslational degradation may serve as a proofreading mechanism for protein folding, degrading nascent chains which fail to fold correctly. Examining cotranslational degradation of a protein with a folding defect could test this possibility. Several derivatives of T4 lysozyme with well characterized folding defects have been shown to be degraded in *E. coli* and in mammalian cells (8, 9). However, preliminary experiments failed to detect cotranslational degradation of several of these folding-defective mutants. There are several reasons why T4 lysozyme mutants may not show cotranslational degradation. First, they are not very rapidly degraded in mammalian cells. T4 lysozyme is a very stable protein, requiring 15 kcal/mol for unfolding (16). This high energy of unfolding may present considerable difficulties for the proteasome, which must unfold a substrate in order to degrade it. Additionally, T4 lysozyme is very small (16 kD), and therefore has little time in which it might be degraded cotranslationally. Undeniably the best approach would be to use a large (≥ 60 kD) endogenous yeast protein with a well characterized folding defect produced by a point mutation.

Regulation of the Ubr1 pathway

Many of the components of biochemical switches and oscillators are short-lived proteins. Their degradation of these proteins is regulated by environmental or cell-intrinsic signals. In most cases studied so far, this regulation is accomplished by modulating the exposure or structure of the degradation signal on the substrate. However, important regulatory mechanisms also govern the activity of the substrate-selecting E3 components of the Ub-proteasome pathway. For example, a complex network of positive and negative regulatory

phosphorylation events regulates the activity of the APC E3 (14). Attachment of Ub-like molecules is required for the activity of at least two members of the SCF family of E3s (21) (22).

The first example of allosteric regulation of an E3 was found in studies of Ubr1, the E3 of the N-end rule pathway (Chapter 3). Ubr1 regulates dipeptide uptake in *S. cerevisiae* by controlling the degradation of Cup9, a homeodomain containing repressor of the dipeptide transporter Ptr2 (3). Dipeptides carrying destabilizing N-terminal residues are allosteric activators of Ubr1 activity. (These are the same destabilizing residues that Ubr1 recognizes in order to target proteins according to the N-end rule pathway). This creates a positive feedback loop (i.e., biochemical switch), where the import of dipeptides stimulates Ubr1, increasing Cup9 degradation, de-repressing Ptr2 expression. Thus, the expression of the machinery for dipeptide uptake is coupled to the availability of dipeptides.

Ubr1-dependent degradation of Cup9 is also regulated by a signal transduction pathway that senses extracellular concentrations of amino acids and induces Ptr2 expression accordingly (Chapter 4). Ssy1, a protein with homology to amino acids transporters, is the putative receptor in this signal transduction pathway (4, 10). Ptr3, a novel protein of unknown function, appears to be a component of the signal transduction apparatus (1, 12). The precise mechanism by which this signal transduction cascade activates Ubr1 has yet to be determined.

These observations raise many interesting questions. A number of preliminarily experiments, and proposals for experiments to address these questions, are described below.

1- ***Why is Cup9 degraded so rapidly ($t_{1/2}$ ~10 min) even in the absence of any identified inducing signal?***

Such rapid degradation seemingly wastes cellular energy in a futile cycle of protein synthesis and degradation. Other substrates of the Ub-proteasome system are all stable under some conditions; presumably the same is true of Cup9. The following experiments would be useful to determine other signals that might induce Cup9 degradation.

i) Is Cup9 stabilized by total nitrogen starvation?

Dipeptide transport is about nitrogen source assimilation. Is Cup9 stable, and Ptr2 completely repressed, when yeast are starved for all sources of nitrogen? If so, this would suggest that any nitrogen source, from ammonium to allantoin, is capable of inducing Cup9 degradation.

ii) Is Cup9 degradation influenced by copper metabolism?

Cup9 also plays a role in copper homeostasis (13). In the absence of the Cup1 metallothionein, *cup9Δ* cells show increased sensitivity to copper when undergoing aerobic respiration. Thus, Cup9 may be turned over under the experimental conditions examined so far for reasons related to copper metabolism. Preliminary experiments have not identified any regulation of Cup9 degradation by copper levels or carbon source. It may be useful to look at Cup9 half-life during transitions in carbon source, or in adapting to quick elevations or reductions in copper concentration, since protein degradation has generally evolved to act during transitional events.

iii) What are other targets of Cup9?

Cup9 may regulate processes other than copper homeostasis and nitrogen assimilation. Identifying other targets of Cup9 regulation may facilitate the search for other pathways that regulate its degradation. The following approaches could be taken:

a) Microarray analysis comparing mRNA profiles in *WT* and *cup9Δ*.

b) Screening for bypass suppressors of the toxicity of Cup9 over-expression. Cup9 is toxic when over-expressed from the *GAL1* promoter (P_{GAL1}) on a high-copy plasmid, suggesting that it completely extinguishes expression of an essential gene under these conditions. Screening a genomic library in a high-copy vector, or a P_{GAL1} driven cDNA library, might identify this target.

It would also be important to examine the terminal phenotype of cells expressing lethal levels of Cup9. *S. cerevisiae* detoxify copper in part by forming insoluble CuS mineralizations on their cell wall (29). Thus, copper homeostasis may be linked to sulphur metabolism. SCF^{MET30} regulates levels of Met4, an activator of the sulphur metabolism pathway (19). *MET30* is essential for cell viability, and *met30Δ* mutants arrest at the G1 to S transition (18, 25). Cup9 shows residual instability in *ubr1Δ* cells, suggesting that it may be targeted by another pathway, possibly SCF^{MET30}. If there is a connection between Cup9 and the sulphur metabolism pathway, Cup9 over-expression may also arrest cells at the G1 to S transition.

2- *What is the mechanism by which amino acids regulate Ubr1-dependent degradation of Cup9?*

Although it is clear that the amino acid induction pathway exerts its effects by modulating Ubr1-dependent degradation of Cup9, it is unclear how Ssy1 and Ptr3 actually activate Ubr1. Several experiments designed to test specific hypotheses are detailed below. I also describe several screens to identify additional components of the amino acid induction pathway, which would provide valuable clues to the mechanism of the regulation.

i) Does purified Ptr3 alter *in vitro* ubiquitination activity of Ubr1?

One possible model is that in the presence of amino acids, Ssy1 releases Ptr3, which is then free to interact with and activate Ubr1. (Of course there are many variations of this scenario, such as Ptr3 inhibiting Ubr1 in the absence of amino acids, but being transported elsewhere in the presence of amino acids, etc.) To test these possibilities, His_6-tagged Ptr3 was expressed in *E. coli*, partially purified and added to *in vitro* ubiquitination reactions containing E1, E2, Ubr1, Ub, ATP and radiolabeled Cup9 substrate. Ptr3 did not alter the rate of Cup9 ubiquitination in these reactions. However, it is possible that *E. coli* expressed Ptr3 is lacking some important posttranslational modification normally carried out in *S. cerevisiae*. Thus it would be of interest to test the ability of partially purified $Ptr3_{HIS6}$ from *S. cerevisiae* to influence ubiquitination activity of Ubr1 *in vitro*.

ii) What are the effects of phosphorylation on Ubr1 activity?

In vivo $^{32}PO_4$ labeling of an *S. cerevisiae* strain expressing N-terminally FLAG-tagged Ubr1 demonstrate that Ubr1 is a phospho-protein (Figure 5.2)

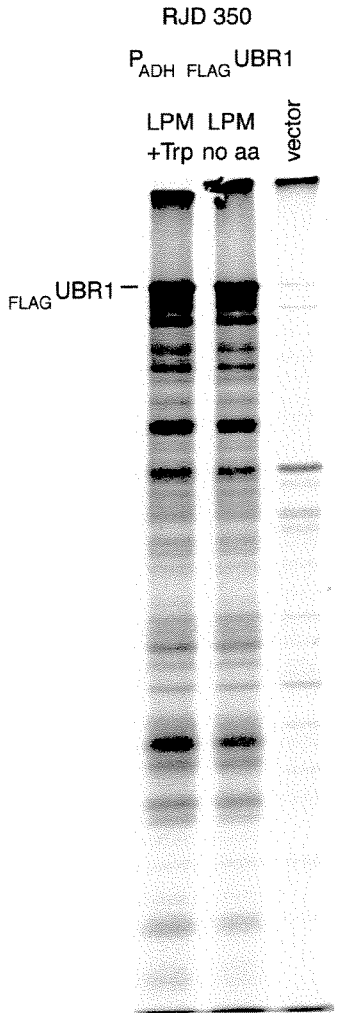


Figure 5.2) *In vivo* $^{32}PO_4$ labeling of cells expressing N-terminally FLAG-tagged Ubr1 from the *ADH1* promoter on a high copy (2μ) plasmid. Cells were exposed to a 30 min labeling with 0.25 mCi $^{32}PO_4$, heated to 95°C for 3 min, and then lysed by vortexing in lysis buffer containing 1% SDS 90mM HEPES pH 7.5 10mM Sodium Fluoride 60 mM β–glycerophosphate 10 mM Sodium Pyrophosphate 2 mM Sodium Vanadate. After dilution to 0.25% SDS in standard Triton lysis buffer, extracts were immunoprecipitated with anti-FLAG and subjected to SDS-PAGE.

The consequences of this phosphorylation could be examined by dephosphorylating purified Ubr1 (using Calf Intestinal Phosphatase, for example) and testing the *in vitro* ubiquitination activity of this dephosphorylated Ubr1. A greater investment of labor, but a far more rigorous approach, would be to map these phosphorylation sites by mass spectrometry, and then test the effects of mutating the phosphorylation sites on Ubr1 activity *in vivo*. Although there was no obvious difference in the extent of Ubr1 phosphorylation in the presence or

absence of inducing amino acids, one could examine this question more carefully using mass spectrometry.

iii) Do inducing amino acids alter the localization of Ubr1, Cup9 or Ptr3? The induction of Ubr1-dependent degradation of Cup9 by amino acids may just involve changes in the localization of the proteins involved. The subcellular localization of each of these four components should be examined in the presence and absence of inducing amino acids.

iv) Do amino acids regulate the conjugation of a Ub-like molecule (Ubl) to Ubr1?

SCF^{SKP2} and $SCF^{\beta TrCP}$ are both activated by conjugation of a Ubl (21) (22). Ubl modification of Ubr1 in response to amino acids could be readily detected by immunoblotting analysis.

v) An over-expression screen for positive regulators of Cup9 degradation. Identifying additional components of the Ssy1- Ptr3-dependent amino acid induction pathway may be necessary in order to understand how this signaling pathway functions mechanistically. Over-expression of the gene products that participate in this cascade may accelerate Cup9 degradation. The best readout for enhanced Cup9 degradation is levels of Ptr2 expression; Northern blot analysis (see Chapters 3 and 4) shows that levels of Ptr2 mRNA can be strongly elevated by even a small increase in the rate of Cup9 degradation. Thus, it should be possible to use a P_{PTR2}*::LacZ* fusion to screen a high copy genomic library, or a P_{GAL1} driven cDNA library, for plasmids that enhance the activity of P_{PTR2}. (It may be important to carry out this screen in a strain carrying a hypomorphic allele of *ssy1*

or *ptr3*.) After this primary screen, the effects on Cup9 degradation should immediately be tested by pulse chase analysis.

vi) Analyze the dominant mutants found in the original screen for suppressors of the block to dipeptide transport in *ubr1Δ* (3)

Screening for relief of repression of dipeptide transport in a *ubr1Δ* strain uncovered 121 dominant mutants, in addition to 201 recessive mutants representing *cup9, tup1* and *ssn6* (see Chapter 4). These mutants could represent gain-of-function alleles in positive regulators of Cup9 degradation. Thus, identifying the gene defects in these mutants might also identify components of the amino acid sensing pathway that cooperate with Ssy1 and Ptr3 to enhance Ubr1 activity. It is possible that simple over-expression of these gene products is not sufficient to accelerate Cup9 degradation, but they can be activated by mutation.

vii) Identify the transcription factor that activates *PTR2* in the absence of Cup9.

There is clearly a potent activator driving *PTR2* expression in *cup9Δ* strains. This activator drives *PTR2* expression during amino acid induction and dipeptide activation. Consequently, it would be valuable to determine the identity of this transcription factor, and test whether it is somehow regulated by the presence of dipeptides or inducing amino acids. This can be easily achieved by screening for mutants (perhaps conditional mutants, i.e., temperature sensitive) that prevent dipeptide uptake in a *cup9Δ* strain.

3- *What are the negative regulators of the Ub-proteasome system?*

The Ub-proteasome system is bound to have important positive and negative regulators. Positive regulation has been explored; however, there have been few systematic attempts to look for negative regulators of protein degradation. The following experiments would initiate such a study.

i) An over-expression screen for negative regulators of N-degron mediated degradation.

Negative regulators could be identified by screening a high copy genomic library, or a P_{GAL1} driven cDNA library, for plasmids capable of enhancing the steady-state level of β-gal or Ura3 based N-end rule substrates. This screen would identify general negative regulators that could act anywhere in the Ub-proteasome pathway, since the degradation signals these proteins carry are not known to be regulated.

ii) An over-expression screen for negative regulators of Cup9 degradation.

This screen may identify regulatory pathways acting specifically on Cup9, in addition to regulators of the ubiquitination/degradation machinery.

iii) A two-hybrid analysis of the interactions of Ub-specific proteases (Ubps).

Ubps cleave Ub off of substrates, and any other proteins they might be attached to. There are 16 Ubps in *S. cerevisiae*, and a specific function has only been assigned to a few. Examining the localization and the interaction partners of Ubps may illuminate their role in the Ub-proteasome system. For example, Ubps may serve as negative regulators of protein degradation by removing multiUb chains from substrates. Ubps may conditionally interact with substrates to exert this effect.

Two-hybrid screening or any other type of physical interaction assay would directly test this possibility.

REFERENCES

1. **Barnes, D., W. Lai, M. Breslav, F. Naider, and J. M. Becker.** 1998. *PTR3*, a novel gene mediating amino acid-inducible regulation of peptide transport in *Saccharomyces cerevisiae*. Mol. Microbiol. **29:**297-310.

2. **Busconi, L., J. Z. Guan, and B. M. Denker.** 2000. Degradation of heterotrimeric $G\alpha_o$ subunits via the proteosome pathway is induced by the Hsp90-specific compound geldanamycin. J. Biol. Chem. **275:**1565-1569.

3. **Byrd, C., G. C. Turner, and A. Varshavsky.** 1998. The N-end rule pathway controls the import of peptides through degradation of a transcriptional repressor. EMBO J. **17:**269-277.

4. **Didion, T., B. Regenberg, M. U. Jørgesen, M. C. Kielland-Brandt, and H. A. Andersen.** 1998. The permease homologue Ssy1p controls the expression of amino acid and peptide transporter genes in *Saccharomyces cerevisiae*. Mol. Microbiol. **27:**643-650.

5. **Frydman, J., and F. U. Hartl.** 1996. Principles of chaperone-assisted protein folding: differences between *in vitro* and *in vivo* mechanisms. Science **272:**1497-1502.

6. **George, R., T. Beddoe, K. Landl, and T. Lithgow.** 1998. The yeast nascent polypeptide-associated complex initiates protein targeting to mitochondria *in vivo*. Proc. Natl. Acad. Sci. USA **95:**2296-2301.

7. **Gottesman, S., S. Wickner, and M. R. Maurizi.** 1997. Protein quality control: triage by chaperones and proteases. Genes Dev. **11:**815-823.

8. **Inoue, I., and M. Rechsteiner.** 1994. On the relationship between the metabolic and thermodynamic stabilities of T4 lysozymes - measurements in *Escherichia coli*. J. Biol. Chem. **269:**29241-29246.

9. **Inoue, I., and M. Rechsteiner.** 1994. On the relationship between the metabolic and thermodynamic stabilities of T4 lysozymes - measurements in eukaryotic cells. J. Biol. Chem. **269:**29247-29251.

10. **Iraqui, I., S. Vissers, F. Bernard, J. O. De Craene, E. Boles, A. Urrestarazu, and B. Andre.** 1999. Amino acid signaling in *Saccharomyces cerevisiae*: a permease-like sensor of external amino acids and F-box protein Grr1p are required for transcriptional induction of the *AGP1* gene, which encodes a broad-specificity amino acid permease. Mol. Cell. Biol. **19:**989-1001.

11. **Johnson, E. S., B. Bartel, W., and A. Varshavsky.** 1992. Ubiquitin as a degradation signal. EMBO J. **11:**497-505.

12. **Klasson, H., G. R. Fink, and P. O. Ljungdahl.** 1999. Ssy1p and Ptr3p are plasma membrane components of a yeast system that senses extracellular amino acids. Mol. Cell. Biol. **19:**5405-5416.

13. **Knight, S. A. B., K. T. Tamai, D. J. Kosman, and D. J. Thiele.** 1994. Identification and analysis of a *Saccharomyces cerevisiae* copper homeostasis gene encoding a homeodomain protein. Mol. Cell. Biol. **14:**7792-7804.

14. **Kotani, S., H. Tanaka, H. Yasuda, and K. Todokoro.** 1999. Regulation of APC activity by phosphorylation and regulatory factors. J. Cell Biol. **146:**791-800.

15. **Madura, K., and A. Varshavsky.** 1994. Degradation of Gα by the N-end rule pathway. Science **265:**1454-1458.

16. **Matthews, B. W.** 1996. Structural and genetic analysis of the folding and function of T4 lysozyme. FASEB J. **10:**35-41.

17. **Nelson, R. J., T. Ziegelhoffer, C. Nicolet, M. Wernerwashburne, and E. A. Craig.** 1992. The translation machinery and 70 Kd heat-shock protein cooperate in protein synthesis. Cell **71:**97-105.

18. **Patton, E. E., C. Peyraud, A. Rouillon, Y. Surdin-Kerjan, M. Tyers, and D. Thomas.** 2000. SCF^{Met30}-mediated control of the transcriptional activator Met4 is required for the G1-S transition. EMBO J. **19:**1613-1624.

19. **Patton, E. E., A. R. Willems, D. Sa, L. Kuras, D. Thomas, K. L. Craig, and M. Tyers.** 1998. Cdc53 is a scaffold protein for multiple Cdc34/Skp1/F-box protein complexes that regulate cell division and methionine biosynthesis in yeast. Genes Dev. **12:**692-705.

20. **Pfund, C., N. Lopez-Hoyo, T. Ziegelhoffer, B. A. Schilke, P. Lopez-Buesa, W. A. Walter, M. Wiedmann, and E. A. Craig.** 1998. The molecular chaperone Ssb from *Saccharomyces cerevisiae* is a component of the ribosome-nascent chain complex. EMBO J. **17:**3981-9.

21. **Podust, V. N., J. E. Brownell, T. B. Gladysheva, R.-S. Luo, C. Wang, M. B. Coggins, J. W. Pierce, E. S. Lightcap, and V. Chau.** 2000. A Nedd8 conjugation pathway is essential for proteolytic targeting of $p27^{Kip1}$ by ubiquitination. Proc. Natl. Acad. Sci. **97:**4579-4584.

22. **Read, M. A., J. E. Brownell, T. B. Gladysheva, M. Hottelet, L. A. Parent, M. B. Coggins, J. W. Pierce, V. N. Podust, R. S. Luo, V. Chau, and V. J. Palombella.** 2000. Nedd8 modification of Cul-1 activates $SCF^{\beta TrCP}$-dependent ubiquitination of IκBα. Mol. Cell. Biol. **20:**2326-2333.

23. **Schneider, C., L. Sepp-Lorenzino, E. Nimmesgern, O. Ouerfelli, S. Danishefsky, N. Rosen, and F. U. Hartl.** 1996. Pharmacologic shifting of a balance between protein refolding and degradation mediated by Hsp90. Proc. Natl. Acad. Sci. USA **93:**14536-14541.

24. **Suzuki, T., and A. Varshavsky.** 1999. Degradation signals in the lysine-asparagine sequence space. EMBO J. **18:**6017-6026.

25. **Thomas, D., L. Kuras, R. Barbey, H. Cherest, P. L. Blaiseau, and Y. Surdinkerjan.** 1995. Met30p, a yeast transcriptional inhibitor that responds to S-adenosylmethionine, is an essential protein with WD40 repeats. Mol. Cell. Biol. **15:**6526-6534.

26. **Thrower, J. S., L. Hoffman, M. Rechsteiner, and C. M. Pickart.** 2000. Recognition of the polyubiquitin proteolytic signal. EMBO J. **19:**94-102.

27. **Wiedmann, B., H. Sakai, T. A. Davis, and M. Wiedmann.** 1994. A protein complex required for signal-sequence-specific sorting and translocation. Nature **370:**434-440.

28. **Xu, Y., M. A. Singer, and S. Lindquist.** 1999. Maturation of the tyrosine kinase c-src as a kinase and as a substrate depends on the molecular chaperone Hsp90. Proc. Natl. Acad. Sci. USA **96:**109-114.

29. **Yu, W., R. A. Farrell, D. J. Stillman, and D. R. Winge.** 1996. Identification of *SLF1* as a new copper homeostasis gene involved in copper sulfide mineralization in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **16:**2464-2472.

Appendix

The N-end rule pathway controls the import of peptides through degradation of a transcriptional repressor

Christopher Byrd, Glenn C. Turner, and Alexander Varshavsky

The EMBO Journal Vol.17 No.1 pp.269–277, 1998

The N-end rule pathway controls the import of peptides through degradation of a transcriptional repressor

Christopher Byrd, Glenn C.Turner and Alexander Varshavsky[1]

Division of Biology, California Institute of Technology, 1200 East California Boulevard, Pasadena, CA 91125, USA

[1]Corresponding author
e-mail: avarsh@cco.caltech.edu

Ubiquitin-dependent proteolytic systems underlie many processes, including the cell cycle, cell differentiation and responses to stress. One such system is the N-end rule pathway, which targets proteins bearing destabilizing N-terminal residues. Here we report that Ubr1p, the main recognition component of this pathway, regulates peptide import in the yeast *Saccharomyces cerevisiae* through degradation of Cup9p, a 35 kDa homeodomain protein. Cup9p was identified using a screen for mutants that bypass the previously observed requirement for Ubr1p in peptide import. We show that Cup9p is a short-lived protein ($t_{1/2}$ ~5 min) whose degradation requires Ubr1p. Cup9p acts as a repressor of *PTR2*, a gene encoding the transmembrane peptide transporter. In contrast to engineered N-end rule substrates, which are recognized by Ubr1p through their destabilizing N-terminal residues, Cup9p is targeted by Ubr1p through an internal degradation signal. The Ubr1p–Cup9p–Ptr2p circuit is the first example of a physiological process controlled by the N-end rule pathway. An earlier study identified Cup9p as a protein required for an aspect of resistance to copper toxicity in *S.cerevisiae*. Thus, one physiological substrate of the N-end rule pathway functions as both a repressor of peptide import and a regulator of copper homeostasis.

Keywords: *CUP9*/N-end rule/peptide import/proteolysis/*PTR2*/*UBR1*

Introduction

Many regulatory proteins are short-lived *in vivo* (Schwob *et al.*, 1994; King *et al.*, 1996; Varshavsky, 1996). This metabolic instability makes possible rapid adjustment of the protein's concentration (or subunit composition) through changes in the rates of its synthesis or degradation. Protein degradation plays a role in a multitude of processes, including cell growth, division, differentiation and responses to stress. In eukaryotes, a large fraction of intracellular proteolysis is mediated by the ubiquitin system. Ubiquitin (Ub) is a 76-residue protein whose covalent conjugation to other proteins marks them for processive degradation by the 26S proteasome—an ATP-dependent, multisubunit protease (Jentsch and Schlenker, 1995; Hilt and Wolf, 1996; Hochstrasser, 1996; Rubin *et al.*, 1997).

Features of proteins that confer metabolic instability are called degradation signals (degrons). One of the degradation signals recognized by the ubiquitin system is called the N-degron. It comprises two essential determinants: a destabilizing N-terminal residue and an internal lysine of a substrate (Bachmair *et al.*, 1986; Varshavsky, 1996). The Lys residue is the site of formation of a substrate-linked multiubiquitin chain (Bachmair and Varshavsky, 1989; Chau *et al.*, 1989). A set of N-degrons bearing different N-terminal residues that are destabilizing in a given cell type yields a rule, called the N-end rule, which relates the *in vivo* half-life of a protein to the identity of its N-terminal residue. Similar but distinct versions of the N-end rule operate in all organisms examined, from mammals to fungi and bacteria (Varshavsky, 1996).

The N-end rule pathway is organized hierarchically. In eukaryotes such as *Saccharomyces cerevisiae*, Asn and Gln are tertiary destabilizing N-terminal residues in that they function through their conversion, by the *NTA1*-encoded N-terminal amidase (Nt-amidase), into the secondary destabilizing residues Asp and Glu (Baker and Varshavsky, 1995). Secondary residues, in turn, function through their conjugation to Arg by the *ATE1*-encoded Arg-tRNA–protein transferase (R-transferase) (Balzi *et al.*, 1990). Arg is one of several primary destabilizing N-terminal residues which are bound directly by N-recognin, a 225 kDa E3 protein encoded by the *UBR1* gene (Bartel *et al.*, 1990). Ubr1p, together with the associated ubiquitin-conjugating (E2) enzyme Ubc2p, mediates the formation of a substrate-linked multiubiquitin chain (Varshavsky, 1996).

The N-end rule pathway was first encountered in experiments that explored, in *S.cerevisiae*, the metabolic fate of a fusion between Ub and a reporter such as *Escherichia coli* β-galactosidase (β-gal) (Bachmair *et al.*, 1986). While such engineered N-end rule substrates have been extensively characterized (Varshavsky, 1996), little is known about their physiological counterparts. The few identified so far include the *GPA1*-encoded Gα subunit of the *S.cerevisiae* heterotrimeric G protein, which mediates the pheromone response in this fungus, and RNA polymerases of alphaviruses whose hosts include mammalian and insect cells (de Groot *et al.*, 1991; Madura and Varshavsky, 1994). Physiological functions of the instability of these proteins remain to be understood (Varshavsky, 1996). Inactivation of the N-end rule pathway in *S.cerevisiae*—through deletion of the *UBR1* gene—results in cells which grow slightly slower than their wild-type counterparts, and are impaired in sporulation (increased frequency of asci containing fewer than four spores), but otherwise appear to be normal (Bartel *et al.*, 1990).

Recently, Becker and colleagues (Alagramam *et al.*, 1995) have reported that *ubr1*Δ cells are deficient in the

A

Strain	Genotype	Peptide import	Strain	Genotype	Peptide import
JD53	wt	+	MHY599	*pas2Δ*	+
JD83-1A	*ubr1Δ*	–	CBY8	*ubc2Δ, ubc1Δ*	+
KM207-1	*ate1Δ*	+	CBY11	*ubc2Δ, cdc34-1*[1]	+
SGY6	*nta1Δ*	+	EJY105/6	*ubc2Δ, ubc4Δ*	–
CBY20	*ptr2Δ*	+	CBY4	*ubc2Δ, ubc5Δ*	+
EJ2	*ubc1Δ*	+	CBY5	*ubc2Δ, ubc6Δ*	+
BBY67	*ubc2Δ*	+/–	CBY6	*ubc2Δ, ubc7Δ*	+
JD34	*ubc2Δ*	+/–	CBY7	*ubc2Δ, ubc8Δ*	+
EJY102	*ubc2Δ*	+/–	CBY9	*ubc2Δ, ubc9-1*[1]	+
RJD549	*cdc34-1*[1]	+	CBY10	*ubc2Δ, pas2Δ*	+
RJD795	*cdc34-2*[1]	+	EJ10	*ubc4Δ, ubc5Δ*	+
EJ3	*ubc4Δ*	+	MHY503	*ubc4Δ, ubc6Δ*	+
EJ1	*ubc5Δ*	+	MHY554	*ubc4Δ, ubc7Δ*	+
MHY495	*ubc6Δ*	+	MHY552	*ubc6Δ, ubc7Δ*	+
MHY507	*ubc7Δ*	+	MHY550	*ubc4Δ, 5Δ, 6Δ*	+
MHY601	*ubc8Δ*	+	MHY557	*ubc4Δ, 6Δ, 7Δ*	+
YWO55	*ubc9-1*[1]	+	MHY570	*ubc4Δ, 5Δ, 6Δ, 7Δ*	+

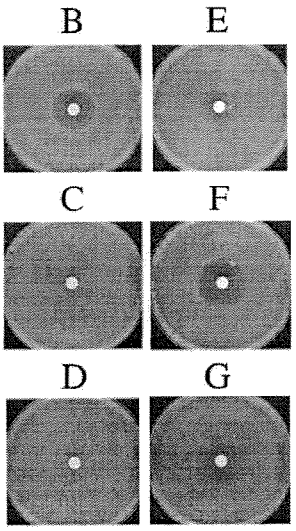


Fig. 1. The import of peptides is decreased in the absence of Ubc2p and virtually abolished in the absence of Ubc2p and Ubc4p. *Saccharomyces cerevisiae* mutants deficient in one or more ubiquitin-conjugating (E2) enzymes or other components of the ubiquitin system were tested for their ability to import peptides, using the halo assay and a toxic dipeptide L-leucyl-L-ethionine (Leu-Eth) (see Materials and methods). '+', '±' and '-' denote, respectively, the apparently wild-type, significantly reduced, and undetectable levels of peptide import. A superscript '1' refers to strains that carried a *ts* allele of an essential E2 enzyme, either Cdc34p (Ubc3p) or Ubc9p; the 30°C temperature of the test was semi-permissive for these strains. (**A**) Summary of the results. (B–F) Examples of the actual halo assays, with wild-type (**B**), *ptr2Δ* (**C**), *ubr1Δ* (**D**), *ubc2Δ* (**E**), *ubc4Δ* (**F**) and *ubc2Δ ubc4Δ* (**G**) strains of *S.cerevisiae*. Elimination of some E2 enzymes in the *ubc2Δ* background restored halo formation, presumably because such strains were growth-impaired in a way that made them hypersensitive to the toxicity of ethionine. By contrast, although *ubc2Δ ubc4Δ* cells were also growth-impaired, they were import-defective and therefore grew in the immediate vicinity of the filter.

import of di- and tripeptides, suggesting that this process, which is universal among living cells, requires the N-end rule pathway. In the present work, we identified the underlying regulatory mechanism and discovered a new physiological substrate of the N-end rule pathway, the homeodomain protein Cup9p. This short-lived protein is targeted for degradation by Ubr1p, and acts as a transcriptional repressor of *PTR2*, a gene that encodes a transmembrane peptide transporter. The Ubr1p–Cup9p–Ptr2p circuit is the first example of a physiological process controlled by the N-end rule pathway.

Results

The involvement of Ubc2p and Ubc4p E2 enzymes in the control of peptide import

We began by asking whether components of the N-end rule pathway other than Ubr1p were also necessary for the import of peptides. Previous work has shown that Ubc2p, one of 13 ubiquitin-conjugating (E2) enzymes of *S.cerevisiae*, is required for the degradation of engineered N-end rule substrates, and is physically associated with the E3 protein Ubr1p (N-recognin) (Jentsch, 1992; Madura *et al.*, 1993).

To test for the ability of *S.cerevisiae* to import peptides, we used a halo assay, in which a filter soaked in the toxic dipeptide L-leucyl-L-ethionine (Leu-Eth) is placed on a plate, inhibiting the growth of import-competent cells near the filter. By this test, the elimination of *UBC2* impaired, but did not abolish, the import of peptides (Figure 1A, B and E). To determine which of the other E2 enzymes, if any, were required for the residual peptide import observed in *ubc2Δ* cells, a number of single and multiple mutants in *UBC* genes were examined (Figure 1). We found that the elimination of both *UBC2* and *UBC4* virtually abolished the import of peptides (Figure 1A, B and E–G). Elimination of Ubc4p, one of the more abundant E2 enzymes (Bachmair *et al.*, 1986; Jentsch, 1992), had previously been noticed to decrease slightly the activity of the N-end rule pathway (Bartel, 1990). Cells lacking either Nta1p or Ate1p—the 'upstream' components of this pathway—were also examined and found unimpaired in the import of peptides, in contrast to *ubr1Δ* and *ubc2Δ ubc4Δ* cells (Figure 1A).

Identification of Cup9p as a negative regulator of peptide import

Previous work (Alagramam *et al.*, 1995) has shown that deletion of *UBR1* greatly reduces the level of *PTR2* mRNA, which encodes the transmembrane peptide transporter. This result, and our observation that peptide import requires the presence of at least one of two specific ubiquitin-conjugating enzymes (Figure 1), suggested a model in which expression of the Ptr2p transporter is regulated by a short-lived repressor that is degraded by the N-end rule pathway. In cells lacking *UBR1*, the repressor would be expected to accumulate, thereby blocking peptide import. One prediction of this model is that inactivation of this repressor would bypass the requirement for Ubr1p in peptide import. A screen for such 'bypass' mutations (see Materials and methods) yielded 199 recessive isolates, of which 101 defined one complementation group, termed

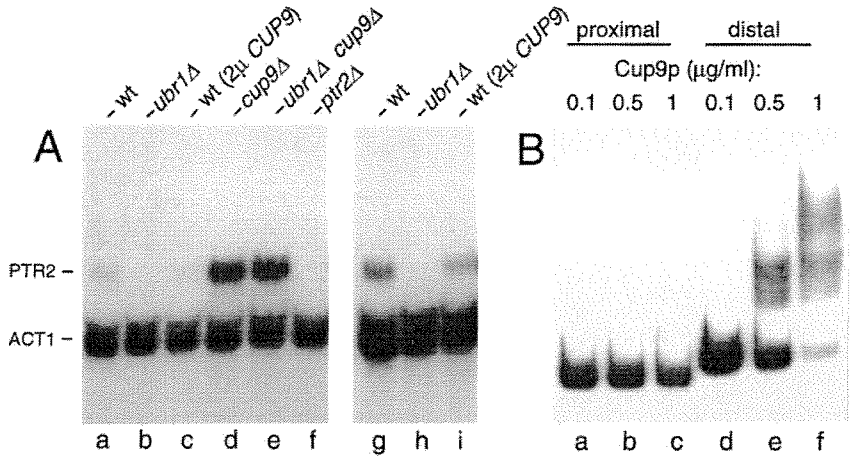


Fig. 2. Cup9p is a repressor of the *PTR2* gene. (**A**) Expression of the *S.cerevisiae PTR2* gene in different genetic backgrounds. Equal amounts of total RNA isolated from different strains were analyzed by Northern hybridization (see Materials and methods), using the *PTR2* (peptide transporter) and *ACT1* (actin) genes as ^{32}P-labeled probes. Lane a, JD52 (*CUP9 UBR1*) (wild-type) transformed with pCB201 (empty vector). Lane b, JD55 (*CUP9 ubr1*Δ) transformed with pCB201. Lane c, JD52 (*CUP9 UBR1*) transformed with the high-copy plasmid pCB209 that expressed *CUP9* from its natural promoter. Lane d, CBY18 (*cup9*Δ *UBR1*). Lane e, CBY16 (*cup9*Δ *ubr1*Δ). Lane f, CBY21 (*CUP9 UBR1 ptr2*Δ). Lanes g–i, same as lanes a–c, but a longer autoradiographic exposure to highlight the tight repression of *PTR2* by Cup9p in *ubr1*Δ cells (b, h) and the difference between levels of *PTR2* mRNA in the wild-type cells (a, g) and their counterparts that overexpressed Cup9p (c, i). (**B**) Cup9p specifically binds to a site in the P_{PTR2} promoter. A gel shift assay with Cup9-H_6, poly-dI·dC and ^{32}P-labeled DNA fragments of the *PTR2* promoter hybridization (see Materials and methods). Lanes a–c, with a fragment (–1 to –447) proximal to the inferred start codon of the *PTR2* ORF. Lanes d–f, same as lanes a–c, but with a more distal DNA fragment (–448 to –897). Concentrations of Cup9-H_6 (in μg/ml) are indicated above the lanes.

sub1 (suppressor of a block to peptide import in *ubr1*Δ). In agreement with the model's prediction, *sub1* mutants acquired the ability to express *PTR2* in the absence of *UBR1* (Figure 2A, lane b versus lane e). The *sub1* locus was cloned by complementation (see Materials and methods), and was found to be the *CUP9* gene.

CUP9 was originally identified by Knight *et al.* (1994) as a gene whose disruption impairs the copper resistance of *S.cerevisiae* growing on lactate, a non-fermentable carbon source. Under these conditions, Cup9p plays a major (but mechanistically obscure) role in copper homeostasis (Knight *et al.*, 1994). *CUP9* encodes a 35 kDa protein that contains a homeodomain, an ~60-residue helix–turn–helix DNA-binding motif present in many eukaryotic regulatory proteins (Wolberger, 1996). Outside the homeodomain region, the sequence of Cup9p is not similar to sequences in databases.

To verify that *CUP9* and *SUB1* were the same gene, complementation tests were carried out. Two independently derived *ubr1*Δ *cup9::LEU2* strains (CBY16 and CBY17) were crossed to *ubr1*Δ *sub1-1* (CBY15), and the resulting diploids (CBY23 and CBY24, respectively) were tested for their ability to import dipeptides (see Materials and methods). As would be expected of allelic loci, *cup9::LEU2* and *sub1-1* failed to complement one another: both diploid strains remained import-competent (data not shown). In another test, CBY23 and CBY24 were sporulated, and the segregants were analyzed for the presence of the *LEU2* gene (integrated at the *CUP9* locus) and for the ability to import peptides. Among the eight tetrads tested, *LEU2* was present in two of the four segregants, whereas all four segregants were invariably import-competent, a pattern expected if *CUP9* and *SUB1* were one and the same gene.

To examine the regulation of peptide import by Cup9p and Ubr1p, congenic *S.cerevisiae* strains that lacked, expressed or overexpressed Cup9p and/or Ubr1p were constructed and assayed for peptide import by growth on selective media. Suspensions of cells (auxotrophic for lysine) were serially diluted and plated on either rich media, minimal media lacking lysine and containing Lys-Ala dipeptide (selecting for peptide import), or minimal media containing the toxic dipeptide Leu-Eth (selecting against peptide import). All strains grew at comparable rates on rich media (Figure 3A). On minimal media that supplied the essential lysine as the Lys-Ala dipeptide, the *CUP9 UBR1* (wild-type), *cup9*Δ *UBR1* and *cup9*Δ *ubr1*Δ strains grew at comparable rates (Figure 3B), whereas the *CUP9 ubr1*Δ strain failed to grow (Figure 3B), in agreement with the observation that *UBR1* is required for peptide import (Figure 1). Opposite growth patterns were observed on media containing toxic dipeptide (Figure 3C). Comparison of the data in Figure 3 with *PTR2* mRNA levels (Figure 2A) suggested that *CUP9* exerts its effect on the import of peptides by repressing transcription of *PTR2*. For example, wild-type (*CUP9 UBR1*) cells overexpressing Cup9p exhibited reduced levels of *PTR2* mRNA (Figure 2A) and decreased sensitivity to toxic dipeptide (Figure 3C), whereas strains lacking *CUP9* (*cup9*Δ *UBR1* and *cup9*Δ *ubr1*Δ) overexpressed *PTR2* (Figure 2A) and were hypersensitive to toxic dipeptide (Figure 3C).

Cup9p is a repressor of the PTR2 gene

To address the mechanism of repression by Cup9p, we asked whether purified Cup9p (see Materials and methods) could selectively bind to specific regions of the *PTR2* promoter. Gel shift assays in the presence of poly-dI·dC competitor DNA were performed with labeled DNA

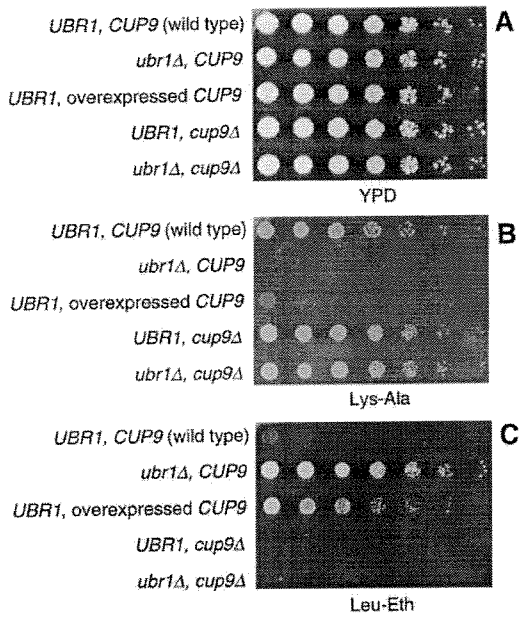


Fig. 3. Relative capacity for peptide import in congenic *S.cerevisiae* strains that contained, lacked or overexpressed Cup9p and/or Ubr1p. Serial dilutions of the indicated strains were deposited by a 48-pin applicator onto either rich (YPD) medium (**A**), minimal medium containing 66 μM Lys-Ala dipeptide as the sole source of lysine (**B**), or minimal medium containing both lysine (at 110 μM) and the toxic dipeptide Leu-Eth (at 55 μM) (**C**). The plates were incubated at 30°C for 1–2 days. The relevant genetic loci are shown on the left. The '*UBR1*, overexpressed *CUP9*' strain (JD52-2μ-CUP9) carried the high-copy plasmid pCB209 that expressed Cup9p from its natural promoter.

fragments of the *PTR2* promoter and His_6-tagged Cup9p (Cup9p-H_6) purified from *E.coli*. Cup9p-H_6 bound to a site within a distal region of the *PTR2* promoter (positions −448 to −897 relative to the inferred *CUP9* start codon), but did not bind to the proximal region of the *PTR2* promoter (positions −1 to −447) under the same conditions (Figure 2B).

The transcriptional repressor function of Cup9p was further suggested by the finding that the co-repressor complex Tup1p/Ssn6p plays a role in the control of peptide import. The Tup1p/Ssn6p complex inhibits transcription of many yeast genes through interactions with gene-specific DNA-binding repressors such as Matα2p (Chen *et al.*, 1993; Smith *et al.*, 1995), a homeodomain homolog of Cup9p. We found that most of our *sub* mutants that were not *CUP9* mutants could be complemented by low-copy plasmids bearing *SSN6* or *TUP1* (G.Turner, S.Saha and A.Varshavsky, unpublished data). In addition, deletion of *SSN6*, like deletion of *CUP9*, restored the ability of *ubr1*Δ cells to import peptides (data not shown).

Ubr1p-dependent degradation of Cup9p

The fact that deletion of *CUP9* renders *PTR2* transcription independent of Ubr1p (Figure 2A), and the observation that overexpression of Ubr1p enhances the import of peptides in Cup9p-expressing strains (data not shown) suggested that the N-end rule pathway regulates peptide import by targeting Cup9p for degradation. To test this conjecture, we carried out pulse–chase experiments with a C-terminally FLAG-tagged Cup9p in *UBR1* and *ubr1*Δ cells. Cup9p-FLAG was a very short-lived protein ($t_{1/2}$ ~5 min) in *UBR1* cells (Figure 4). By contrast, Cup9p was much longer-lived ($t_{1/2}$ >30 min) in *ubr1*Δ cells (Figure 4). Degradation of Cup9p was also found to depend upon *UBC2* and *UBC4* (data not shown), in agreement with the observation that a *ubc2*Δ *ubc4*Δ double mutant failed to import dipeptides (Figure 1G). The residual instability of Cup9p in *ubr1*Δ cells (Figure 4) suggested the presence of a second, Ubr1p-independent degron; this pattern is reminiscent of another homeodomain repressor, Matα2p, which also contains at least two distinct degradation signals (Hochstrasser and Varshavsky, 1990; Chen *et al.*, 1993).

Ubr1p recognizes engineered N-end rule substrates through their destabilizing N-terminal residues (Varshavsky, 1996). To determine the N-terminal residue of Cup9p, we overexpressed and purified Cup9p-FLAG from *ubr1*Δ *S.cerevisiae* and subjected it to N-terminal sequencing. Cup9p-FLAG was found to have a blocked (presumably acetylated) N-terminus (see Materials and methods), suggesting that Ubr1p targets Cup9p through a degron distinct from the N-degron. Independent evidence for this conjecture was produced through the analysis of GST-Cup9p-ha_2, a fusion of glutathione transferase (GST) and C-terminally ha-tagged Cup9p. Pulse–chase analysis of GST-Cup9p-ha_2 revealed that the fusion protein was nearly as short-lived *in vivo* as the N-terminally unmodified Cup9p-FLAG (Figure 4, lanes b–e; compare with Figure 5, lanes b–e). If Cup9p were targeted for processive degradation through a destabilizing N-terminal residue, a preliminary proteolytic cleavage(s) of Cup9p would be required to expose such a residue (Varshavsky, 1996). In the case of GST-Cup9p-ha_2, this cleavage would generate a proteolytic fragment consisting of GST and an N-terminal portion of Cup9p preceding the cleavage site. Since free GST has been found to be long-lived when expressed in yeast (data not shown), accumulation of such a proteolytic fragment would be expected to accompany the degradation of GST-Cup9p-ha_2.

Pulse–chase analysis of GST-Cup9p-ha_2, using glutathione–agarose beads to isolate GST-containing proteins (see Materials and methods), indicated that the degradation of GST-Cup9p-ha_2 by the N-end rule pathway was not accompanied by the appearance of a fragment containing the N-terminal GST moiety (Figure 5). This finding strongly suggested that Cup9p bears an 'internal' degron recognized by Ubr1p. Additional support for this conjecture was provided by truncation analysis of a Cup9p–DHFR–myc fusion protein. These experiments indicated that the N-terminal 81 residues of the 306-residue Cup9p are dispensable for its Ubr1p-dependent degradation, strongly suggesting that the relevant degradation signal resides in the C-terminal two-thirds of Cup9p (C.Byrd, I.Davydov and A.Varshavsky, unpublished data). Although these results are fully consistent with the presence of an internal Ubr1p-dependent degron in Cup9p, there remains the less parsimonious possibility that Cup9p is degraded via *trans*-targeting (Johnson *et al.*, 1990). In this process, the two determinants of the N-degron (a destabilizing N-terminal residue and a ubiquitin-accepting internal Lys

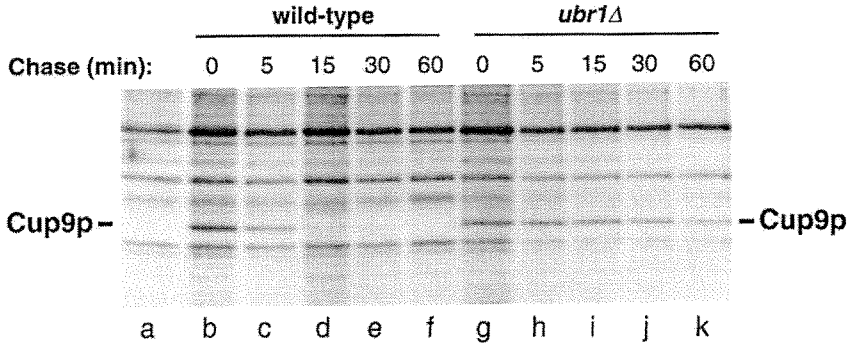


Fig. 4. Degradation of Cup9p by the N-end rule pathway. *Saccharomyces cerevisiae* strains carrying pCB210, a low-copy plasmid that expressed Cup9p-FLAG from the P_{CUP9} promoter, were labeled at 30°C with [^{35}S]methionine/cysteine for 5 min, followed by a chase for 0, 5, 15, 30 and 60 min, preparation of extracts, immunoprecipitation of Cup9p-FLAG with a monoclonal anti-FLAG antibody, SDS–PAGE and autoradiography/quantitation (see Materials and methods). Lane a, JD55 (*ubr1*Δ) cells that carried pCB200 (vector alone). Lanes b–f, JD52 (*UBR1*) (wild-type) cells that carried pCB210, expressing Cup9p-FLAG. Lanes g–k, same as lanes b–f, but with JD55 (*ubr1*Δ) cells.

residue) reside in two different polypeptides, whose interaction yields an active N-degron, leading to ubiquitinylation and degradation of the polypeptide bearing the Lys residue (Johnson *et al.*, 1990). At present, little is known about the protein ligands of Cup9p, save for the likely possibility that Cup9p interacts with the Tup1p/Ssn6p repressor complex (see above), similarly to the previously established interaction of this complex with Matα2, a homeodomain-containing homolog of Cup9p (Smith *et al.*, 1995).

Discussion

The discovery that Ubr1p controls the import of peptides through degradation of the Cup9p repressor (Figure 6) identifies the first clear physiological function of the N-end rule pathway. The existence of mammalian, plant and bacterial homologs of the yeast Ptr2p transporter suggests that the import of peptides in these organisms may also be regulated by the N-end rule pathway.

Why was this pathway, rather than another Ub-dependent proteolytic system, recruited in the course of evolution for the regulation of peptide import? A plausible explanation is suggested by the ability of Ubr1p to bind peptides bearing destabilizing N-terminal residues (Varshavsky, 1996). Since more than half of the 20 amino acids are destabilizing in the yeast and mammalian N-end rules (Varshavsky, 1996), a significant fraction of imported peptides would be expected to compete with Cup9p for binding to Ubr1p. This competition may decrease the rate of Cup9p degradation. [Dipeptides added to a culture of *S.cerevisiae* have been shown to inhibit the N-end rule pathway (Baker and Varshavsky, 1991).] The ensuing increase in the level of Cup9p repressor would in turn decrease the production of the Ptr2p transporter, diminishing the rate of peptide import. This 'peptide-sensing' negative feedback loop would maintain the intracellular concentration of short peptides within a predetermined range—potentially a useful feature, made possible by the substrate-binding properties of Ubr1p. Experiments to verify this model are under way.

Another interesting aspect of the Ubr1p–Cup9p–Ptr2p

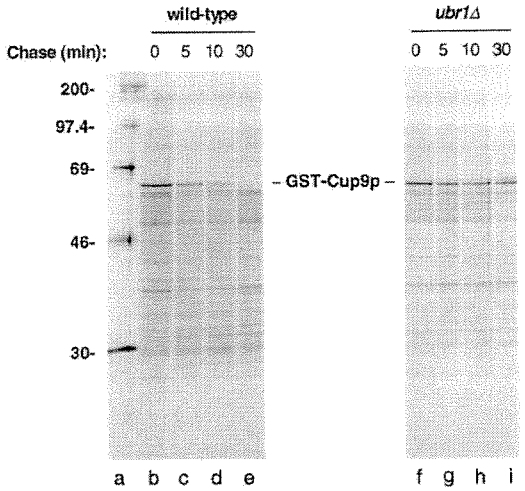


Fig. 5. Degradation of GST-Cup9p-ha$_2$ is not preceded by a processing that releases a GST-containing fragment. *Saccharomyces cerevisiae* strains carrying pCB120, a low-copy plasmid that expressed GST-Cup9p-ha$_2$ from the P_{GAL1} promoter, were labeled at 30°C with [^{35}S]methionine/cysteine for 5 min, followed by a chase for 0, 5, 10 and 30 min, preparation of extracts, isolation of GST-Cup9p-ha$_2$ on glutathione–agarose beads, SDS–PAGE and autoradiography/quantitation (see Materials and methods). Lanes b–e, JD52 (*UBR1*) (wild-type) cells expressing GST-Cup9p-ha$_2$. Lanes f–i, same as lanes b–e, but with JD55 (*ubr1*Δ) cells. Lane a, molecular mass markers (in kDa).

regulatory circuit is its possible relevance to copper homeostasis. In addition to functioning as a repressor of peptide transport, Cup9p also contributes to the resistance of *S.cerevisiae* to copper toxicity during growth on lactate, a non-fermentable carbon source (Knight *et al.*, 1994). Copper homeostasis is mediated by a complex set of circuits, some of which also regulate iron metabolism (Zhou and Thiele, 1993; Ooi *et al.*, 1996). The double role of Cup9p in regulating both peptide import and copper homeostasis may signify a physiological connection between these seemingly unrelated processes.

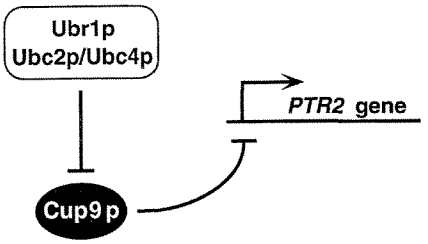


Fig. 6. A model for regulation of peptide import in *S.cerevisiae*. The expression of *PTR2*, which encodes a transmembrane peptide transporter, is regulated by the short-lived, homeodomain-containing transcriptional repressor Cup9p. The concentration of Cup9p is controlled in part through its degradation by the N-end rule pathway, whose targeting components include Ubr1p and either Ubc2p or Ubc4p (see the main text).

Our work adds Cup9p to the list of short-lived regulatory proteins whose degradation is mediated by the ubiquitin system. Many of these proteins are negative regulators. For example, Matα2p, a homeodomain-containing homolog of Cup9p, controls the mating type of *S.cerevisiae* (**a** or α) through repression of **a**-specific genes (Herskowitz, 1989). Matα2p appears to be constitutively short-lived in haploid cells (Hochstrasser and Varshavsky, 1990). Therefore, cessation of Matα2p synthesis upon the conversion of an α cell into an **a** cell results in rapid disappearance of Matα2p and the establishment of **a**-specific circuits (Hochstrassser, 1996). Progression of the cell cycle is also controlled by short-lived negative regulators, in particular by Sic1p, an inhibitor of CDK, the cyclin-dependent kinase (Schwob *et al.*, 1994). In this case, however, a rapid 'on-switch' is based on the phosphorylation-induced degradation of the previously stable Sic1p (King *et al.*, 1996). Whether the Cup9p repressor (Figure 6) is constitutively short-lived or whether its stability is regulated by external conditions such as, for example, different nitrogen sources, remains to be determined.

The finding that Cup9p lacks a destabilizing N-terminal residue indicates that Ubr1p, the recognition component of the N-end rule pathway, is able to target substrates bearing either internal degradation signals or N-degrons. The Gα subunit of the G protein—the other known physiological substrate of Ubr1p in *S.cerevisiae*—also lacks a destabilizing N-terminal residue (Madura and Varshavsky, 1994). Thus, a substantial fraction of naturally short-lived proteins targeted by the N-end rule pathway may bear internal degrons rather than N-degrons, a possibility that the identification of other physiological substrates of this pathway will address.

Materials and methods

Strains, media and genetic techniques

Saccharomyces cerevisiae strains were grown in rich (YPD) medium containing 2% peptone, 1% yeast extract and 2% glucose or in synthetic yeast media, containing 0.67% yeast nitrogen base without amino acids (Difco), auxotrophic nutrients at concentrations specified by Sherman *et al.* (1986) and either 2% glucose (SD medium), 2% raffinose (SM–raffinose medium) or 2% galactose (SM–galactose medium) as carbon sources. SHM–glucose medium used in halo assays was prepared according to Island *et al.* (1987) and was identical to SD medium except that it lacked methionine and contained allantoin (1 mg/ml) and yeast nitrogen base (Difco, 1.7 mg/ml) without amino acids and ammonium sulfate. *Escherichia coli* strain DH5α was used for plasmid propagation and cloning steps. For induction of the P_{GAL1} promoter, cells were grown to an A_{600} of 0.5–1 in SM–raffinose medium, pelleted and transferred to SM–galactose medium for 3 h.

Halo and dilution assays

Peptide import was assayed using the halo and dilution methods. For halo assays (Island *et al.*, 1987), cells were grown to an A_{600} of ~1 in SHM–glucose medium with auxotrophic supplements. Cells were pelleted by centrifugation and resuspended in water to ~5×10^7 cells/ml. Then 0.1 ml of cell suspension was mixed with 10 ml of 0.8% noble agar (Difco) at 55°C, and spread on plates containing 20 ml of SHM medium. Sterile filter disks containing the toxic dipeptide L-leucyl-L-ethionine (Leu-Eth; 5 μmol) were placed in the middle of each plate, followed by incubation at 30°C for 1–2 days.

For dilution assays, strains were grown (under selection for plasmids) in SD medium to an A_{600} of ~1. Cells from each sample (1.5×10^7) were spun down and resuspended in 1 ml of water. The samples were serially diluted by 4-fold in a microtiter plate (150 μl/well) to generate eight different initial concentrations of cells that ranged from 1.5×10^7 to 915 cells/ml. The suspensions were spotted to various media, using a 48-pin applicator. The plates were incubated at 30°C for 1–2 days.

Leu-Eth was synthesized using standard methods of organic chemistry. Briefly, L-ethionine methyl ester (Eth-OMe) was produced from L-ethionine and methanol. Eth-OMe was then coupled with *N-tert*-butoxy-carbonyl-L-leucine-*p*-nitrophenyl ester (N-*t*-BOC-L-leucine-PNP), yielding N-*t*-BOC-L-leucyl-L-ethionine methyl ester, which was purified by flash chromatography on a silica column. N-*t*-BOC-L-leucyl-L-ethionine methyl ester was then converted into N-*t*-BOC-L-leucyl-L-ethionine by treatment with KOH. N-*t*-BOC-L-leucyl-L-ethionine was deprotected with trifluoroacetic acid, yielding Leu-Eth.

A screen for import-competent mutants in the ubr1Δ background

Thirty 5-ml cultures of JD83-1A (*leu2-3 ubr1Δ*), auxotrophic for leucine, were grown to an A_{600} of ~1, and ~1.5×10^7 cells from each culture were plated onto SD medium lacking leucine and histidine but containing the leucyl-histidine dipeptide at 0.23 μM. Plates were incubated at 30°C for 2 days, selecting for mutants able to grow on this medium. Of the 320 sub mutants ('suppressors of a block to peptide import in *ubr1Δ*') thus obtained, 199 were found by complementation tests to be clearly recessive. Among these, 101 mutants belonged to one complementation group, termed *sub1*.

Isolation of the CUP9 (SUB1) gene

A 50 ml culture of *S.cerevisiae* CBY15 (*ubr1Δ sub1-1*) was grown to an A_{600} of ~1 in SHM–glucose. Cells were made competent with lithium acetate (Ausubel *et al.*, 1992), and 25 0.1-ml samples (~7×10^7 cells/ml) were transformed with a yeast genomic DNA library (American Type Culture Collection; #77164) in the *TRP1*, *CEN6*-based vector pRS200 (Sikorski and Hieter, 1989). The cells were pelleted, and each sample was resuspended in 1.5 ml of SHM–glucose and incubated at 30°C for 3 h. The cells were pelleted again, each sample was resuspended in 0.1 ml of water, added to 10 ml of 0.8% Noble agar at 55°C, and spread onto SHM–glucose plates lacking Trp and containing Leu-Eth at 37 μM. Of the ~2.5×10^4 Trp^+ transformants plated, 14 could grow in the presence of Leu-Eth. Of these, two yielded the library-derived plasmids, pSUB1-1 and pSUB1-6, that complemented the *sub1* mutation of CBY15. The insert of pSUB1-6 contained the insert of pSUB1-1. Partial sequencing of the ~8.2 kb insert of pSUB1-1 identified it as a region of the *S.cerevisiae* chromosome XVI. Deletion analysis (not shown) localized the complementing activity to an ~2.8 kb *Hin*dIII–*Kpn*I fragment, whose sequence revealed the presence of two ORFs (*CUP9* and SCYPL178W). Further deletion analysis (not shown) localized the complementing activity to the previously isolated (Knight *et al.*, 1994) *CUP9* gene. Verification that *CUP9* and *SUB1* were one and there same gene was carried out as described in Results.

Construction of null cup9 mutants

The ~2.8 kb *Hin*dIII–*Kpn*I genomic DNA fragment containing *CUP9* was ligated to *Hin*dIII–*Kpn*I-cut pRS306ΔSpeI, yielding pCB117. pRS306ΔSpeI was derived from the *URA3*-bearing pRS306 (Sikorski and Hieter, 1989) through elimination of its *Spe*I site. The *CUP9* ORF was disrupted by inserting an ~2 kb, blunt-ended, *LEU2*-containing *Sal*I fragment from pJJ283 into the *Spe*I site of *CUP9*, yielding pCB119, in which the *LEU2* and *CUP9* ORFs were oriented in opposite directions. An ~5 kb *Hin*dIII–*Pvu*II fragment of pCB119 containing the *cup9::LEU2*

Table I. *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype	References
DF5	*MATa/MATα trp1-1/trp1-1 ura3-52/ura3-52 his3-Δ200/his3-Δ200 leu2-3,112/leu2-3,112 lys2-801/lys2-801 gal/gal*	Finley *et al.* (1987)
EJ1	*MATα ubc5Δ::LEU2 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 gal*	Derivative of DF5[a]
EJ2	*MATα ubc1Δ::URA3 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 gal*	Derivative of DF5[a]
EJ3	*MATα ubc4Δ::HIS3 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 gal*	Derivative of DF5[a]
EJ10	*MATα ubc4Δ::HIS3 ubc5Δ::LEU2 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 gal*	Derivative of DF5[a]
EJY102	*MATα ubc2Δ::LEU2 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 gal*	Derivative of DF5[a]
EJY105	*MATa ubc2Δ::LEU2 ubc4Δ::HIS3 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 gal*	Derivative of DF5[a]
EJY106	*MATα ubc2Δ::LEU2 ubc4Δ::HIS3 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 gal*	Derivative of DF5[a]
MHY495	*MATα ubc6-Δ1::HIS3 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801*	Derivative of DF5[b]
MHY503	*MATα ubc4-Δ1::HIS3 ubc6-Δ1::HIS3 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801*	Derivative of DF5[b]
MHY507	*MATα ubc7::LEU2 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801*	Derivative of DF5[b]
MHY550	*MATα ubc4-Δ2::TRP1 ubc5-Δ1::LEU2 ubc6-Δ1::HIS3 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801*	Derivative of DF5[b]
MHY552	*MATα ubc6-Δ1::HIS3 ubc7::LEU2 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801*	Derivative of DF5[b]
MHY554	*MATα ubc4-Δ1::HIS3 ubc7::LEU2 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801*	Derivative of DF5[b]
MHY557	MATα ubc4-Δ1::HIS3 ubc6-Δ1::HIS3 ubc7::LEU2 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801	Derivative of DF5[b]
MHY570	*MATα ubc4-Δ2::TRP1 ubc5-Δ1::LEU2 ubc6-Δ1::HIS3 ubc7::LEU2 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801*	Derivative of DF5[b]
MHY599	*MATα pas2 trp ura3-52 ade1 leu2-3*	Chen *et al.* (1993)
MHY601	*MATα ubc8::URA3 trp1-1 ura3-1 ade2-1 his3-11 leu2-3,112 can1-100*	Chen *et al.* (1993)
YWO55	*MATα ubc9-1 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801*	Derivative of DF5[c]
YPH500	*MATα trp1-Δ63 ura3-52 ade2-101 his3-Δ200 leu2-Δ1 lys2-801*	Sikorski and Hieter (1989)
BBY67	*MATα ubc2Δ::LEU2 trp1-Δ63 ura3-52 ade2-101 his3-Δ200 leu2-Δ1 lys2-801*	Derivative of YPH500[d]
KM207-1	*MATα ate1Δ::TRP1 trp1-Δ63 ura3-52 ade2-101 his3-Δ200 leu2-Δ1 lys2-801*	Derivative of YPH500[e]
JD34	*MATa ubc2Δ::URA3 trp1-Δ63 ura3-52 ade2-101 his3-Δ200 leu2-Δ1 lys2-801*	Derivative of YPH500[f]
JD51	*MATa/MATα trp1-Δ63/trp1-Δ63 ura3-52/ura3-52 his3-Δ200/his3-Δ200 leu2-3,112/leu2-3,112 lys2-801/lys2-801*	Dohmen *et al.* (1995)
JD52	*MATa trp1-Δ63 ura3-52 his3-Δ200 leu2-3,112 lys2-801*	Johnson *et al.* (1995)
JD53	*MATα trp1-Δ63 ura3-52 his3-Δ200 leu2-3,112 lys2-801*	Dohmen *et al.* (1995)
JD55	*MATa ubr1Δ::HIS3 trp1-Δ63 ura3-52 his3-Δ200 leu2-3,112 lys2-801*	Madura and Varshavsky (1994)
JD83-1A	*MATα ubr1Δ::HIS3 trp1-Δ63 ura3-52 his3-Δ200 leu2-3,112 lys2-801*	Derivative of JD51[f]
RJD549	*MATa cdc34-1 trp1 ura3-52 leu2-3*	R.Deshaies[g]
RJD795	*MATa cdc34-2 trp1 ura3-52 leu2-3*	R.Deshaies[g]
SGY6	*MATα nta1Δ::TRP1 trp1-Δ63 ura3-52 ade2-101 his3-Δ200 leu2-3,112 lys2-801*	S.Grigoryev[h]
CBY4	*MATα ubc2Δ::URA3 ubc5Δ::LEU2 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 gal*	Derivative of EJ1
CBY5	*MATα ubc2Δ::LEU2 ubc6-Δ1::HIS3 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801*	Derivative of MHY495
CBY6	*MATα ubc2Δ::URA3 ubc7::LEU2 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801*	Derivative of MHY507
CBY7	*MATα ubc2Δ::LEU2 ubc8::URA3 trp1-1 ura3-1 ade2-1 his3-11 leu2-3,112 can1-100*	Derivative of MHY601
CBY8	*MATα ubc2Δ::LEU2 ubc1Δ::URA3 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 gal*	Derivative of EJ2
CBY9	*MATα ubc2Δ::LEU2 ubc9-1 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801*	Derivative of YWO55
CBY10	*MATα ubc2Δ::LEU2 pas2 trp ura3-52 ade1 leu2-3*	Derivative of MHY599
CBY11	*MATa ubc2Δ::LEU2 cdc34-1 trp1 ura3-52 leu2-3*	Derivative of RJD549
CBY15	*MATα sub1-1 ubr1Δ::HIS3 trp1-Δ63 ura3-52 his3-Δ200 leu2-3,112 lys2-801*	Derivative of JD83-1A
CBY16	*MATa cup9::LEU2 ubr1Δ::HIS3 trp1-Δ63 ura3-52 his3-Δ200 leu2-3,112 lys2-801*	Derivative of JD55
CBY17	*MATa cup9::LEU2 ubr1Δ::HIS3 trp1-Δ63 ura3-52 his3-Δ200 leu2-3,112 lys2-801*	Derivative of JD55
CBY19	*MATa cup9::LEU2 trp1-Δ63 ura3-52 his3-Δ200 leu2-3,112 lys2-801*	Derivative of JD52
CBY23	*MATa/MATα sub1-1/cup9Δ::LEU2 ubr1Δ::HIS3/ubr1Δ::HIS3 trp1-Δ63/trp1-Δ63 ura3-52/ura3-52 his3-Δ200/his3-Δ200 leu2-3,112/leu2-3,112 lys2-801/lys2-801*	Produced by mating CBY15 and CBY16
CBY24	*MATa/MATα sub1-1/cup9Δ::LEU2 ubr1Δ::HIS3/ubr1Δ::HIS3 trp1-Δ63/trp1-Δ63 ura3-52/ura3-52 his3-Δ200/his3-Δ200 leu2-3,112/leu2-3,112 lys2-801/lys2-801*	Produced by mating CBY15 and CBY17

[a]Johnson *et al.* (1992, 1995). A gift from E.Johnson, the Rockefeller University, New York, NY 10021-6399, USA.
[b]Chen *et al.* (1993). A gift from M.Hochstrasser, Department of Biochemistry and Molecular Biology, University of Chicago, Chicago, IL 60637, USA.
[c]A gift from S.Jentsch, ZMBH, Universität Heidelberg, 69120 Heidelberg, Germany.
[d]Dohmen *et al.* (1990).
[e]A gift from K.Madura, Department of Biochemistry, UMDNJ-Johnson Medical School, Piscataway, NJ 08854, USA.
[f]A gift from J.Dohmen, Heinrich-Heine-Universität, Institut für Mikrobiologie, 40225 Düsseldorf, Germany.
[g]A gift from R.Deshaies, Division of Biology, Caltech, Pasadena, CA 91125, USA.
[h]A gift from S.Grigoryev, Department of Biology, University of Massachusetts, Amherst, MA 01003, USA.

disruption allele was used to replace the wild-type *CUP9* alleles of JD52 (wild-type) and JD55 (*ubr1Δ*) by homologous recombination (Rothstein, 1991), generating strains CBY19 and CBY17, respectively.

CUP9-expressing plasmids

The plasmid pCB116, which expressed Cup9p-FLAG from the P_{GAL1} promoter, was constructed by subcloning an ~1 kb *Bam*HI–*Eco*RI fragment containing the *CUP9-FLAG* ORF into the *Bam*HI–*Eco*RI site(s) of p416GAL1 (Mumberg *et al.*, 1994). The *CUP9-FLAG*-containing fragment was constructed by PCR amplification of the *CUP9* ORF of plasmid pCB111 using primers PCB1 (5′-CGCGGATCCGAATAGTTACATTCGAAGATG-3′) and PCB6 (5′-CCGGAATTCTCATTTATCATCATCGTCTTTGTAATCATTCATATCAGGGTTGGATAG-3′), resulting in the addition of the 8-residue FLAG epitope, DYKDDDDK, to the C-terminus of Cup9p. pCB111 was constructed by subcloning the ~2.8 kb *Hin*dIII–*Kpn*I fragment of the *CUP9*-containing pSUB1-1 (see above) into *Hin*dIII–*Kpn*I-cut pRS316 (Sikorski and Hieter, 1989). pCB202 was constructed by subcloning an ~1 kb fragment containing the P_{CUP9} promoter into *Hin*dIII–*Bam*HI-cut pCB201. [The ~1 kb fragment was produced by PCR from pCB111 using primers PCB8

(5′-GTGTTAGTAAGCTTGTAAAGGAATGCACGTATT-3′) and PCB9 (5′-CCCGCGGATCCGCATGCAACTATTCTCGAAGGTTGT-3′).] pCB200 (*ARS-CEN, LEU2*) and pCB201 (2μ, *LEU2*) were constructed by replacing the 517 bp *Sca*I–*Eco*RI fragment of pBR322 (Ausubel *et al.*, 1992) with, respectively, the 3822 bp *Sca*I–*Nae*I fragment of pRS415 (Sikorski and Hieter, 1989) and the 4650 bp *Sca*I–*Nae*I fragment of pRS425 (Christianson *et al.*, 1992).

The plasmid pCB209 (2μ, *LEU2*), which expressed *CUP9* from the P_{CUP9} promoter, was constructed by replacing the *Sph*I–*Sal*I fragment of pCB202 with an ~1 kb fragment containing the *CUP9* ORF that was produced by PCR from pCB111, using primers PCB10 (5′-CCC-GCGGATCCGCATGCGAAGATGAATTATAACTGC-3′) and PCB12 (5′-CCCGCGCGGTCGACCTCAATTCATATCAGGGTTGGATAG-3′). pCB210 (*ARS-CEN, LEU2*) that expressed Cup9p-FLAG from the P_{CUP9} promoter was constructed by replacing the *Sph*I–*Sal*I fragment of pCB202 with an ~1 kb fragment containing the *CUP9-FLAG* ORF, which was produced from pCB111 using primers PCB10 (see above) and PCB13 (5′-CCCGCGCGGTCGACCTCATTTATCATCATCGTCTTTGTA-ATCATTCATATCAGGGTTGGATAG-3′), yielding pCB211. The ~2 kb *Hin*dIII–*Sal*I fragment of pCB211 containing P_{CUP9} and the *CUP9-FLAG* ORF was subcloned into pCB200, yielding pCB210.

Plasmid pCB120, expressing GST-Cup9p-ha_2 from the P_{GAL1} promoter, was constructed by subcloning the ~1.6 kb *Xba*I–*Eco*RI fragment, containing the *GST-CUP9-*ha_2 ORF, into the *Xba*I–*Eco*RI site(s) of p416GAL1. The *Xba*I–*Eco*RI fragment was produced by PCR amplification of the *GST-CUP9-*ha_2 ORF of pGEX-2T–CUP9-ha_2, using the primers PCB3 (5′-CCGGAATTCTCAAGCGTAATCTGGAACATC-GTATGGGTAAGCGTAATCTGGAACATCGTATGGGTAATTCATA-TCAGGGTTGGATAG-3′) and PCB5 (5′-TGCTCTAGAACAGT-ATTCATGTCCCCTATA-3′). pGEX-2T–CUP9-ha_2 was constructed by subcloning an ~1 kb fragment containing the *CUP9-*ha_2 ORF into the *Bam*HI–*Eco*RI site(s) of pGEX-2T (Pharmacia), resulting in an in-frame fusion of the sequence encoding 26 kDa glutathione *S*-transferase (GST) domain of *Saccharomyces japonicum* (Smith and Johnson, 1988) to the second codon of *CUP9*. The *CUP9-*ha_2-containing fragment was produced by PCR amplification of the *CUP9* ORF of pCB111 using the primers PCB3 (see above) and PCB4 (5′-CGCGGATCCAATTAT-AACTGCGAAATACAAAAC-3′). This step added to the C-terminus of Cup9p a sequence encoding a tandem repeat of the 9-residue sequence YPYDVPDYA, derived from hemagglutinin (ha) of influenza virus.

Northern hybridization

RNA was isolated from *S.cerevisiae* as described (Schmitt *et al.*, 1990). Electrophoresis of the RNA samples was carried out on a formaldehyde RNA gel (Ausubel *et al.*, 1992). An ~50-μg RNA sample was loaded on a 1% agarose gel containing 1× MOPS buffer, 0.74% (v/v) formaldehyde, 1.9 mg/ml iodoacetamide and 0.5 μg/ml ethidium bromide. Electrophoresis was carried out in 1× MOPS buffer at 5 V/cm. RNA was transferred to BrightStar-Plus membrane (Ambion) using TurboBlotter (Schleicher & Schuell) and Ambion RNA transfer buffer. RNA was crosslinked to the air-dried membranes using 254 nm light (Ausubel *et al.*, 1992).

DNA probes were prepared by the random priming method (Ausubel *et al.*, 1992) using [^{32}P]dCTP and a DNA labeling kit (Pharmacia). Hybridization was carried out for 8–16 h at 42°C in Prehybridization/Hybridization Solution (Ambion). Filters were washed according to the manufacturer's protocol and subjected to autoradiography.

Gel shift assay

PCR was used to extend the Cup9p ORF with a sequence encoding Ser-Gly-Gly-Thr-His_6, yielding Cup9p-H_6, and to engineer flanking restriction sites (*Nde*I and *Bam*HI) for insertion into pET-11c (Novagen). Cup9p-H_6 was overexpressed in *E.coli* BL21 (DE3) (Novagen) (Ausubel *et al.*, 1992) and purified on a 3 ml Ni-NTA column (Qiagen), using a linear gradient of imidazole. Cup9p-H_6 eluted at ~0.25 M imidazole (~90% pure at this step); it was dialyzed at 4°C against 10% glycerol, 0.1 M KCl, 1 mM EDTA, 0.5 mM dithiothreitol, 20 mM HEPES, pH 7.9, and then snap-frozen in multiple samples in liquid N_2, and stored at –80°C. The proximal (–1 to –447) and distal (–448 to –897) *PTR2* promoter probes for the gel shift assay were constructed by PCR amplification in the presence of [α-^{32}P]dCTP, and were purified using spin columns (Qiagen). The gel shift reactions (20 μl) contained 50 μg/ml poly-dI·dC (Pharmacia); ~1.5 μg/ml (500 c.p.m.) DNA probe; 1 mg/ml acetylated serum albumin (New England Biolabs) and either 0.1, 0.5 or 1 μg/ml of Cup9p-H_6 in 10% glycerol, 0.1 M KCl, 2.5 mM $MgCl_2$, 1 mM EDTA, 20 mM HEPES, pH 7.9. The samples were incubated for 30 min at room temperature, then loaded onto a 4% polyacrylamide gel (40:1, acrylamide:*bis*-acrylamide) in 0.5× TBE (Ausubel *et al.*, 1992), and electrophoresed at 10 V/cm for 3 h at 4°C, followed by autoradiography.

Pulse–chase analysis of Cup9p

One hundred ml cultures of *S.cerevisiae* JD52 (*UBR1*) and JD55 (*ubr1*Δ) carrying either pCB210 (expressing Cup9p-FLAG from the *CUP9* promoter) or pCB200 (vector alone) were grown to an A_{600} of ~1 in SD(–Leu) medium. Cells (50 A_{600} units total) from each of the four cultures were gently pelleted by centrifugation, washed with 5 ml of SD(–Leu), pelleted again, resuspended in 2 ml of SD(–Leu), and incubated at 30°C for 10 min. Each sample was labeled for 5 min with 1.4 mCi of [^{35}S]methionine/cysteine (EXPRESS, New England Nuclear) at 30°C, followed by pelleting in a microfuge for ~15 s. The cells were resuspended in 2.6 ml of SD(–Leu), 5 mM L-methionine, 5 mM L-cysteine, and incubated at 30°C. Samples of 0.5 ml were withdrawn during the incubation, pelleted and resuspended in 0.15 ml of 0.5 M NaCl-Lysis Buffer (1% Triton X-100, 0.5 M NaCl, 5 mM EDTA, 50 mM Na-HEPES, pH 7.5) containing a mixture of protease inhibitors (Ghislain *et al.*, 1996). Glass beads (0.5 mm) were added, and cells were disrupted by vortexing (six times, for 30 s each, with 1 min incubations on ice in between), followed by the adjustment of NaCl concentration to 0.15 M through the addition of 75 mM NaCl-Lysis Buffer, further vortexing for 30 s, and centrifugation at 12 000 *g* for 10 min. The volumes of supernatants were adjusted to equalize the amounts of 10% trichloroacetic acid-insoluble ^{35}S. Cup9p-FLAG was immunoprecipitated by the addition of 20 μl of the monoclonal anti-FLAG M2 antibody conjugated to agarose beads (Kodak). Suspensions were incubated at 0°C for 1 h, with rotation, then centrifuged at 12 000 *g* for 30 s, and washed four times with 0.8 ml of 0.15 M NaCl-Lysis Buffer. The pellets were resuspended in SDS-sample buffer, heated at 100°C for 3 min, and subjected to SDS–12% PAGE, followed by autoradiography and quantitation using a PhosphorImager (Molecular Dynamics).

Pulse–chase analysis of GST-Cup9p-ha_2 was carried out as described by Bartel *et al.* (1990). Approximately 10 A_{600} units of galactose-induced cells were labeled for 5 min with 0.3 mCi of [^{35}S]EXPRESS in 400 μl SM–galactose (–Ura) at 30°C. The cells were then transferred to microfuge tubes, pelleted and resuspended in 500 μl of SD (–Ura), 5 mM L-methionine, 5 mM L-cysteine. Samples of 0.1 ml were withdrawn during the incubation, pelleted and lysed as above. The ^{35}S-labeled GST-Cup9p-ha_2 was purified using glutathione–agarose beads (Sigma) which had been blocked with bovine serum albumin (BSA; 10 mg/ml). Twenty μl of glutathione–agarose beads were added to each sample and the suspensions were incubated at 0°C for 60 min, with rotation, followed by washes and electrophoretic analyses as described for Cup9p-FLAG.

Purification and N-terminal sequencing of Cup9p-FLAG

Four 2-l cultures of JD55 (*ubr1*Δ) carrying pCB116 that expressed Cup9p-FLAG from the *GAL1* promoter were grown under selection in SM–raffinose to an A_{600} of ~0.8, followed by transfer to SM–galactose and incubation at 30°C for 3 h. Longer induction times resulted in a Cup9p-mediated cytotoxicity and lower yields of Cup9p-FLAG. The cells (~1×10^{11}) were harvested and lysed at 4°C as described by Burgers (1995). The extract was fractionated by precipitation with 0.4% Polymin P (Sigma) and then further by precipitation with 48% saturated ammonium sulfate. The pellet was dissolved in 3 ml of TBS buffer (0.15 M NaCl, 50 mM Tris–HCl, pH 7.5), and passed through Sephadex G-25 in TBS. The resulting sample (8.3 ml, ~70 mg/ml of protein) was applied to a column (1 ml) of the monoclonal anti-FLAG M2 antibody (Kodak). The column was washed three times with 10 ml of TBS, and Cup9p-FLAG was eluted by the addition of five 1-ml samples of TBS containing, respectively, 50, 100, 100, 200 and 200 μg/ml of the FLAG peptide (Kodak). Peak Cup9p-FLAG fractions (detected by immunoblotting) were concentrated by partial lyophilization, followed by precipitation with methanol (Wessel and Flügge, 1984) in the presence of human insulin (Sigma, 0.3 mg/ml) as a carrier. The resulting sample was fractionated by SDS–12% PAGE and electroblotted onto Pro-Blot membrane (Perkin-Elmer). After a brief staining with Coomassie, the band of the 37 kDa Cup9p-FLAG (~15 pmol) was excised from the membrane. Half of the sample was used to determine the amino acid composition; the other half was subjected to N-terminal sequencing for seven cycles, using the Applied Biosystems 476A protein sequencer at the Caltech Microchemistry Facility.

Acknowledgements

We thank R.Deshaies, R.J.Dohmen, S.Grigoryev, M.Hochstrasser, S.Jentsch, E.S.Johnson, K.Madura, I.Ota and C.Trotta for the gifts of

strains and plasmids; S.Carter for his guidance in the synthesis of Leu-Eth; N.Johnsson, R.Deshaies, R.J.Dohmen and A.Webster for helpful discussions; L.Peck, Y.T.Kwon, A.Webster and F.Du for comments on the manuscript; S.Saha for help in overexpressing Cup9p; and N.Riley for technical assistance. This work was supported by grants to A.V. from the National Institutes of Health (DK39520 and GM31530).

References

Alagramam,K., Naider,F. and Becker,J.M. (1995) A recognition component of the ubiquitin system is required for peptide transport in *Saccharomyces cerevisiae*. *Mol. Microbiol.*, **15**, 225–234.

Ausubel,F.M., Brent,R., Kingston,R.E., Moore,D.D., Smith,J.A., Seidman,J.G. and Struhl,K. (1992) *Current Protocols in Molecular Biology*. Wiley-Interscience, New York.

Bachmair,A. and Varshavsky,A. (1989) The degradation signal in a short-lived protein. *Cell*, **56**, 1019–1032.

Bachmair,A., Finley,D. and Varshavsky,A. (1986) *In vivo* half-life of a protein is a function of its amino-terminal residue. *Science*, **234**, 179–186.

Baker,R.T. and Varshavsky,A. (1991) Inhibition of the N-end rule pathway in living cells. *Proc. Natl Acad. Sci. USA*, **87**, 2374–2378.

Baker,R.T. and Varshavsky,A. (1995) Yeast N-terminal amidase: a new enzyme and component of the N-end rule pathway. *J. Biol. Chem.*, **270**, 12065–12074.

Balzi,E., Choder,M., Chen,W.A., Varshavsky,A. and Goffeau,A. (1990) Cloning and functional analysis of the arginyl-tRNA–protein transferase gene *ATE1* of *Saccharomyces cerevisiae*. *J. Biol. Chem.*, **265**, 7464–7471.

Bartel,B. (1990) *Molecular Genetics of the Ubiquitin System: the Ubiquitin Fusion Proteins and Proteolytic Targeting Mechanisms*. PhD Thesis, M.I.T., Cambridge, MA, USA.

Bartel,B., Wünning,I. and Varshavsky,A. (1990) The recognition component of the N-end rule pathway. *EMBO J.*, **9**, 3179–3189.

Burgers,P.M.J. (1995) Preparation of extracts from yeast and avoidance of proteolysis. *Methods Mol. Cell. Biol.*, **5**, 330–335.

Chau,V., Tobias,J.W., Bachmair,A., Marriott,D., Ecker,D.J., Gonda,D.K. and Varshavsky,A. (1989) A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. *Science*, **243**, 1576–1583.

Chen,P., Johnson,P., Sommer,T., Jentsch,S. and Hochstrasser,M. (1993) Multiple ubiquitin-conjugating enzymes participate in the *in vivo* degradation of the yeast MATα2 repressor. *Cell*, **74**, 357–369.

Christianson,T.W., Sikorski,R.S., Dante,M. and Hieter,P. (1992) Multifunctional yeast high-copy-number shuttle vectors. *Gene*, **110**, 119–122.

deGroot,R.J., Rümenapf,T., Kuhn,R.J. and Strauss,J.H. (1991) Sindbis virus RNA polymerase is degraded by the N-end rule pathway. *Proc. Natl Acad. Sci. USA*, **88**, 8967–8971.

Dohmen,R.J., Stappen,R., McGrath,J.P., Forrová,H., Kolarov,J., Goffeau,A. and Varshavsky,A. (1995) An essential yeast gene encoding a homolog of ubiquitin-activating enzyme. *J. Biol. Chem.*, **270**, 18099–18109.

Finley,D., Özkaynak,E. and Varshavsky,A. (1987) The yeast polyubiquitin gene is essential for resistance to high temperatures, starvation and other stresses. *Cell*, **48**, 1035–1046.

Ghislain,M., Dohmen,R.J., Lévy,F. and Varshavsky,A. (1996) Cdc48p interacts with Ufd3p, a WD-repeat protein required for ubiquitin-dependent proteolysis in *Saccharomyces cerevisiae*. *EMBO J.*, **15**, 4884–4899.

Herskowitz,I. (1989) A regulatory hierarchy for cell specialization in yeast. *Nature*, **342**, 749–757.

Hilt,W. and Wolf,D.H. (1996) Proteasomes: destruction as a programme. *Trends Biochem. Sci.*, **21**, 96–102.

Hochstrasser,M. (1996) Ubiquitin-dependent protein degradation. *Annu. Rev. Genet.*, **30**, 405–439.

Hochstrasser,M. and Varshavsky,A. (1990) *In vivo* degradation of a transcriptional regulator: the yeast α2 repressor. *Cell*, **61**, 697–708.

Island,M.D., Naider,F. and Becker,J.M. (1987) Regulation of dipeptide transport in *S.cerevisiae* by micromolar amino acid concentrations. *J. Bacteriol.*, **169**, 2132–2136.

Jentsch,S. (1992) The ubiquitin-conjugating system. *Annu. Rev. Genet.*, **26**, 179–207.

Jentsch,S. and Schlenker,S. (1995) Selective protein degradation: a journey's end within the proteasome. *Cell*, **82**, 881–884.

Johnson,E.S., Gonda,D.K. and Varshavsky,A. (1990) *Cis–trans* recognition and subunit-specific degradation of short-lived proteins. *Nature*, **346**, 287–291.

Johnson,E.S., Bartel,B., Seufert,W. and Varshavsky,A. (1992) Ubiquitin as a degradation signal. *EMBO J.*, **11**, 497–505.

Johnson,E.S., Ma,P.C.M., Ota,I.M. and Varshavsky,A. (1995) A proteolytic pathway that recognizes ubiquitin as a degradation signal. *J. Biol. Chem.*, **270**, 17442–17456.

King,R.W., Deshaies,R.J., Peters,J.M. and Kirschner,M.W. (1996) How proteolysis drives the cell cycle. *Science*, **274**, 1652–1659.

Knight,S.A.B., Tamai,K.T., Kosman,D.J. and Thiele,D.J. (1994) Identification and analysis of a *Saccharomyces cerevisiae* copper homeostasis gene encoding a homeodomain protein. *Mol. Cell. Biol.*, **14**, 7792–7804.

Madura,K. and Varshavsky,A. (1994) Degradation of Gα by the N-end rule pathway. *Science*, **265**, 1454–1458.

Madura,K., Dohmen,R.J. and Varshavsky,A. (1993) N-recognin/Ubc2 interactions in the N-end rule pathway. *J. Biol. Chem.*, **268**, 12046–12054.

Mumberg,G., Müller,R. and Funk,M. (1994) Regulatable promoters of *Saccharomyces cerevisiae*: comparison of transcriptional activity and their use for heterologous expression. *Nucleic Acids Res.*, **22**, 5767–5768.

Ooi,C.E., Rabinovich,E., Dancis,A., Bonifacino,J.S. and Klausner,R.D. (1996) Copper-dependent degradation of the *Saccharomyces cerevisiae* plasma membrane copper transporter Ctr1p in the apparent absence of endocytosis. *EMBO J.*, **15**, 3515–3523.

Rothstein,R. (1991) Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast. *Methods Enzymol.*, **194**, 281–301.

Rubin,D.M., van Nocker,S., Glickman,M., Coux,O., Wefes,I., Sadis,S., Fu,H., Goldberg,A., Vierstra,R. and Finley,D. (1997) ATPase and ubiquitin-binding proteins of the yeast proteasome. *Mol. Biol. Rep.*, **24**, 17–26.

Schmitt,M.E., Brown,T.A. and Trumpower,B.L. (1990) A rapid and simple method for preparation of RNA from *Saccharomyces cerevisiae*. *Nucleic Acids Res.*, **18**, 3091–3092.

Schwob,E., Bohm,T., Mendenhall,M.D. and Nasmyth,K. (1994) The B-type cyclin kinase inhibitor p40 (Sic1) controls the G1 to S transition in *Saccharomyces cerevisiae*. *Cell*, **79**, 233–244.

Sherman,F., Fink,G.R. and Hicks,J.B. (1986) *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sikorski,R.S. and Hieter,P. (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *S. cerevisiae*. *Genetics*, **122**, 19–27.

Smith,D.B. and Johnson,K.S. (1988) Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene*, **67**, 31–38.

Smith,R.L., Redd,M.J. and Johnson,A.D. (1995) The tetratricopeptide repeats of Ssn6 interact with the homeodomain of α2. *Genes Dev.*, **9**, 2903–2910.

Varshavsky,A. (1996) The N-end rule: functions, mysteries, uses. *Proc. Natl Acad. Sci. USA*, **93**, 12142–12149.

Wessel,D. and Flügge,U.I. (1984) A method for quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal. Biochem.*, **138**, 141–143.

Wolberger,C. (1996) Homeodomain interactions. *Curr. Opin. Struct. Biol.*, **6**, 62–68.

Zhou,P. and Thiele,D.J. (1993) Copper and gene regulation in yeast. *Biofactors*, **4**, 105–115.

Received August 13, 1997; revised October 13, 1997; accepted October 14, 1997