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The *Saccharomyces cerevisiae* ubiquitin–proteasome system

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Our studies of the yeast ubiquitin–proteasome pathway have uncovered a number of general principles that govern substrate selectivity and proteolysis in this complex system. Much of the work has focused on the destruction of a yeast transcription factor, MATα2. The α2 protein is polyubiquitinated and rapidly degraded in α-haploid cells. One pathway of proteolytic targeting, which depends on two distinct endoplasmic reticulum-localized ubiquitin-conjugating enzymes, recognizes the hydrophobic face of an amphipathic helix in α2. Interestingly, degradation of α2 is blocked in **a**/α-diploid cells by heterodimer formation between the α2 and **a**1 homeodomain proteins. The data suggest that degradation signals may overlap protein–protein interaction surfaces, allowing a straightforward steric mechanism for regulated degradation. Analysis of α2 degradation led to the identification of both 20S and 26S proteasome subunits, and several key features of proteasome assembly and active-site formation were subsequently uncovered. Finally, it has become clear that protein (poly)ubiquitination is highly dynamic *in vivo*, and our studies of yeast de-ubiquitinating enzymes illustrate how such enzymes can facilitate the proteolysis of diverse substrates.

Keywords: yeast; ubiquitin; proteasome; proteolysis; mating type

1. INTRODUCTION

Intracellular protein degradation contributes to many cellular regulatory mechanisms, including cell cycle control, DNA repair, the stress response, differentiation, circadian rhythms, signal transduction and metabolic control (Hochstrasser 1996). For many short-lived eukaryotic proteins, conjugation to the polypeptide ubiquitin is a prerequisite for their degradation (figure 1). Ubiquitin is joined reversibly to other proteins via an isopeptide linkage between the C-terminus of ubiquitin and lysine (Lys) ε-amino groups of the acceptor proteins. In order to be attached to proteins, the C-terminus of ubiquitin must first be activated in an energy-dependent reaction by the enzyme E1, to which it becomes linked by a high-energy thiolester bond. Ubiquitin then forms a thiolester with a second protein, E2 or ubiquitin-conjugating (Ubc) enzyme. E2, usually with an additional factor, E3, catalyses isopeptide bond formation between ubiquitin and the substrate. There are multiple isoforms for both E2 and E3 proteins, and these many variants and their combinations underlie the marked substrate specificity of protein ubiquitination. For proteolytic substrates, assembly of a ubiquitin chain(s) on the protein is usually necessary for rapid degradation by the 26S proteasome, a *ca.* 2000 kDa protease complex that consists of a catalytic core called the 20S proteasome and a multisubunit regulatory complex, which confers ATP- and ubiquitin-dependence on substrate proteolysis by the 20S proteasome.

Our studies have focused on the intracellular proteolysis of the MATα2 repressor of *Saccharomyces cerevisiae* (Chen *et al.* 1993; Hochstrasser *et al.* 1995; Johnson *et al.* 1998). This eukaryote has three cell types: two haploid forms, **a** and α, and an **a**/α-diploid, produced by mating of haploid cells of opposite cell type (Herskowitz *et al.* 1992). Cell identity is governed by the mating type, or *MAT*, locus. In homothallic strains, mating type can switch when **a** or α sequences from one of two unexpressed loci are copied into the *MAT* locus. The change in cellular phenotype is apparent within a single cell cycle, suggesting that the transcriptional regulators encoded by the *MAT* loci may be short-lived. In fact, we found that the α2 homeodomain protein, which is encoded by the *MAT*α locus, has an *in vivo* half-life of about 4 min in α-cells. The ubiquitin–proteasome system is responsible for α2 degradation by mechanisms involving at least two degradation signals and four E2/Ubc enzymes. Ubc4 and Ubc5 define one proteolytic pathway, while Ubc6 and Ubc7 define the other, with the Ubc6/Ubc7-containing complex targeting the *Deg1* degradation signal, which resides within the first 67 residues of α2 (Chen *et al.* 1993).

In this paper, we review recent work from our laboratory on the yeast ubiquitin–proteasome system. Although we have concentrated much of our analysis on a single naturally short-lived regulatory protein, we have been able to glean a number of general insights into how

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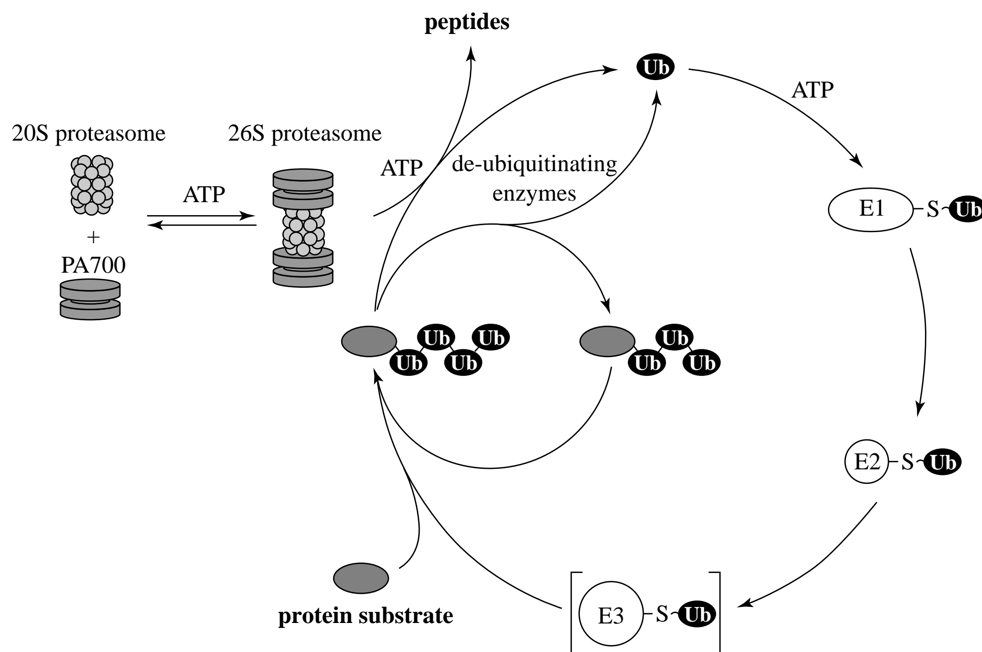


Figure 1. The ubiquitin–proteasome system. It is very likely that most if not all ubiquitin–protein ligation events *in vivo* require an E3 factor. Some of these E3s act as direct donors in ubiquitin transfer to substrate, forming an E3-ubiquitin thiolester (depicted in figure), whereas other E3s appear to act largely as adaptors, bringing substrate and the E2-ubiquitin thiolester conjugate into proper juxtaposition. See text for further details.

proteins are targeted for ubiquitin modification, how such ubiquitinated proteins are destroyed by the 26S proteasome complex, and how substrate de-ubiquitination reactions are integrated into the degradative pathway. Here we will consider several illustrative examples of what we have learned about facets of this complex metabolic regulatory system.

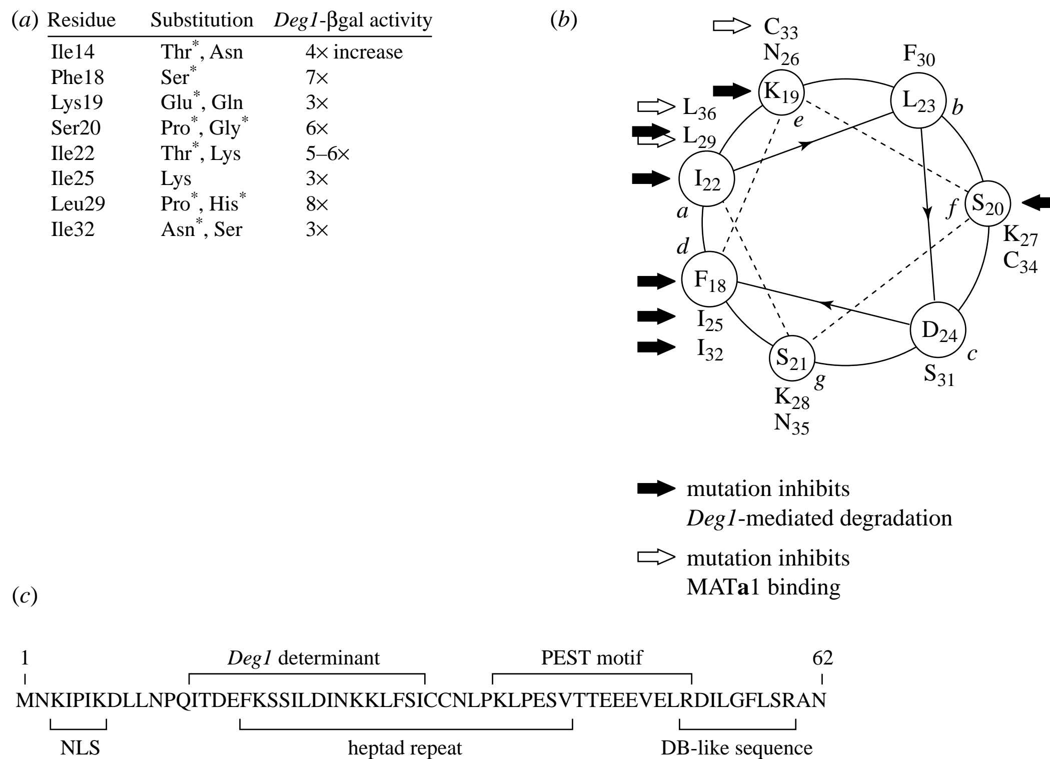
2. RESULTS AND DISCUSSION

(a) *The* **Deg1** *degradation signal of* α*2 and its regulation by protein–protein interaction*

A basic question about the ubiquitin pathway concerns the nature of the substrate features that allow specific recognition by E2s and/or E3s. At present, only fragmentary information on the nature of such degradation signals is available. The first characterized signal was defined with a set of engineered proteins called N-end rule substrates (Varshavsky 1997). The principal components of the N-end rule signal are the N-terminal residue and an accessible Lys residue that can be ligated to ubiquitin. Short-sequence motifs such as the cyclin destruction box (DB) (Glotzer *et al.* 1991) and stretches rich in Pro, Glu/Asp, Ser, and Thr residues called PEST sequences (Rechsteiner & Rogers 1996) are necessary for degradation of certain ubiquitin-dependent substrates, but they are not sufficient. The defining character of PEST elements also remains unclear. For instance, only a subset of site-directed mutations in the PEST motif of the yeast Gcn4 protein inhibited proteolysis (Kornitzer *et al.* 1994).

We have recently conducted an extensive analysis of the *Deg1* degradation signal of α2 (Johnson *et al.* 1998). The initial approach was to systematically delete small segments within the N-terminal 67 residues of the repressor in the context of α2-β-galactosidase (α2-βgal) fusions to localize further the sequences necessary for proteolytic targeting. The first 62 residues were found to be sufficient for maximal rates of proteolytic targeting. However, several regions within this stretch of α2 could be deleted without compromising degradation by the Ubc6/Ubc7-dependent pathway. These regions included a strong PEST sequence. A mutation in the DB-like sequence of α2 also did not impede degradation, whereas the same mutation in the cyclins strongly inhibits their turnover. In addition, the presence of a functional nuclear localization sequence (NLS) facilitates degradation, but is not absolutely required. This latter effect may reflect the fact that the Ubc6 and Ubc7 enzymes are both localized to the endoplasmic reticulum (ER)–nuclear envelope (in yeast, the outer nuclear envelope represents the bulk of the ER). The NLS may help concentrate the *Deg1*-bearing substrate in the vicinity of the ubiquitination complexes.

Deletion analysis provided a low-resolution description of the *Deg1* proteolytic signal. We therefore devised a screen to identify individual residues within *Deg1* that are critical for its function (Johnson *et al.* 1998). The DNA encoding the $\alpha2_{1-67}$ segment of $\alpha2_{1-67}$βbgal was randomly mutagenized by low-fidelity polymerase chain reaction, and plasmids encoding mutagenized *Deg1*-βgal were tested for degradation in wild-type yeast cells. Subsequently, specific portions within the $\alpha2_{1-67}$ segment were mutagenized by cassette mutagenesis and site-specific mutagenesis. The mutations identified that strongly impaired degradation was tightly clustered, being restricted to 19 residues in the stretch from Ile14 to Ile32 in the *Deg1* sequence (figure 2*a*). This region overlaps a prominent 3,4-heptad repeat that is predicted to form part of a coiled-coil structure (Ho *et al.* 1994). When the *Deg1*-inactivating mutations were mapped onto a helical wheel projection of this segment of α2, almost all of the residues affected were on the hydrophobic face of the predicted amphipathic helix (figure 2*b*,*c*). The alterations observed in residues at the hydrophobic *a* and *d* positions of the heptad repeat would be expected to weaken coiled-coil interactions. The K19 mutations affected a residue at the *e* position. Residues at the *e* and *g* positions of heptad repeats often help stabilize the interhelical interface (Lupas 1996). The S20P and S20G mutations mapped to the outside surface of the predicted helix, but Pro and



(*a*)

Residue	Substitution	*Deg1*-βgal activity
Ile14	Thr*, Asn	4× increase
Phe18	Ser*	7×
Lys19	Glu*, Gln	3×
Ser20	Pro*, Gly*	6×
Ile22	Thr*, Lys	5–6×
Ile25	Lys	3×
Leu29	Pro*, His*	8×
Ile32	Asn*, Ser	3×

Figure 2. The ubiquitin-dependent *Deg1* degradation signal of MATα2. (*a*) Summary of point mutations in *Deg1* that cause defects in *Deg1*-βgal degradation. The average fold-increase in βgal activity over wild-type is indicated. Residues marked with an asterisk denote mutants that were tested by pulse chase. (*b*) Helical-wheel representation of region of α2 N-terminus (residues 18–36) predicted to form part of a coiled-coil structure. The hydrophobic surface may extend to residue 14, but the additional sequence does not conform to the 3,4 heptad repeat. Black arrows mark residues with a mutation which inhibited *Deg1*-mediated proteolysis. Open arrows indicate residues where substitutions weaken binding to **a**1 (Ho *et al.* 1994). (*c*) Summary of sequence elements tested for role in *Deg1*-mediated degradation by the ubiquitin–proteasome pathway.

Gly can act as helix breakers. Collectively, the mutagenesis data reveal a striking clustering of inactivating mutations within *Deg1*, and these alterations localize primarily to the hydrophobic face of a predicted amphipathic helix.

Independent evidence for an N-terminal amphipathic helix in α2 was obtained by Ho *et al.* (1994), who showed that mutations predicted to alter this helix disrupted the binding of α2 and **a**1 *in vitro* and *in vivo*. Therefore, overlapping segments of α2 are involved in both ubiquitin-dependent proteolysis and **a**1 interaction. Previous studies of α2 turnover were carried out almost entirely in α-haploid cells. Remarkably, when assayed in **a**/α-diploid cells, α2 degradation was dramatically reduced relative to haploids (figure 3*a*). A detailed kinetic analysis of α2 degradation in **a**/α-diploids revealed an initial period during which degradation approached the rate observed in haploid α-cells, followed by an extended period with very little degradation (figure 3*b*).

By the same logic that α2 was initially predicted to be degraded rapidly in α-cells (see Hochstrasser *et al.* 1995), the **a**1 protein would be expected to be short-lived in **a**-cells. Indeed, we found that **a**1 was destroyed exceptionally rapidly in **a**-haploid cells (figure 3*c*). We do not yet know many details about the mechanism of **a**1 turnover, but in *ubc4 ubc6* and *doa4* mutants (Hochstrasser 1996), degradation of **a**1 was inhibited by at least threefold and sixfold, respectively, relative to the wild-type (not shown). Hence, the ubiquitin–proteasome pathway is also crucial for **a**1 breakdown. Examination of **a**1 degradation in **a**/α-cells revealed that its turnover was regulated by cell type in the same manner as that of α2. The half-life of **a**1 was well below one minute in **a**-haploids and increased dramatically in **a**/α-cells (figure 3*c*).

The biphasic kinetics of α2 degradation in **a**/α-cells (figure 3*b*) suggested the presence of two metabolically distinguishable populations of α2 molecules in these cells. In this view, rapid disappearance of α2 at early chase time points reflects efficient degradation of α2 homodimers (and/or monomers), as occurs in α-haploids. The fraction of α2 bound to **a**1 would represent a second pool of α2 in **a**/α-cells that is only slowly degraded during the chase. This interpretation predicts that changing the relative levels of **a**1 and α2 should lead to changes in the degradation kinetics of α2 (and **a**1). This was precisely what was observed (Johnson *et al.* 1998). Specifically, when more of α2 was forced into heterodimers with **a**1, a greater fraction of α2 became resistant to degradation. The corresponding stabilizing effect was seen for **a**1 when α2 was overexpressed relative to **a**1. We also found that mutations in either α2 or **a**1 that have been shown or are predicted to interfere with **a**1–α2 dimerization led to haploid rates of degradation for both proteins. Finally, we showed that the N-terminal domain of α2 alone could strongly stabilize **a**1 and was itself strongly stabilized by **a**1. This block to degradation was correlated with a strong drop in the level of α2 ubiquitination. Taken together, our

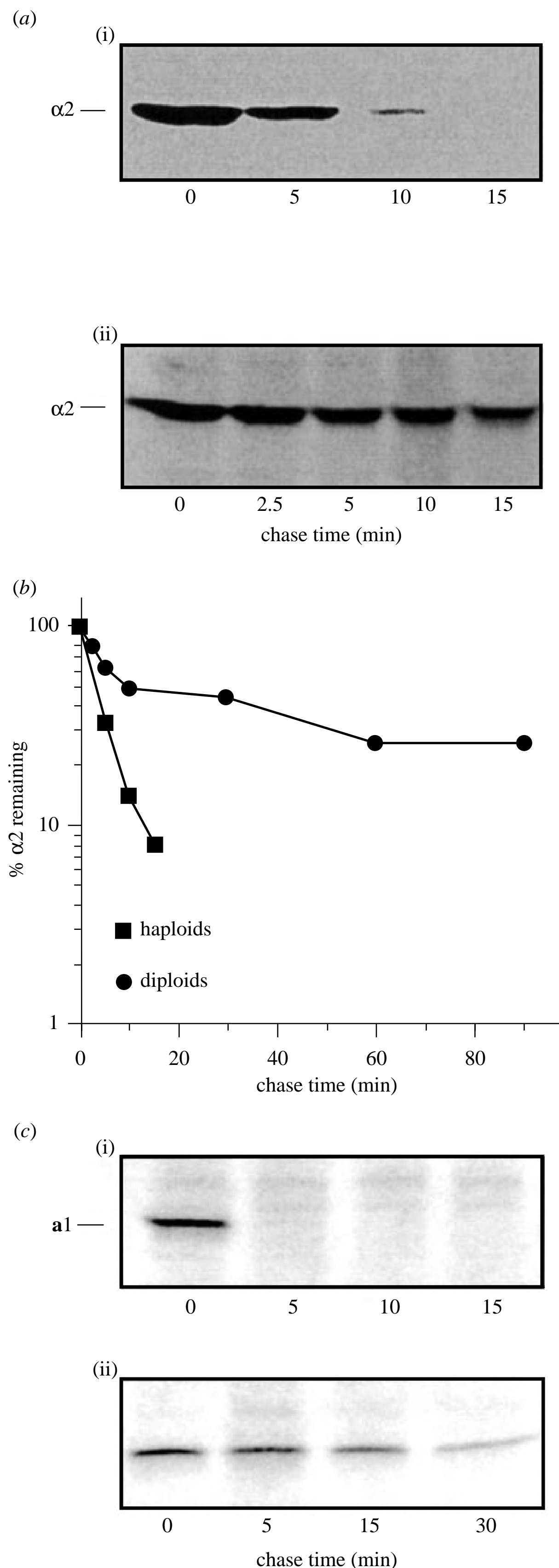


Figure 3. Regulation of α2 by **a**1 binding. (*a*) Pulse-chase analysis of α2 degradation in (i) **a**-haploid and (ii) **a**/α-diploid cells. (*b*) Detailed kinetic analysis of α2 turnover in **a**/α-diploids. (*c*) Rapid degradation of **a**1 in **a**-cells but not **a**/α-diploids. Pulse-chase analysis of endogenous **a**1 in wild-type (i) **a**-haploid and (ii) **a**/α-diploid cells. Proteins were precipitated with an antiserum against **a**1.

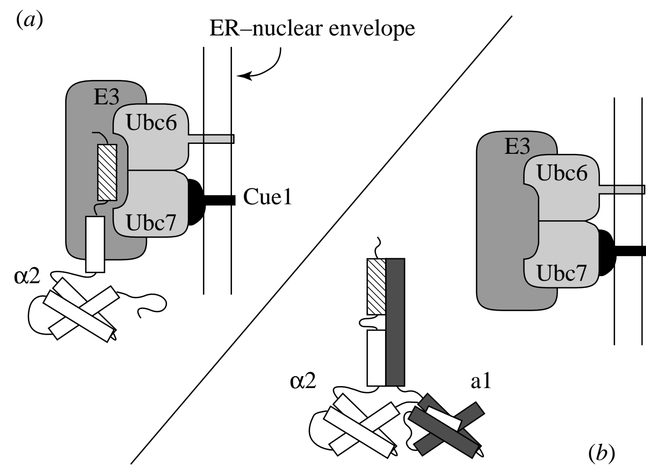


Figure 4. Model for regulated α2 turnover. (*a*) In α-cells the *Deg1* signal of α2 is accessible to the E2s and (predicted) E3 that recognize *Deg1*. (*b*) In **a**/α-diploid cells **a**1 binding to α2 blocks the α2 degradation signals, preventing α2 ubiquitination. At the same time, presumptive **a**1 degradation signals are blocked by α2 binding. Ubc6 and Ubc7 are shown tethered to the ER–nuclear envelope. Ubc6 in anchored by a hydrophobic C-terminal sequence and Ubc7 binds to Cue1, an integral membrane protein (Biederer *et al.* 1997). Both Cue1 and the hydrophobic tail of Ubc6 are required for *Deg1*-mediated degradation.

data demonstrate that degradation of **a**1 and α2 is controlled by the direct physical interaction between these two proteins, with the most critical interactions occurring between their N-terminal domains.

The most straightforward interpretation of the mutagenesis data is that this hydrophobic surface of the *Deg1* determinant is a primary recognition element for the Ubc6/Ubc7-containing ubiquitination complex (figure 4). Additional residues also contribute to degradation, although they may not participate directly in E2/E3 recognition. The presence of **a**1 greatly retards the degradation of α2 in **a**/α-cells, and a symmetrical regulation of **a**1 by α2 is observed in these same cells. Although we cannot completely exclude the possibility that **a**1 binding somehow alters the conformation of α2 in a way that inactivates *Deg1* irrespective of exposure of the signal in the heterodimer, the direct overlap of segments of α2 implicated in both **a**1 binding and *Deg1* function makes it far more likely that binding to **a**1 directly masks at least a portion of the *Deg1* signal from the ubiquitin pathway.

The coiled coil or leucine zipper is among the most widespread protein–protein interaction motifs (Lupas 1996). Coiled coils mediate interactions between a number of proteins that are known to be short-lived, including c-Jun homodimers, Fos-Jun heterodimers, and Gcn4 homodimers (Treier *et al.* 1994; Kornitzer *et al.* 1994). Whether any of the degradation signals in these proteins involves the heptad repeats is not yet clear. Coiled coils may also be involved in the degradation of normally long-lived proteins. For example, keratin filaments are made up of two heterologous subunits that assemble into coiled-coil structures, but when one subunit is produced in excess over its partner, the excess protein is degraded (Magin *et al.* 1998). By analogy to the data on regulated α2 degradation, turnover of keratin monomers

may depend on accessibility of a hydrophobic surface in an unpaired amphipathic helix.

In support of this analogy, Ubc6/Ubc7-dependent ubiquitination has been implicated in the degradation of another protein that is normally part of a multisubunit complex. The Ndc10 protein forms part of the yeast kinetochore, but in *ndc10-2* mutant cells, it appears that the Ndc10-2 protein is not efficiently incorporated into kinetochore complexes at the restrictive temperature, apparently causing the unincorporated protein to be rapidly degraded (Kopski & Huffaker 1997). Mutation of *UBC6* or *UBC7* suppresses this defect. We suggest that exposure of a cryptic degradation signal overlapping a kinetochore interaction site of Ndc10 is responsible for the rapid degradation of the unincorporated subunit. More generally, 'aberrant' proteins, which are often rapidly destroyed by the ubiquitin pathway, may be identified as such by the cell based on their exposure of hydrophobic surfaces normally buried in protein–protein interfaces or within protein interiors. Our data on **a**1–α2 suggest that the principles of substrate recognition for natural and aberrant substrates of the ubiquitin–proteasome pathway may often be similar.

(b) *20S proteasome architecture: the example of Pup1–Pup3*

Four heptameric rings stack into a hollow cylinder to form the 20S proteasome (Peters 1994). In the archaeon *Thermoplasma acidophilum*, a 20S proteasome has been described that has only two kinds of subunits, α and β, with seven α-subunits in each of the outer two rings and seven β-subunits in each inner ring (Rubin & Finley 1995). All eukaryotic 20S proteasome subunits are related in sequence to the archaeal subunits. In yeast, there are 14 genes encoding 20S proteasome subunits, with seven different α-type subunits and seven different β-type subunits. Yeast 20S proteasomes are present as a uniform population of complexes in which each particle has the same 14 different subunits (Chen & Hochstrasser 1995). The *Thermoplasma* proteasome has a single type of active site, whereas the eukaryotic particle has at least three (Rubin & Finley 1995). The archaeal enzyme efficiently cleaves model peptides only after hydrophobic residues (a 'chymotrypsin-like' activity); the eukaryotic protease can, in addition, cleave peptides after basic ('trypsin-like' activity) and acidic residues ('peptidylglutamyl peptide hydrolysing' (PGPH) activity).

Solution of the *Thermoplasma* proteasome crystal structure revealed that the active sites are in the β-subunits within a central chamber of the particle (Löwe *et al.* 1995). Each β-subunit is synthesized with a short N-terminal propeptide; the N-terminal Thr side-chain of the processed β-subunit is responsible for nucleophilic attack on substrates (Löwe *et al.* 1995). We recently showed that the yeast *S. cerevisiae* 20S proteasome is likely to use the same catalytic mechanism (Chen & Hochstrasser 1996; Arendt & Hochstrasser 1997). Consistent with this, when mammalian proteasomes are treated with lactacystin, an irreversible proteasome inhibitor, one of the two rapidly modified sites in the β-subunit X/MB1 is the N-terminal Thr (Fenteany *et al.* 1995). Interestingly, only three out of the seven different β-type subunits in a given eukaryotic 20S particle appear to have all the residues required for formation of such an N-terminal active site (Seemüller *et al.* 1995) (figure 5).

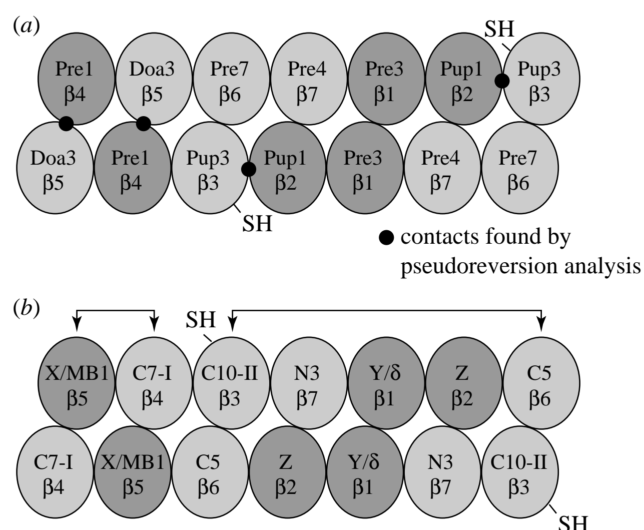


Figure 5. Proposed β-subunit arrangements in yeast and human 20S proteasomes. The central two β-subunit rings are shown opened and splayed. (*a*) The yeast subunit arrangement and β1–β7 nomenclature are from Groll *et al.* (1997). Also indicated are the subunit contacts (black dots) determined initially by pseudoreversion studies (Chen & Hochstrasser 1996; Arendt & Hochstrasser 1997). (*b*) Arrangement of the human subunits is from Kopp *et al.* (1997), with the apparent positional switches between the human and yeast subunits indicated. Subunits bearing the protease active sites are shown as darker grey. The subunit with the Cys residue that can be modified by NEM to inhibit the trypsin-like active site is shown with a sulfhydryl (SH) group.

Our earlier work led to a model in which we proposed that interactions between specific subunits across the central β-subunit rings were critical for establishment of individual proteasome active sites (Chen & Hochstrasser 1995). This model received strong support from both the *Thermoplasma* proteasome-inhibitor structure (Löwe *et al.* 1995) and experiments in yeast (Chen & Hochstrasser 1996). However, it seems likely that specific interactions between heterologous β-subunits within each β-subunit ring will also be important for active-site formation (Hochstrasser *et al.* 1995). Dick *et al.* (1992) identified a specific Cys residue in the C10-II β-subunit of the bovine proteasome the modification of which by N-ethylmaleimide (NEM) specifically inhibited trypsin-like activity (figure 5). This NEM modification could be blocked by preincubation with leupeptin, a competitive inhibitor of the trypsin-like activity (Dick *et al.* 1992). The residue in the archaeal enzyme that apparently corresponds to the NEM-modified Cys (figure 6*a*) is at the interface between subunits within the β-subunit ring (Hochstrasser *et al.* 1995). The C10-II subunit, which is homologous to yeast Pup3, does not have the N-terminal Thr residue shown to be required for catalysis in the *Thermoplasma* proteasome, so C10-II modification by NEM may interfere with substrate binding or cleavage in a neighbouring subunit in the same ring.

We determined whether yeast 20S proteasomes were also inhibited by NEM (Arendt & Hochstrasser 1997). Treatment of purified proteasomes with 5 mM NEM

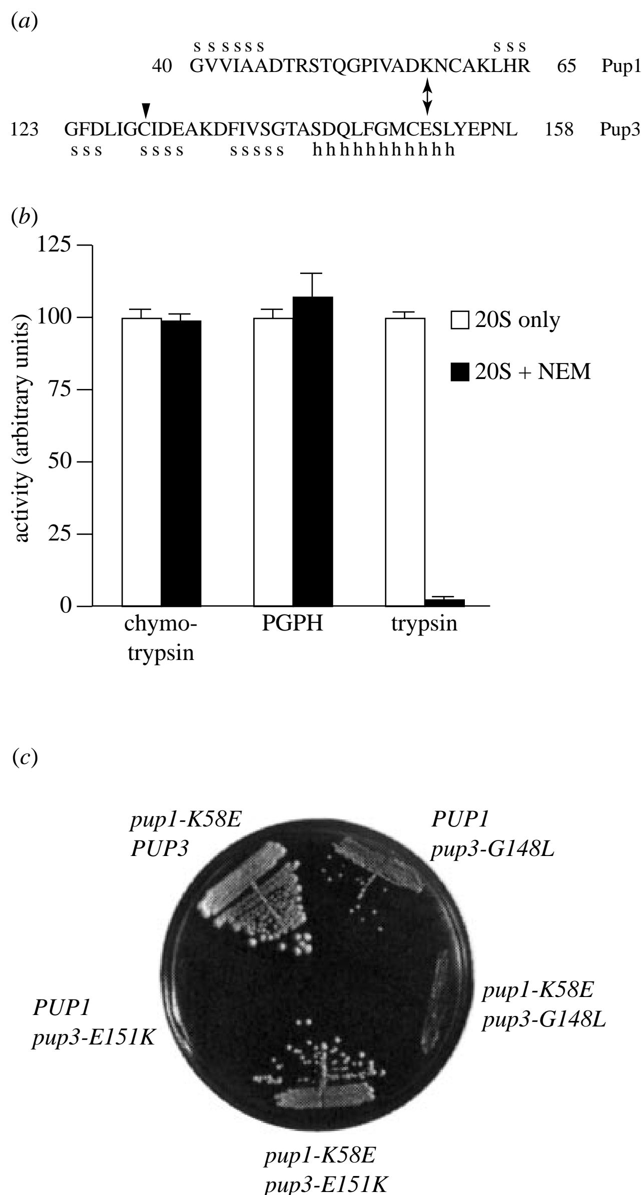


Figure 6. Active-site formation and subunit arrangement in the 20S proteasome. (*a*) Pup1 and Pup3 β-subunits in the regions surrounding the residues involved in the putative salt bridge (double arrow) and the region of Pup3 with the conserved Cys residue (arrowhead) whose modification by NEM in bovine C10-II inhibits trypsin-like activity. Secondary structures expected based on the *Thermoplasma* structure (Löwe *et al.* 1995) are also shown: s, β-strand; h, helix. (*b*) Inhibition by NEM of peptidase activities of purified yeast 20S proteasomes. Values are the mean of three measurements. Proteasomes were incubated with 5 mM NEM for 20 min at 30 °C. (*c*) Structure-based pseudoreversion analysis of the interaction between Pup1 and Pup3. Cells transformed with the indicated plasmid-borne alleles were streaked on FOA plates and grown at 30 °C.

almost completely eliminated the trypsin-like activity, but PGPH and chymotrypsin-like activities were unaffected (figure 6*b*). The Cys in C10-II whose modification by NEM correlates with loss of trypsin-like activity in bovine proteasomes is conserved in the yeast Pup3 subunit (figure 6*a*), suggesting that modification of this Cys in Pup3 is responsible for inactivation of the trypsin-like sites in the yeast enzyme.

Because the catalytic residues for the trypsin-like active sites reside in the Pup1 subunits (Arendt & Hochstrasser 1997), the inhibition of trypsin-like activity by NEM would be predicted to be due to perturbation of the active-site pocket of Pup1. We therefore guessed that Pup1 and Pup3 might be direct neighbours. Previous pseudo-reversion studies in yeast that were based on the *Thermoplasma* proteasome structure were used to demonstrate a direct interaction of Doa3 with Pre1 between the different β-subunit rings that is critical for formation of the chymotrypsin-like sites (Chen & Hochstrasser 1996). We used an analogous strategy to test for an interaction between Pup1 and Pup3. A salt bridge is observed between Lys29 of each *Thermoplasma* β-subunit with Glu139 in each neighbouring subunit in the same ring. Sequence alignments with eukaryotic β-subunits suggested that this interaction might be conserved in a subset of proteasome pairings. For the putative Pup1–Pup3 contact, the salt bridge was predicted to form between Lys58 of Pup1 (numbering refers to the unprocessed subunit) and Glu151 of Pup3 (figure 6*a*). We reasoned that if this electrostatic interaction existed, a Lys-to-Glu mutation in Pup1 or a Glu-to-Lys mutation in Pup3 might be strongly deleterious, but proteasome function might be at least partially restored in a strain carrying both mutations.

Low- or high-copy plasmids carrying the *pup1-K58E* and *pup3-E151K* alleles were transformed into yeast strains bearing chromosomal deletions of either or both *PUP1* and *PUP3*, with the corresponding wild-type genes present on plasmids that also carried the *URA3* gene. Transformants were then plated on 5-fluoroorotic acid, a compound toxic to cells expressing *URA3*, to determine if colonies were able to form in the absence of the wild-type proteasome gene(s). Mutant *pup1-K58E* cells grew at wild-type rates at 30 °C (figure 6*c*) and grew essentially like wild-type cells at high temperature (37 °C), low temperature (14 °C), or in the presence of 1 μg ml^{-1} canavanine. No defect in MATα2 degradation was seen, and glycerol gradient analysis revealed only a minor defect in trypsin-like activity (data not shown).

In contrast, the *pup3-E151K* allele did not allow colony formation even when expressed from a high-copy plasmid (figure 6*c*). Strikingly, however, the *pup1-K58E* allele in either a low- or high-copy plasmid rescued the lethality of the *pup3-E151K* mutation, although growth was slower than that of wild-type cells. This suppression was only seen when the *pup3-E151K* allele was on a high-copy plasmid, suggesting that the mutant protein was expressed poorly or did not fold or assemble into the proteasome as efficiently as its wild-type counterpart. To test whether suppression by *pup1-K58E* was allele-specific, we combined it with the *pup3-G148L* allele. The *pup1-K58E pup3-G148L* mutant failed to form colonies (figure 6*c*); this synthetic lethality contrasted with the suppression of lethality seen with the *pup1-K58E pup3-E151K* double mutant. Because these pseudoreversion tests were structure based, we conclude that Pup1 and Pup3 are neighbours within each central ring of the 20S proteasome.

A crystal structure for the yeast 20S proteasome has recently been determined (Groll *et al.* 1997), allowing the full subunit arrangement of the complex to be determined (figure 5). These structural data confirm our genetic

demonstration of the Pup1–Pup3 interaction as well as the earlier demonstration of an inter-ring contact between Doa3 and Pre1 (Chen & Hochstrasser 1996). Using immunoelectron microscopy and protein–protein cross-linking, the subunit arrangement within the human 20S proteasome has also been described recently (Kopp *et al.* 1997). However, the Z and C10-II subunits, which correspond, respectively, to yeast Pup1 and Pup3, were not inferred to be neighbours with the β-subunit rings. This could indicate either that the data for the proposed human subunit arrangement are not yet definitive or that yeast and human 20S proteasomes have a different organization. Our data on Pup1–Pup3 suggest that the former explanation is more likely (see figure 5). For both yeast and mammalian proteasomes, NEM specifically ablates trypsin-like activity, and the Cys in C10-II, the modification of which leads to this inactivation, is conserved in yeast Pup3. In the proposed human subunit arrangement, the within-ring neighbours of C10-II are both subunits lacking catalytic sites. Thus, it would be difficult to explain the similar NEM effects on yeast and mammalian proteasomes and the highly specific perturbation of just a single activity unless in both cases Pup1/Z and Pup3/C10-II were direct neighbours. We expect that further analysis of the human proteasome will reveal that the subunits are arranged in the same way as their yeast counterparts.

(c) *Protein degradation and the disassembly of polyubiquitin chains*

Ubiquitination of proteins is reversible. De-ubiquitination is catalysed by specialized proteases called de-ubiquitinating or DUB enzymes, which are thiol proteases that hydrolyse the amide bond between Gly76 of ubiquitin and the substrate protein (Wilkinson & Hochstrasser 1998). One of the two known classes of DUB enzymes is the so-called ubiquitin-specific processing protease or UBP class. The UBP family is extremely divergent, but all members contain several short consensus sequences, the Cys and His boxes, that are likely to form part of the active site (Baker *et al.* 1992; Papa & Hochstrasser 1993). These sequence motifs include absolutely conserved Cys and His residues, respectively, which are critical for catalytic activity. Several additional short sequences show some conservation as well (Papa & Hochstrasser 1993; Wilkinson & Hochstrasser 1998). As a group, DUB enzymes are the largest known family of enzymes in the ubiquitin system. There are 17 genes encoding such enzymes in *S. cerevisiae*, out of which 16 are of the UBP class (Hochstrasser 1996). However, very little is known about their physiological functions.

At the enzymological level, the best understood UBP enzyme is isopeptidase T (IsoT) from mammalian cells (Hadari *et al.* 1992; Falquet *et al.* 1995; Wilkinson *et al.* 1995; Melandri *et al.* 1996). IsoT acts largely, if not exclusively, on unanchored ubiquitin chains, i.e. oligomers with a free ubiquitin C-terminus (Wilkinson *et al.* 1995). *In vitro* studies suggest that IsoT could facilitate protein degradation by the proteasome, possibly by preventing accumulation of ubiquitin chains generated as intermediates in substrate degradation (Hadari *et al.* 1992; Beal *et al.* 1996). A number of enzymes can also synthesize unanchored ubiquitin chains from free ubiquitin (Chen & Pickart 1990; Van Nocker & Vierstra 1991), and these

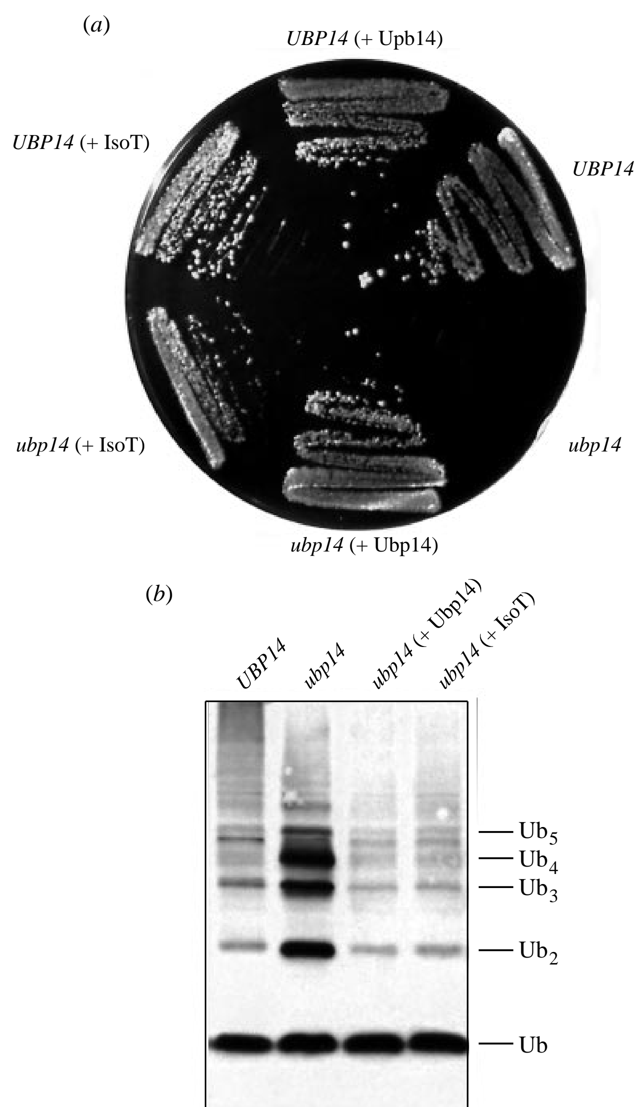


Figure 7. Dynamics of ubiquitin–protein conjugates and proteolysis by the proteasome. (*a*) Human IsoT complements an *S. cerevisiae ubp14Δ* mutation. Growth on canavanine. Wild-type and *ubp14* cells carrying the indicated plasmids were streaked onto selective media containing 1.2 μg ml^{-1} canavanine sulphate. The plates were incubated at 30 °C for three days. (*b*) Reduction of unanchored ubiquitin chains to wild-type levels in *ubp14* mutants by expression of human IsoT. Anti-ubiquitin immunoblot analyses of cells shown in panel (*a*). Positions of free ubiquitin and ubiquitin multimers are indicated.

chains may need to be disassembled to avoid accumulation to levels that inhibit proteolysis.

We have recently completed a study on a yeast UBP-type enzyme, Ubp14, that is 31% identical to human IsoT (Amerik *et al.* 1997). Ubp14 and IsoT have similar enzymatic properties, e.g. neither can disassemble a ubiquitin dimer terminating with *des*GlyGly-ubiquitin (UbΔGG), a truncated ubiquitin missing the last two residues. A yeast mutant lacking Ubp14 has defects in ubiquitin-dependent protein degradation and accumulates free ubiquitin chains. The *ubp14* proteolytic defect can be 'phenocopied' by expression of UbΔGG in wild-type cells; *in vivo* expression of UbΔGG also creates an overabundance of ubiquitin chains, suggesting that the

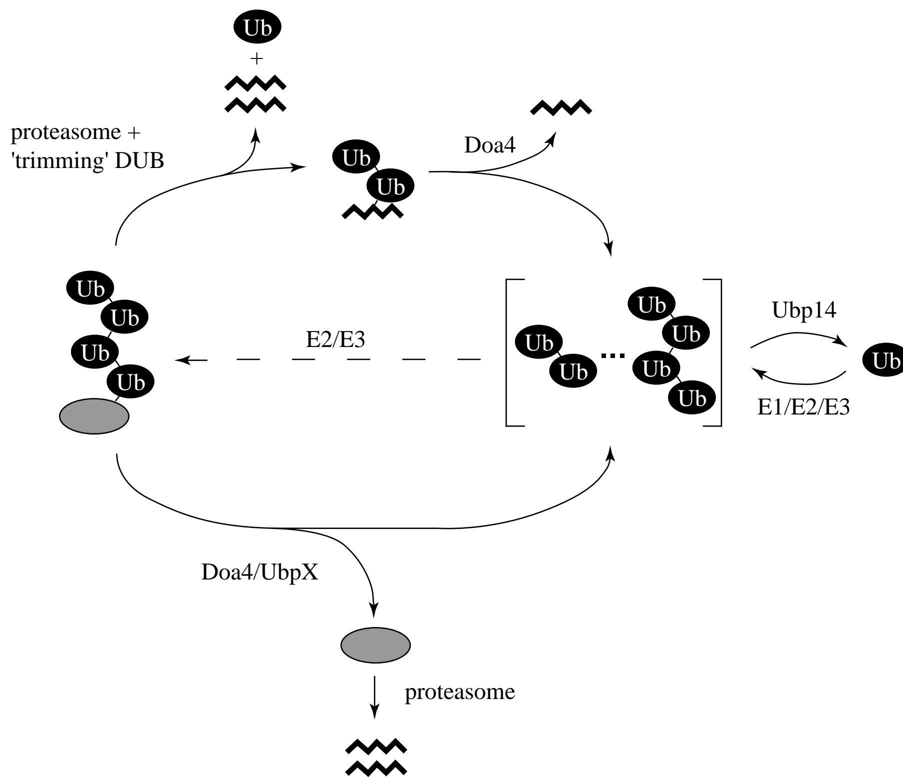


Figure 8. Model for Ubp14 action and its integration into the ubiquitin-dependent proteolytic system. Release of complete ubiquitin chains from either proteasome-bound protein or peptide is shown, but removal of ubiquitins by 'trimming' DUB enzymes (Lam *et al.* 1997) is not precluded and may actually occur concurrently with degradation, particularly in the upper pathway where the protein is 'committed' to proteolysis while still ubiquitinated and may have more extended access to a slow-acting trimming DUB. Ubiquitin oligomers may also become severed from polyubiquitinated substrates that are not bound to proteasomes. The hypothesized ligation of preformed polyubiquitin chains by E2 and/or E3 enzymes to protein is indicated by the dashed arrow.

proteolytic defects resulting from loss of Ubp14 are due to the accumulation of ubiquitin chains.

The sequence similarity of the yeast Ubp14 and mammalian IsoT proteins and their similar substrate specificities suggest they may be functionally related. To test this conjecture, the cDNA for human IsoT (Wilkinson *et al.* 1995) was expressed in yeast *ubp14* cells, and the phenotype of the transformants was characterized. Expression of IsoT under these conditions almost completely suppressed the canavanine hypersensitivity of *ubp14* cells (figure 7*a*). At the same time, the level of unanchored ubiquitin chains was reduced to amounts comparable with those observed in wild-type cells (figure 7*b*). This suppression occurred despite there being only low levels of IsoT expressed; levels were below our detection limit in immunoblots with an antibody that allowed the detection of nanogram amounts of recombinant IsoT. In contrast, overexpression of Ubp1, Ubp2 or Doa4 did not correct the phenotypic abnormalities of the *ubp14* mutant, indicating that the suppression was not due to a general increase in intracellular ubiquitin isopeptidase activity; conversely, IsoT expression in *doa4* cells did not alter their mutant phenotype (data not shown). From these data, we conclude that Ubp14 and IsoT are functional homologues.

Recently, we have found that the *Dictyostelium* UbpA protein can substitute for Ubp14 in yeast cells as well (Lindsey *et al.* 1998). Interestingly, a *D. discoideum* strain lacking UbpA grows normally but has a specific defect in multicellular development when induced to differentiate. Given its functional homology with Ubp14 and the demonstration that yeast *ubp14Δ* cells have a defect in ubiquitin-dependent proteolysis, the developmental defect in *ubpA* mutants is very likely due to an impairment of ubiquitin-dependent proteolysis, perhaps of only one or a few (negative) regulators of multicellular development.

How is the enzymatic activity of Ubp14 mechanistically coupled to ubiquitin–proteasome-dependent proteolysis? We propose that by restricting the intracellular accumulation of ubiquitin chains, Ubp14 limits their binding to and inhibition of proteasomes and potentially other enzymes of the ubiquitin system (figure 8). This would be consistent with the inhibition of ubiquitin-lysozyme conjugate degradation observed when high concentrations of ubiquitin chains were added to a crude fraction of rabbit reticulocyte lysates (Beal *et al.* 1996). The model is also consistent with the original proposal of Hadari *et al.* (1992), who suggested that IsoT disassembles polyubiquitin chains generated during substrate breakdown by the proteasome and thereby stimulates ubiquitin-dependent proteolysis by preventing inhibition by these end-products; however, our data indicate that *in vivo*, inhibitory chains may not derive solely from proteasome action. Ubp14 action is likely also to be linked to that of Doa4, the other major DUB enzyme required for normal rates of ubiquitin-dependent proteolysis in yeast (Papa & Hochstrasser 1993) (figure 8). Based on analysis of *doa4* mutants, Doa4 is likely to act by cleaving ubiquitin or polyubiquitin chains from substrates that have been targeted to the proteasome. Following the release of the chains, Ubp14 can disassemble them to monomeric ubiquitin. Whether Ubp14 can interact with the proteasome to target such Doa4/proteasome products is not yet clear. We observed that a catalytically inactive derivative of Ubp14 behaved as a dominant-negative mutant, a finding that is consistent with the possibility of a Ubp14–proteasome interaction.

Our data suggest that the concentration of free ubiquitin chains must be carefully controlled *in vivo*. An

excess of unanchored chains can inhibit proteolysis, e.g. by competing for substrate-binding sites on the proteasome, but a level that is too low may reduce the efficiency of protein degradation as well, at least for certain substrates. This latter suggestion is based on the finding that some ubiquitin-dependent proteolytic substrates are stabilized in yeast by the overproduction of wild-type Ubp14 and this correlates with a reduction in intracellular levels of polyubiquitin chains (Amerik *et al.* 1997). The control of cellular ubiquitin-chain levels by regulation of ubiquitin chain assembly and/or disassembly rates by Ubp14 or its functional homologues may thus provide a mechanism for changing the relative degradation rates of different proteins during changing environmental or developmental conditions. The developmental defect associated with inactivation of the *Dictyostelium* homologue of Ubp14 provides an apparent example of what can occur when such a regulatory mechanism fails to work properly in a multicellular eukaryote. It is also noteworthy that human IsoT isozymes are encoded by two different genes in addition to being subject to alternative splicing. Ubiquitin-dependent processes could therefore be regulated by differential control of the activity and/or localization of individual Ubp14-related isozymes.

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Discussion

M. Yanagida (*CREST Research Project, Kyoto University, Kyoto, Japan*). What is the phenotype of UBC6 and UBC7 knockout?

M. Hochstrasser. They are a little different: UBC6 has very little in the way of a phenotype. It does seem to be important for the degradation of ER proteins—particularly aberrant ER proteins. The only 'plate' phenotype for either mutant is that UBC7 mutants are sensitive to cadmium.

K. A. Nasmyth (*Research Institute for Molecular Pathology, Vienna, Austria*). You mentioned at the beginning that the nuclear localization sequence (NLS) might be important for degradation. Do you have any evidence that your signal is really a ubiquitination signal as opposed to, let's say, a nuclear export signal?

M. Hochstrasser. First of all, we don't have an *in vitro* system to show that these are direct effects on ubiquitination. It is possible that at least a component of the signal functions in nuclear export. We have found using a trans-acting mutant which is defective in importing proteins into the nucleus that the *Deg1*-βgal fusion does stay in the cytoplasm but is still rapidly degraded, and the rapid degradation still requires the ER-localized UBC6 and UBC7 enzymes. So we think the NLS might help to concentrate the protein in the nuclear periphery but a block to nuclear import does not inhibit UBC6/7-dependent degradation. How (or whether) the nuclear protein might be translocated out of the nucleus we don't know. We don't see an obvious nuclear export sequence in α2.

M. Tyers (*Mount Sinai Hospital, Toronto, Ontario, Canada*). Have you identified compensatory mutations in **a**1 that allow the complex to be restored and see the appropriate effects on stabilization? The second question is that it is potentially dangerous to make mutations in regulatory proteins that alter cell type. Are you sure that there aren't subtle changes in cell type that might have some unforeseen downstream effect on stabilization?

M. Hochstrasser. In principle, it might be possible to make such compensatory mutations. We have tried to do this by making specific mutations in the amphipathic helix of **a**1, however, we found no compensatory mutations. We haven't tried to randomly mutagenize **a**1 looking specifically for compensatory mutations. As for the second question, all I can say is we are not altering cell type in any way that we can measure; we have looked very carefully for differences in sporulation in diploids, we've looked at haploid mating type efficiency and at changes in expression of **a**1-α2 and α2 operator lacZ fusions, so I think the data argue against this explanation.

R. T. Hunt (*ICRF Clare Hall Laboratories, Hertfordshire, UK*). I was intrigued by your destruction box which did, indeed, look fairly good. I thought early on you said that if you delete those 10–12 residues, you did stabilize the protein. Then you made a point mutant which didn't stabilize the protein so you gave up on the destruction point. Is that correct?

M. Hochstrasser. That is roughly what I said. We could make one mutation which leaves the destruction box intact which ends at residue 57 and that protein is partially stabilized (two- to threefold). If we cut off the destruction box it does stabilize the protein. So there is something important in the C-terminal part of this signal which contains the destruction box-like sequence, yet the point mutagenesis suggests the destruction box itself might not be important. We have also tried to see if there is any change in the half-life of α2 during the cell cycle and so far it looks like the rate of degradation does not dramatically change during the cell cycle.

J. Diffley (*ICRF Clare Hall Laboratories, Hertfordshire, UK*). You didn't say very much about the C-terminal sequence. Is it affected the same way by all of the *doa* mutants?

M. Hochstrasser. It is definitely not affected by the *doa* mutants which include proteasome mutants. We haven't done much work with this for technical reasons. We don't know whether this *Deg2* sequence contributes to α2 stability at all.

J. Diffley (*ICRF Clare Hall Laboratories, Hertfordshire, UK*). Why do you think mutation of a ubiquitin deconjugating enzyme stabilizes α2? This seems somewhat counter-intuitive.

M. Hochstrasser. We've found that the mutant in the ubiquitin deconjugating enzyme (Doa4p) stabilizes most proteins. Doa4p seems to act late in the pathway to recycle ubiquitin from proteasome-targets substrates, and *doa4* mutants are also depleted of free ubiquitin.