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In vivo disassembly of free polyubiquitin chains by yeast Ubp14 modulates rates of protein degradation by the proteasome

Alexander Yu.Amerik, Sowmya Swaminathan, Bryan A.Krantz[1], Keith D.Wilkinson[1] and Mark Hochstrasser[2]

University of Chicago, Department of Biochemistry and Molecular Biology, 920 East 58th Street, Chicago, IL 60637 and [1]Department of Biochemistry, Emory University School of Medicine, Atlanta, GA 30322, USA

[2]Corresponding author
e-mail: hoc1@midway.uchicago.edu

Degradation of many eukaryotic proteins requires their prior ligation to polyubiquitin chains, which target substrates to the 26S proteasome, an abundant cellular protease. We describe a yeast deubiquitinating enzyme, Ubp14, that specifically disassembles unanchored ('free') ubiquitin chains *in vitro*, a specificity shared by mammalian isopeptidase T. Correspondingly, deletion of the *UBP14* gene from yeast cells results in a striking accumulation of free ubiquitin chains, which correlates with defects in ubiquitin-dependent proteolysis. Increasing the steady-state levels of ubiquitin chains in wild-type cells (by expressing a derivative of ubiquitin with an altered C-terminus) inhibits protein degradation to a degree comparable with that observed in *ubp14*Δ cells. Inhibition of degradation is also seen when an active site mutant of Ubp14 is overproduced *in vivo*. Surprisingly, overproduction of wild-type Ubp14 can inhibit degradation of some proteins as well. Finally, Ubp14 and human isopeptidase T are shown to be functional homologs by complementation analysis. We propose that Ubp14 and isopeptidase T facilitate proteolysis *in vivo* by preventing unanchored ubiquitin chains from competitively inhibiting polyubiquitin–substrate binding to the 26S proteasome.
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Introduction

Covalent modification of proteins by the 76 residue ubiquitin polypeptide is involved in many aspects of protein metabolism and cell function. Among the cellular processes affected by ubiquitin-dependent reactions are cell cycle progression, chromosome structure and segregation, endocytosis, gene expression, organelle biogenesis, viral pathogenesis and the stress response (reviewed in Ciechanover, 1994; Hochstrasser, 1995, 1996; Wilkinson, 1995). Attachment of a polyubiquitin chain(s) to a protein frequently serves to target the modified protein for proteolysis by the proteasome. The ubiquitin molecules in these chains are most often linked to one another by isopeptide bonds between the carboxy-terminus of one ubiquitin and the ε-amino group of Lys48 of the next ubiquitin (Chau *et al.*, 1989; Hochstrasser *et al.*, 1991). There is also evidence that ubiquitin chains with other isopeptide linkages can be formed (Arnason and Ellison, 1994; Spence *et al.*, 1995; Baboshina and Haas, 1996).

Ubiquitination of proteins is reversible. Deubiquitination is catalyzed by specialized proteases called deubiquitinating or DUB enzymes (Wilkinson and Hochstrasser, 1997). These thiol proteases hydrolyze the amide bond between Gly76 of ubiquitin and the substrate protein. 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 and 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 (Wilkinson and Hochstrasser, 1997). As a group, DUB enzymes comprise the largest known family of enzymes in the ubiquitin system. There are 17 genes encoding such enzymes in the yeast *Saccharomyces cerevisiae*, of which 16 are of the UBP class (Hochstrasser, 1996). However, very little is known about their physiological functions.

We have been analyzing protein degradation by the ubiquitin–proteasome pathway in *S.cerevisiae* (Hochstrasser *et al.*, 1995) and had previously discovered a UBP enzyme, Doa4, that plays a critical role in the degradation of substrates of this pathway (Papa and Hochstrasser, 1993). Doa4 can remove ubiquitin both from N-terminal ubiquitin–protein fusions, in which the ubiquitin is linked by a standard peptide bond, and from ubiquitinated species in which the ubiquitin(s) is attached by an isopeptide bond to a lysine side chain of another protein. Although Doa4 is similar in sequence to the human *TRE17* proto-oncogene product and both are active DUBs (Papa and Hochstrasser, 1993), functional homology between the two enzymes has not been established. The function of many of the other UBP enzymes has been difficult to demonstrate using genetic approaches. Many yeast *ubp* deletion strains, for instance, have minimal phenotypic abnormalities (Hochstrasser, 1996).

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 carboxy-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 and Pickart, 1990; Van Nocker and Vierstra, 1991), and these chains may need to be disassembled to avoid accumulation to levels that inhibit proteolysis.

Here we describe a yeast UBP-type enzyme, Ubp14, that is 31% identical to human IsoT. 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 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 proteolytic defects resulting from loss of Ubp14 are due to the accumulation of ubiquitin chains. Finally, we show that the defects of the yeast *ubp14* mutant can be reversed by expression of human IsoT, indicating that the yeast and human proteins are functional homologs.

Results

The UBP14 gene encodes a deubiquitinating enzyme

The *UBP14* gene (ORF YBR058c) on chromosome II was sequenced as part of the *S.cerevisiae* genome project. That it may encode a UBP enzyme was suggested by the presence of recognizable Cys and His boxes in the predicted protein. The *UBP14* ORF is predicted to encode a protein of 803 amino acids (91 kDa). The sequences of yeast Ubp14 and the four most closely related protein sequences in the current sequence databases (three human IsoT sequences and a *Dictyostelium discoideum* entry called UbpA) are shown in Figure 1. In contrast to alignments involving other UBP enzymes, sequence similarity between the proteins shown in Figure 1 extends along their entire lengths, suggesting the possibility of similarity in their physiological roles as well.

To test the deubiquitinating activity of Ubp14, the protein was expressed in *Escherichia coli* as a fusion with glutathione-*S*-transferase (GST). GST–Ubp14 was purified by glutathione–agarose affinity chromatography. Several ubiquitin derivatives were synthesized and tested as substrates for GST–Ubp14 and IsoT. The substrate specificity of GST–Ubp14 closely paralleled that of IsoT (Table I). Most strikingly, a Lys48-linked ubiquitin dimer lacking the free C-terminal two glycines (Ub–UbΔGG) was completely resistant to both enzymes even after extended incubation, whereas a normal Lys48-linked dimer (Ub–Ub) was the best substrate tested. The same preparation of Ub–UbΔGG was disassembled efficiently by another UBP (GenBank D38378) (data not shown). When a purified and radiolabeled ubiquitin-β-galactosidase (Ub–βgal) fusion was used as a substrate, no ubiquitin cleavage was detected in a 1 h incubation at 37°C, even with an excess of enzyme, whereas in a control reaction, a crude yeast extract caused >90% cleavage within 10 min (data not shown).

From these data, we conclude that the *S.cerevisiae* Ubp14 protein is a deubiquitinating enzyme with close similarities in both primary protein sequence and *in vitro* enzymatic properties to mammalian IsoT.

Deletion of UBP14 leads to abnormalities common to mutants of the ubiquitin system

A null allele of the *UBP14* gene was generated by a PCR-based procedure (Baudin *et al.*, 1993); the resulting *ubp14-Δ1::HIS3* allele was transformed into the wild-type diploid MHY606 strain, and heterozygous mutants were identified. Heterozygous diploids were sporulated and tetrads dissected. No growth defect was noted for *ubp14* cells growing on rich medium at 30 or 37°C (see Figure 5A). Cells deleted for *UBP14* also grew well in the presence of 30 μM cadmium, a heavy metal; however, *ubp14* cells were hypersensitive to canavanine, an arginine analog, and they exhibited a strong sporulation defect (Table II; see also Figure 7A). Similar defects have been documented for a variety of ubiquitin system mutants (Jentsch, 1992). To examine the role of Ubp14 in the ubiquitin–proteasome pathway more directly, we measured the degradation rates of several known proteolytic substrates of the pathway. Degradation of the naturally short-lived MATα2 repressor (Hochstrasser and Varshavsky, 1990) was inhibited ~2-fold in *ubp14* cells (Figure 2). More pronounced proteolytic defects (3- to 5-fold) were observed with two well-characterized artificial substrates of the ubiquitin system, L-βgal, an N-rule substrate (Varshavsky, 1992), and Ub–P-βgal, a substrate whose turnover depends on both Lys48 and Lys29 of the N-terminal ubiquitin moiety (Johnson *et al.*, 1995) (Figure 2).

Previously, mutations in enzymes with general roles in ubiquitin-dependent proteolysis were shown to cause characteristic changes in bulk ubiquitin–conjugate profiles (e.g. Seufert and Jentsch, 1990; Papa and Hochstrasser, 1993; Chen and Hochstrasser, 1995). Intracellular levels of free ubiquitin and ubiquitin-containing species were determined by anti-ubiquitin immunoblot analysis in *ubp14* cells. A novel pattern of ubiquitin-containing species was observed in the mutant strain (Figure 3A); these species migrate on SDS–polyacrylamide gels with mobilities very similar to those of purified Lys48-linked ubiquitin chains. When wild-type and mutant cell extracts were resolved on two-dimensional gels followed by anti-ubiquitin immunoblot analysis, the abundant ubiquitinated species were found to align in the isoelectric focusing dimension directly over the position of free ubiquitin (Figure 3B). These species were seen in wild-type cells as well, but in much smaller amounts. Thus, all the detectable ubiquitin-containing species that accumulate in *ubp14* cells have molecular weights and isoelectric points identical to unanchored ubiquitin chains, strongly suggesting that these species are oligomers of ubiquitin that are not attached to other proteins. It is also important to note that had a significant fraction of monomeric ubiquitin in *ubp14* cells accumulated as conjugates of small intracellular nucleophiles, e.g. polyamines, species of differing isoelectric point should have been observed in Figure 3B.

Defective processing of the primary *UBI4* gene product, which consists of five ubiquitin monomers linked in head-to-tail fashion (Özkaynak *et al.*, 1984), would be one possible explanation for the accumulation of ubiquitin chains (with five or fewer ubiquitins) in *ubp14* cells. To examine this, a *ubi4Δ ubp14Δ* double mutant was constructed. Anti-ubiquitin immunoblot analysis demonstrated that ubiquitin chains accumulate to comparable

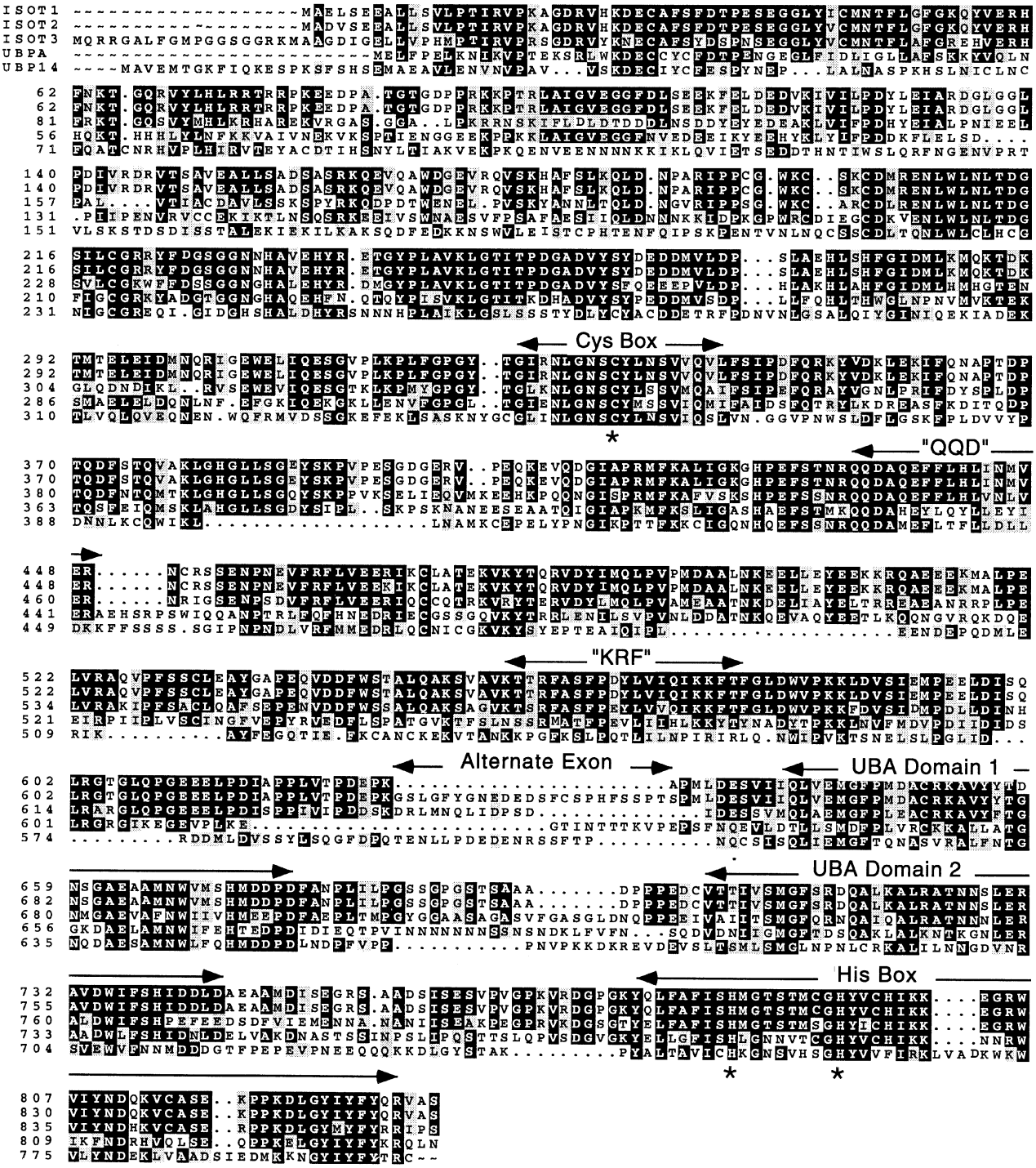

Fig. 1. Multiple sequence alignment of Ubp14 and related enzymes: *S.cerevisiae* Ubp14 (SwissProt P38237), human isopeptidase T isozymes (ISOT1, GenBank U35116; ISOT2, SwissProt P45974; ISOT3, GenBank U75362) and *D.discoideum* UbpA (GenBank U48271). Residues that are identical are shaded black; those that are conserved are shaded gray. The putative catalytic Cys and His residues are in the 'Cys' and 'His' boxes respectively. These motifs and the 'QQD' and 'KRF' regions are conserved between all members of the UBP family. The three isoforms of human isopeptidase T are the products of two independent genes (*ISOT1/2* and *ISOT3*) and an apparently tissue-specific alternative splicing event, as indicated (compare ISOT1 and ISOT2).

levels in the *ubp14* and *ubi4 ubp14* mutants (data not shown). We note that there was no reduction in free ubiquitin levels in *ubp14* cells (Figure 3) and little if any suppression of the canavanine hypersensitivity of *ubp14* cells by overproduction of ubiquitin (data not shown). Thus, it is very unlikely that there is a deficiency in processing of ubiquitin precursors in the mutant. Importantly, these results also indicate that the defects of *ubp14* cells are not due to depletion of free ubiquitin.

Yeast lacking *UBP14* still have 16 other genes encoding DUB enzymes, including enzymes capable of cleaving ubiquitin chains (e.g. Papa and Hochstrasser, 1993), yet *ubp14* cells accumulate high levels of ubiquitin chains. A potential explanation for this paradox is that the accumulat-

ing ubiquitin chains are compartmentalized, e.g. by binding to proteasomes, and Ubp14, unlike other DUBs, can associate with or is part of this compartment. Alternatively, Ubp14 may simply have the highest specific activity of any DUB toward ubiquitin chains. [There does not appear to be a high level of Ubp14 protein relative to other DUBs (see below).] To help distinguish between these hypotheses, we measured Lys48-linked Ub–Ub dimer cleavage in wild-type and *ubp14* yeast cell extracts (Figure 3C). A striking difference in cleavage rates was observed. Longer incubations (30–60 min) of the *ubp14* extracts resulted in a substantial fraction of dimer cleavage, indicating that other active DUB enzymes were present, which is consistent with the efficient cleavage of Ub–βgal in yeast extracts noted above. In contrast, Ub–UbΔGG cleavage rates in the two extracts were the same and approximately equal to the rate of Ub–Ub cleavage in the *ubp14* extract (data not shown). These data suggest that Ubp14 provides the major yeast DUB activity acting on the cellular pool of free isopeptide-linked ubiquitin chains and argue against a limitation of Ubp14 action to proteasome-bound substrates [a >1750-fold molar excess of Ub–Ub relative to proteasomes was used in the reactions, assuming proteasomes represent ~0.5% of soluble cell protein (Chen and Hochstrasser, 1995)].

We conclude that Ubp14 is required *in vivo* for maximal rates of degradation of ubiquitin–proteasome pathway substrates and for disassembling unanchored, isopeptide-linked ubiquitin chains. The accumulation of ubiquitin chains in *ubp14* cells provides a striking parallel to the *in vitro* substrate specificity of Ubp14 (and IsoT) (Table I).

Table I. Hydrolysis of various substrates catalyzed by yeast Ubp14 and human isopeptidase T[a]

Substrate	v_{obs} (min^{-1})	
	IsoT	GST–Ubp14
Ub-ε-Ub	177	56
Ub-ethyl ester	48	37
Ub-ε-*N*-acetyl-L-lysine	12	0.8
Ub-ε-Ub *des*GG	0[b]	0[b]

[a]Turnover numbers (v_{obs}) were determined under initial rate conditions, i.e. when the plot of substrate versus time was linear using high concentrations of substrate (5–15 mM). Enzyme concentrations varied from subnanomolar to 40 nM.
[b]Incubation for 16 h at 37°C with 250 nM isopeptidase T or with 350 nM GST–Ubp14.

Expression of UbΔGG in yeast causes accumulation of ubiquitin chains and inhibits proteolysis

The correlation between ubiquitin chain accumulation and defects in ubiquitin-dependent proteolysis in *ubp14* cells might be due to such chains binding to, and thereby inhibiting, enzymes of the ubiquitin system. Ubiquitin chains bind avidly to a subunit of the 26S proteasome called S5a (Deveraux *et al.*, 1994), and high levels of ubiquitin chains can inhibit proteolysis by the proteasome *in vitro*, presumably because of competition with poly-ubiquitinated protein substrates (Beal *et al.*, 1996). To investigate this issue further, we attempted to phenocopy the *ubp14* proteolytic defect in wild-type yeast cells by

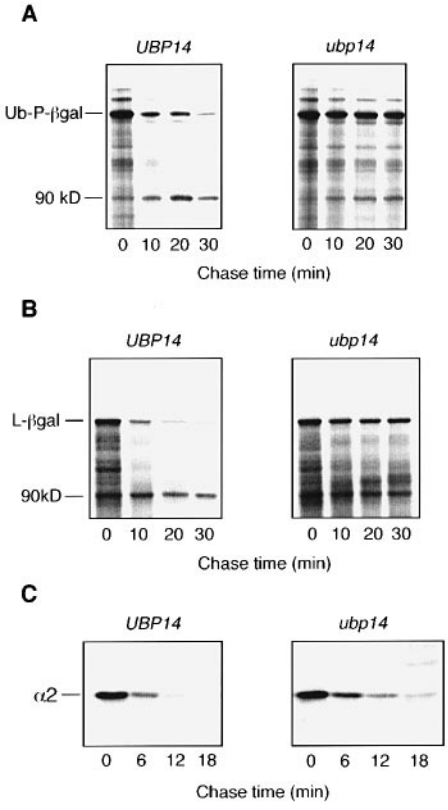

Fig. 2. Mutant *ubp14* cells are defective in ubiquitin-dependent protein degradation. Pulse–chase analysis of Ub–P-βgal (**A**), L-βgal (**B**) and MATα2 (**C**) degradation in wild-type (MHY501) and *ubp14* (MHY840) cells. For analysis of α2 degradation, cells expressed the repressor from the chromosomal *MAT*α locus. For analysis of L-βgal and Ub–P-βgal degradation, expression of plasmid-derived ubiquitin–βgal fusion proteins was induced with galactose.

Table II. Phenotype of *ubp14* mutants

	Wild-type[a]	*ubp14*[b]	*ubp14* [pUBP14]	*ubp14* [pIsoT]
Growth at 37°C	+	+	ND	ND
Growth on Cd^{2+} (30 mM)	+	+	ND	ND
Growth on canavanine (0.8 μg/ml)	+ (96%)[c]	– (0.01%)[c]	+	+
Sporulation of homozygous diploid (%)[d]	20–40	1–4	30–40	20–40

[a]Wild-type, MHY501, except for sporulation assays, which used the MHY606 diploid.
[b]*ubp14*, MHY840, except for sporulation assays, which used the MHY1053 diploid.
[c]Plating efficiencies on canavanine relative to SD minimal medium.
[d]Sporulation at 30°C; at 25°C, *ubp14* homozygotes sporulate at near wild-type frequency.

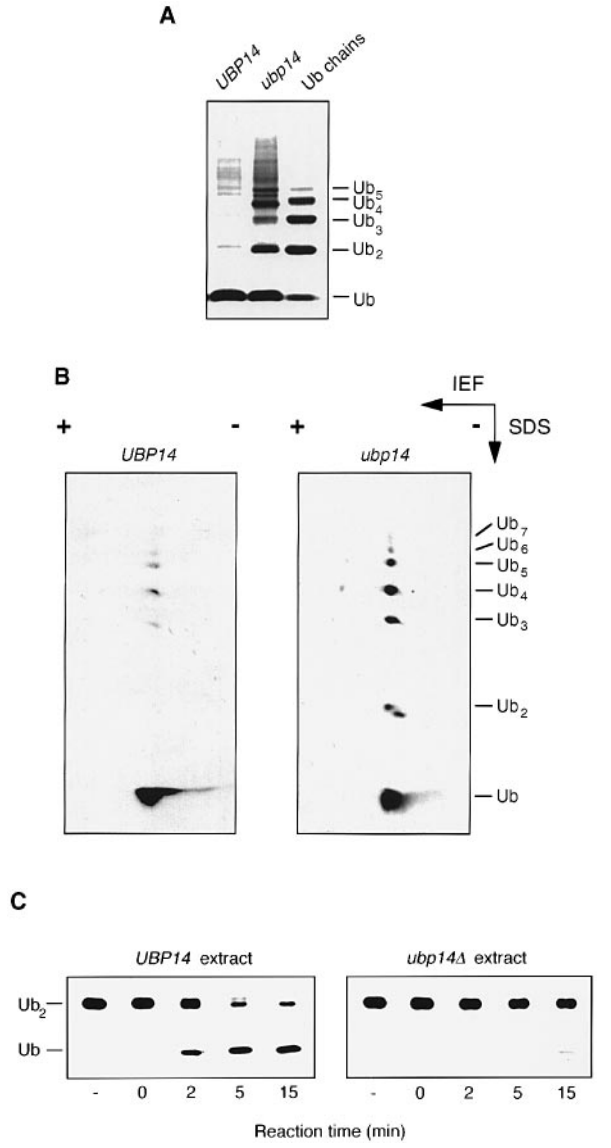

Fig. 3. Unanchored ubiquitin chains accumulate in *ubp14* mutants. All panels show results of anti-ubiquitin immunoblot analyses of *ubp14* mutant (MHY840) and wild-type (MHY501) cells. (**A**) One-dimensional gel. (**B**) Two-dimensional O'Farrell gels. The positions of free ubiquitin and ubiquitin multimers are indicated. A pair of spots is observed at the position of diubiquitin (Ub_2) in both mutant and wild-type cells. These two species are likely to be either free ubiquitin dimers with different amide linkages or dimers differing by protein modification. The antibodies used for the experiment shown in (B) were affinity-purified anti-ubiquitin antibodies provided by A.Haas. The purified unanchored Lys48-linked ubiquitin chains used as size standards were synthesized *in vitro* from bovine ubiquitin and were a gift from C.Pickart. Bovine chains are known to migrate slightly differently from yeast chains (Van Nocker and Vierstra, 1993). (**C**) Kinetics of cleavage of a Lys48-linked ubiquitin dimer in yeast cell-free extracts.

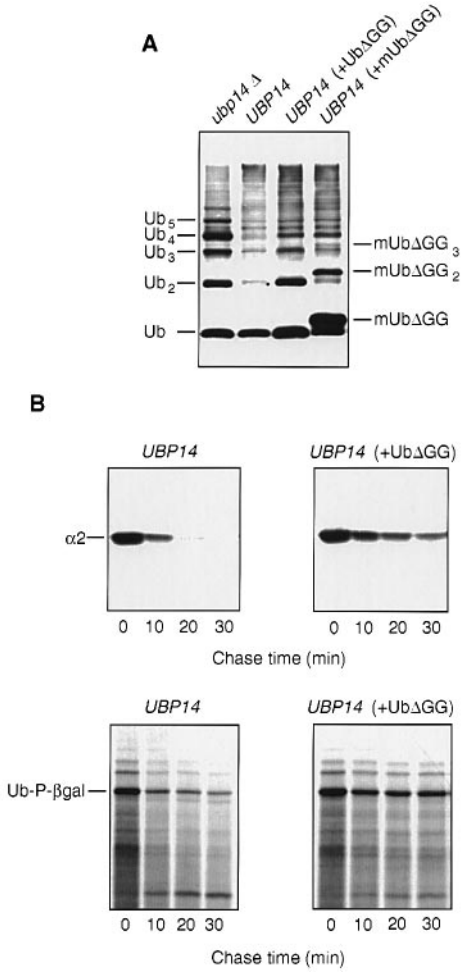

Fig. 4. Expression of *des*GG-ubiquitin (UbΔGG) inhibits ubiquitin-dependent proteolysis and causes accumulation of unanchored ubiquitin chains in wild-type (MHY501) cells. (**A**) Expression of UbΔGG derivatives leads to accumulation of ubiquitin chains. Anti-ubiquitin immunoblot analysis of wild-type cells expressing various UbΔGG derivatives. The positions of free ubiquitin and ubiquitin multimers are indicated. Cells in which chains were synthesized in the presence of myc-tagged UbΔGG (mUbΔGG) contain ubiquitin chains of reduced mobility, as marked. (**B**) Pulse–chase analysis of MATα2 and Ub–P-βgal degradation in cells expressing UbΔGG. Yeast cells carried the following plasmids: pES12 (vector), pES10 (UbΔGG) and pES3 (mUbΔGG).

expression of UbΔGG. The rationale of the experiment is as follows. Because the carboxy-terminal glycines are missing in UbΔGG, it cannot be conjugated via its carboxy-terminus to other proteins. However, yeast cells contain enzymes capable of assembling unanchored ubiquitin chains, so endogenous ubiquitin can be ligated to Lys residues of UbΔGG (Arnason and Ellison, 1994). If UbΔGG-terminated chains, which are not substrates for Ubp14, accumulate and a proteolytic defect is also observed, it would suggest that ubiquitin chain accumulation and defective proteasome-dependent proteolysis are related.

UbΔGG was expressed in wild-type cells from a plasmid-borne allele under the control of the copper-inducible *CUP1* promoter (no copper was added to the medium; under these conditions, expression from the *CUP1* promoter results in a level of the plasmid-encoded ubiquitin near that of endogenous ubiquitin; Ellison and Hochstrasser, 1991). Anti-ubiquitin immunoblot analysis (Figure 4A) revealed a pattern of ubiquitin chains under these conditions similar to that seen in *ubp14* strains. The electrophoretic mobility of at least the dimer and trimer species was reduced when an allele expressing a myc peptide-tagged derivative of UbΔGG replaced the UbΔGG-expressing allele (Figure 4A). Thus, at least a large fraction of the accumulated ubiquitin chains must contain the UbΔGG polypeptide. As expected, overexpression of

Table III. Synergistic inhibitory effect of ubiquitin and UbΔGG on IsoT activity

Inhibitor added	Rate (μM/min)	Activity (%)
None	0.075	100
UbΔGG(211 μM)	0.045	60
Ubiquitin (130 μM)	0.039	52
Ubiquitin + UbΔGG	<0.001	<2

Reactions conditions: 50 mM Tris–HCl, pH 7.6; 10 mM DTT; 0.1 mg/ml ovalbumin; 1.5 nM IsoT; 5.5 μM Ub–Ub; 37°C.

Ubp14 failed to reduce the levels of UbΔGG-terminated chains (data not shown).

Rates of ubiquitin-dependent proteolysis in UbΔGG-expressing cells were measured using MATα2 and Ub–P-βgal as test substrates. Degradation of both proteins was impaired (Figure 4B). Expression of wild-type ubiquitin from the same expression vector and under the same conditions caused little if any accumulation of ubiquitin chains above that seen in wild-type cells and did not lead to significant alteration in the degradation rate of either protein (data not shown). In comparison with *ubp14* cells, the proteolytic deficiency was slightly but reproducibly more severe in the phenocopy experiments. This may reflect an additional effect of UbΔGG on ubiquitin-dependent proteolysis beyond the creation of inhibitory UbΔGG-terminated chains. Indeed, we found that a UbΔGG derivative in which all the Lys residues were converted to Arg also caused chain accumulation and inhibition of proteolysis, although the effects were significantly weaker than those seen with UbΔGG (data not shown).

These latter results could be explained by inhibition of cellular Ubp14 by UbΔGG. Consistent with this interpretation, inhibition by excess ubiquitin or UbΔGG of diubiquitin cleavage *in vitro* by IsoT showed a striking synergism when both were added together (Table III). Because wild-type ubiquitin was also present in the yeast cells expressing UbΔGG, inhibition of Ubp14 would also be predicted to occur *in vivo*. Although the consequences of UbΔGG expression *in vivo* are clearly complex (see also Arnason and Ellison, 1994), the data reinforce the possibility that ubiquitin chain accumulation can inhibit ubiquitin- and proteasome-dependent proteolysis in yeast. Moreover, these results indicate that UbΔGG can be useful as a dominant-negative inhibitor of cellular proteolysis.

Genetic interactions between ubp14 and mutations in the 26S proteasome

The defect in ubiquitin-dependent proteolysis in *ubp14* cells, as measured by pulse–chase experiments, is relatively mild when compared with many proteasome mutants. If ubiquitin chain binding to proteasomes is indeed the basis of the *ubp14* proteolytic defect, it could be that such binding competes relatively poorly with substrate binding to the protease. However, if proteasomes are only partially impaired by ubiquitin chain binding, *ubp14* cells should be very sensitive to any further deficiency in proteasome function. We tested this prediction by constructing double mutants involving *ubp14*Δ and different proteasome alleles. The double mutants tested were *doa3-1 ubp14*Δ and *doa5-1 ubp14*Δ. Doa3 and Doa5 are essential β and α subunits, respectively, of the 20S proteasome (Chen and Hochstrasser, 1995). The *doa3-1* and *doa5-1* missense alleles are not lethal but cause significant phenotypic abnormalities. We also tested a *doa4*Δ *ubp14*Δ mutant; the Doa4 enzyme appears to function in conjunction with the proteasome (Papa and Hochstrasser, 1993), and a fraction of Doa4 co-purifies with the 26S proteasome during multiple chromatographic steps (F.R.Papa and M.Hochstrasser, in preparation).

The *doa3-1 ubp14*Δ and *doa5-1 ubp14*Δ mutants were much more sensitive to elevated temperatures than were the corresponding single mutants, and they had an enhanced growth defect at 30°C (Figure 5A). The *doa4*Δ *ubp14*Δ mutant also had an enhanced mutant phenotype, e.g. the double mutant was unable to form colonies at 37°C. Anti-ubiquitin immunoblot analysis revealed a massive accumulation of unanchored ubiquitin chains in the *doa3-1 ubp14*Δ and *doa5-1 ubp14*Δ double mutants; the level of unanchored chains observed significantly exceeded that seen in *ubp14*Δ single mutants (Figure 5B). Based on an analogous set of experiments using [^{125}I]protein A immunoblotting and PhosphorImager quantitation, ubiquitin chains in the double mutants accumulate to levels higher than the sum of their levels in the corresponding single mutants. Levels of the Ub_2, Ub_3 and Ub_4 species were 66, 77 and 290% higher, respectively, in the *doa3-1 ubp14*Δ cells relative to the levels expected for a simple additive effect of the two mutations, and 33, 38 and 330% higher in *doa5-1 ubp14*Δ cells.

When levels of Ub_2, Ub_3 and Ub_4 were summed and compared between strains, it was found that the *ubp14*Δ mutation resulted in an ~3.1-fold increase relative to wild-type (Ub_4 increased the most: ~4.5-fold), while in *doa3-1* and *doa5-1* cells, these levels were ~1.3 and ~1.0 times that of wild-type, respectively. In *doa3-1 ubp14*Δ and *doa5-1 ubp14*Δ cells, the sum of these species increased dramatically, reaching levels >11- and 10-fold higher, respectively, than in wild-type cells. Monomeric ubiquitin levels in all the strains varied <50% from wild-type. Hence, a large increase in total ubiquitin levels occurs in mutant cells with only relatively minor changes in free ubiquitin levels, suggesting the operation of a homeostatic mechanism that maintains relatively constant intracellular levels of free ubiquitin (for a discussion of ubiquitin pool dynamics, see Haas, 1988). Because ubiquitin is a stable protein in yeast (S.Swaminathan and M.Hochstrasser, unpublished data), it is likely that the increase in total ubiquitin levels in these mutants results from an increase in rates of ubiquitin synthesis.

Previous work demonstrated that *doa4* cells accumulate small ubiquitin-containing species, which may represent ubiquitin chains (mostly dimers) linked to substrate-derived oligopeptides generated by proteasomes (Papa and Hochstrasser, 1993). The electrophoretic mobility of these species is slightly slower than that of unanchored ubiquitin chains. A simple model for the integration of Doa4 and Ubp14 action would be one in which Doa4 cleaves the ubiquitin chains still attached to peptide remnants remaining after substrate breakdown, creating unanchored ubiquitin chain substrates for Ubp14 (see Figure 8). If this were an obligatory pathway for Ubp14 substrate generation in the cell, it would predict that the ubiquitin–conjugate profile of the *doa4*Δ *ubp14*Δ double mutant

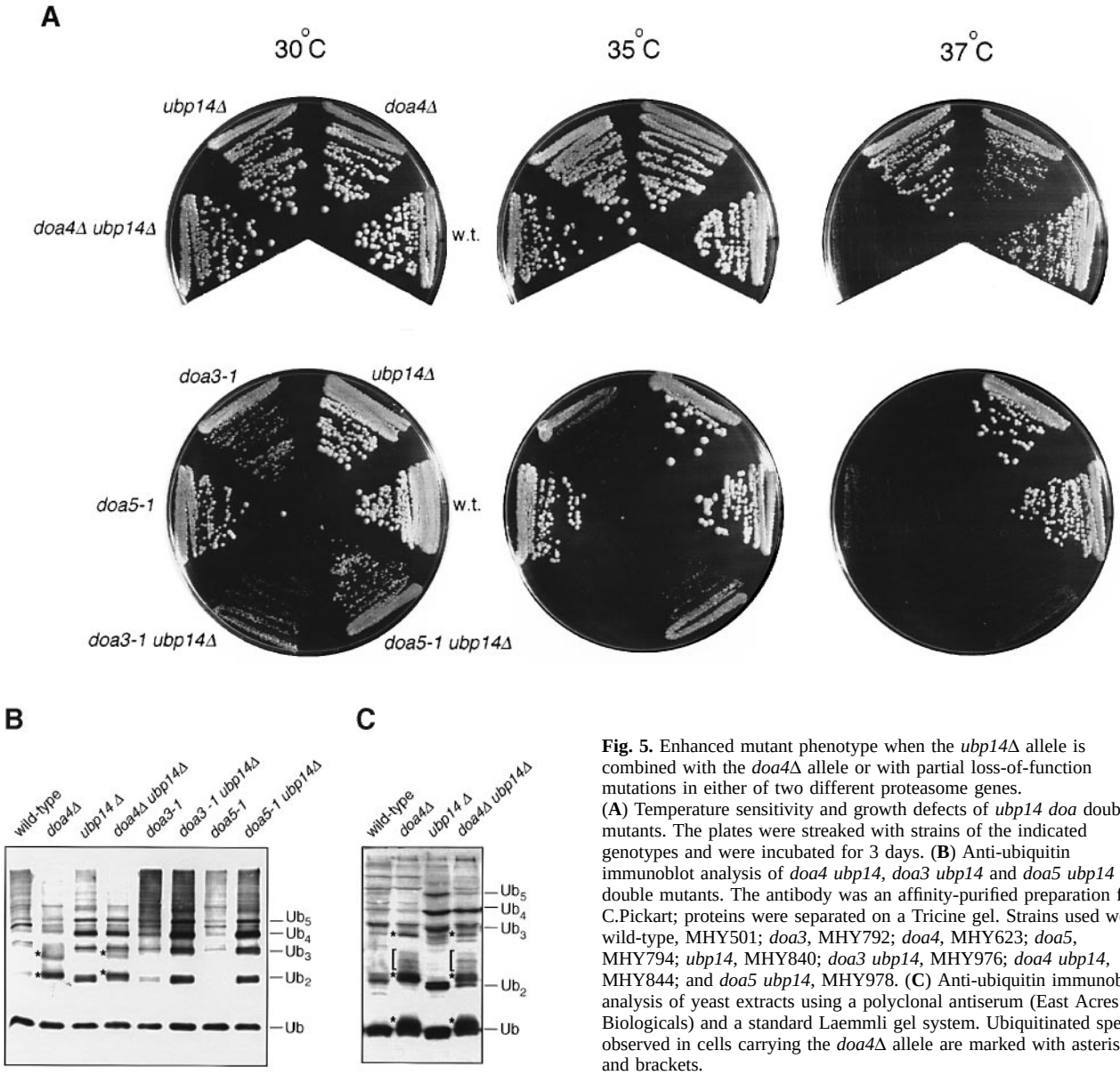

Fig. 5. Enhanced mutant phenotype when the *ubp14*Δ allele is combined with the *doa4*Δ allele or with partial loss-of-function mutations in either of two different proteasome genes. (**A**) Temperature sensitivity and growth defects of *ubp14 doa* double mutants. The plates were streaked with strains of the indicated genotypes and were incubated for 3 days. (**B**) Anti-ubiquitin immunoblot analysis of *doa4 ubp14*, *doa3 ubp14* and *doa5 ubp14* double mutants. The antibody was an affinity-purified preparation from C.Pickart; proteins were separated on a Tricine gel. Strains used were: wild-type, MHY501; *doa3*, MHY792; *doa4*, MHY623; *doa5*, MHY794; *ubp14*, MHY840; *doa3 ubp14*, MHY976; *doa4 ubp14*, MHY844; and *doa5 ubp14*, MHY978. (**C**) Anti-ubiquitin immunoblot analysis of yeast extracts using a polyclonal antiserum (East Acres Biologicals) and a standard Laemmli gel system. Ubiquitinated species observed in cells carrying the *doa4*Δ allele are marked with asterisks and brackets.

should be the same as that of the *doa4*Δ single mutant since Doa4 would act prior to Ubp14.

Instead, anti-ubiquitin immunoblots of *doa4*Δ *ubp14*Δ cell extracts revealed a pattern of ubiquitinated species resembling a combined profile of the two single mutants, indicating that neither mutation is fully epistatic to the other (Figure 5B and C). However, there was a substantial reduction in the level of the unanchored ubiquitin dimer species in *doa4*Δ *ubp14*Δ cells relative to the *ubp14*Δ single mutant. These results indicate that Doa4 affects a subset of unanchored chain substrates (dimers) for Ubp14 but that ubiquitin chains are generated by more than one mechanism *in vivo*.

A dominant-negative allele of UBP14

Mammalian IsoT is monomeric (Wilkinson *et al.*, 1995), but if it (or Ubp14) must associate with another protein(s) to function *in vivo*, then high levels of a catalytically inactive derivative might compete with endogenous wild-type enzyme for binding. Alternatively, inactive Ubp14 may titrate ubiquitin chains and inhibit proteolysis (this would imply that pre-formed ubiquitin chains contribute significantly to protein ubiquitination *in vivo*). We created a *ubp14* allele that encoded a defective Ubp14 enzyme in which Cys354 of the Cys box (Figure 1) was changed to Ala. Both wild-type and mutant alleles were expressed from the strong *ADH1* promoter on high-copy plasmids (pUBP14 and pUBP14-Ala354, respectively). Substitution of the conserved cysteine of the Cys box of several other Ubps has been shown to eliminate catalytic activity (Papa and Hochstrasser, 1993; Huang *et al.*, 1995; Zhu *et al.*, 1996).

Transformation of wild-type cells with the pUBP14 plasmid caused a small but reproducible decrease in the level of unanchored ubiquitin chains normally present in wild-type cells (Figure 6A), suggesting that the wild-type enzyme is being overexpressed under these conditions. The pUBP14 plasmid, but not pUBP14-Ala354, suppressed the canavanine hypersensitivity of *ubp14* cells (Figure 7A and data not shown), consistent with Cys354 being critical for

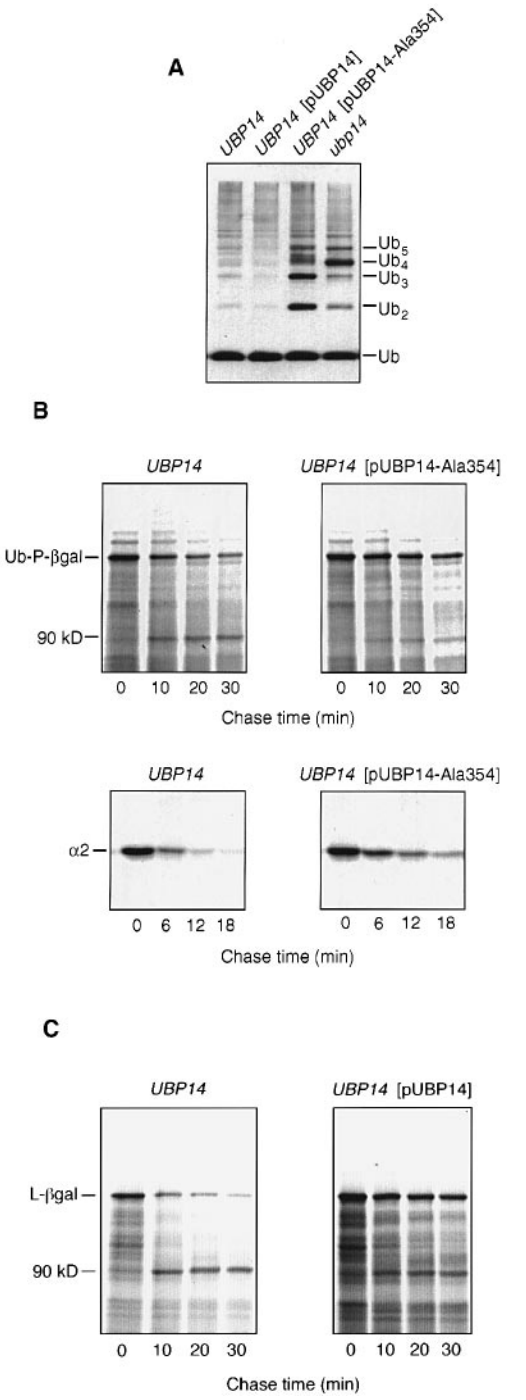

Fig. 6. A dominant-negative effect of the catalytically inactive *ubp14-Ala354* allele. Wild-type Ubp14 and the mutant Ubp14^{Ala354} proteins were overexpressed in wild-type yeast cells (MHY501) from pUBP14 and pUBP14-Ala354, respectively. (**A**) Anti-ubiquitin immunoblot analysis. The positions of free ubiquitin and ubiquitin oligomers are indicated. (**B**) Pulse–chase analysis of Ub–P-βgal and MATα2 degradation in cells overproducing Ubp14^{Ala354}. The half-life of MATα2 is increased ~2-fold by overproduction of the inactive protein. (**C**) Pulse–chase analysis of L-βgal degradation in cells overproducing wild-type Ubp14.

Ubp14 enzymatic activity and *in vivo* function. Overexpression of Ubp14^{Ala354} in wild-type cells inhibited degradation of MATα2 and Ub–P-βgal to an extent approaching that seen in *ubp14* cells (Figure 6B) and caused an accumulation of ubiquitinated species compar-

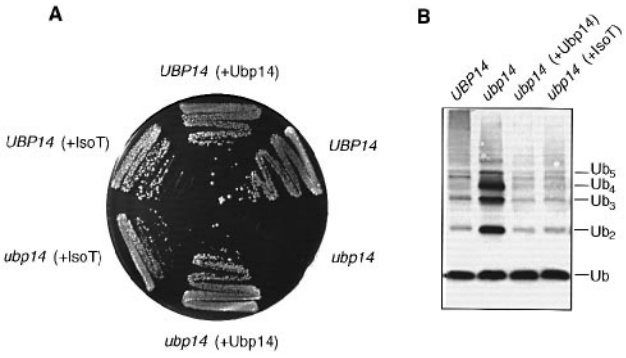

Fig. 7. Human IsoT complements an *S.cerevisiae ubp14*Δ mutation. (**A**) Growth on canavanine. Wild-type and *ubp14* cells carrying the indicated plasmids were streaked onto selective media containing 1.2 μg/ml canavanine sulfate. The plates were incubated at 30°C for 3 days. (**B**) Reduction of unanchored ubiquitin chains to wild-type levels in *ubp14* mutants by expression of human IsoT. Complementation tests and anti-ubiquitin immunoblot analyses were performed using wild-type cells and *ubp14* mutant cells carrying pVT-U vector, pISOT and YEplac195UBP14. The positions of free ubiquitin and ubiquitin multimers are indicated.

able with what was observed in *ubp14* cells (Figure 6A). However, unlike the *ubp14* null mutant, these cells amass mostly short ubiquitin oligomers (dimers and trimers). Mammalian IsoT has been shown to be a non-processive enzyme, with full ubiquitin chain disassembly requiring multiple rounds of enzyme release from and rebinding to substrate (Wilkinson *et al.*, 1995). The preponderance of ubiquitin dimers and trimers in cells overproducing Ubp14^{Ala354} probably reflects a low net activity provided by endogenous Ubp14.

Two observations made with cells overproducing wild-type Ubp14 deserve comment. First, the rate of α2 degradation was unaffected, indicating that Ubp14 levels are not rate-limiting for α2 proteolysis *in vivo* (data not shown). This contrasts with overexpression of Doa4, which accelerates α2 degradation (Papa and Hochstrasser, 1993). Second, elevated levels of Ubp14 substantially inhibited L-βgal degradation; for example, in Figure 6C, 11% of L-βgal remained after a 30 min chase in cells carrying empty vector while 38% remained at this time in pUBP14-bearing cells. Elevated Ubp14 also had a very slight inhibitory effect on Ub–P-βgal turnover. The unanticipated inhibition of N-end rule substrate proteolysis suggests that attachment of pre-assembled ubiquitin chains to these substrates might occur *in vivo* (see Discussion).

Yeast Ubp14 and human isopeptidase T are functional homologs

The sequence similarity of the yeast Ubp14 and mammalian IsoT proteins and their similar substrate specificities suggest that they may be functionally related. To test this conjecture, the cDNA for human IsoT (Wilkinson *et al.*, 1995) was cloned into a yeast expression plasmid. The resulting plasmid, pISOT, was transformed into *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 7A). The sporulation efficiency of *ubp14*/*ubp14* homozygous diploid transformants was close to wild-type levels (Table II). At the same time, the level of unanchored ubiquitin chains was reduced to amounts

comparable with those observed in wild-type cells (Figure 7B). 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 detection of nanogram amounts of recombinant IsoT (data not shown). 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 functionally homologous.

Discussion

The results presented here show a close concordance between the *in vitro* substrate specificity of the Ubp14/IsoT enzymes and the *in vivo* profile of ubiquitin species that accumulate in a yeast *ubp14* mutant. Ubp14 provides the first example of a DUB enzyme whose substrate specificity has been determined by a combination of detailed *in vivo* and *in vitro* analyses. Accumulation of unanchored ubiquitin chains is associated with a defect in ubiquitin-dependent protein degradation in mutant cells, and the proteolytic defect can be mimicked in wild-type cells by expression of UbΔGG. These findings have a number of implications for the regulation of intracellular ubiquitin chain levels and of ubiquitin-dependent proteolysis.

The Ubp14/IsoT subfamily of deubiquitinating enzymes

Sequence comparisons between human IsoT and members of the *S.cerevisiae* UBP family show that Ubp14 is the most closely related of the 16 yeast enzymes to the mammalian protein (31% identity) (Figure 1). Correspondingly, the purified yeast Ubp14 and human IsoT deubiquitinating enzymes have extremely similar substrate specificities (Table I). That these two proteins are functionally homologous was directly confirmed by complementation analysis (Figure 7), the first example of interspecies complementation between DUB enzymes. Recently, we have found that the *Dictyostelium* UbpA protein can substitute for Ubp14 in yeast cells as well (D.Lindsey, A.Amerik, R.Gomer and M.Hochstrasser, unpublished data). These studies suggest that similar functional constraints have resulted in the sequence conservation of these enzymes.

Interestingly, a *D.discoideum* strain lacking UbpA grows normally but has a specific defect in multicellular development when induced to differentiate (D.Lindsey, A.Amerik, W.Deery, J.Bishop, M.Hochstrasser and R.Gomer, in preparation). Given its functional homology with Ubp14 and the demonstration that yeast *ubp14*Δ 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.

Formation and disassembly of unanchored ubiquitin chains in vivo

As shown previously and confirmed here, yeast (and all other tested organisms; Van Nocker and Vierstra, 1993) contain significant amounts of unanchored ubiquitin chains. In principle, such chains could be generated by *de novo* assembly from free ubiquitin and/or by ubiquitin–conjugate disassembly or degradation. Because ubiquitin oligomers terminating on UbΔGG can form in yeast (Figure 4A), *de novo* synthesis of at least short chains (mostly dimers) must be possible. On the other hand, the level of the unanchored ubiquitin dimers was reproducibly reduced in the *doa4 ubp14* double mutant versus the *ubp14* single mutant (Figure 6), suggesting that Doa4 contributes to formation of unanchored dimers *in vivo* as well (Figure 8).

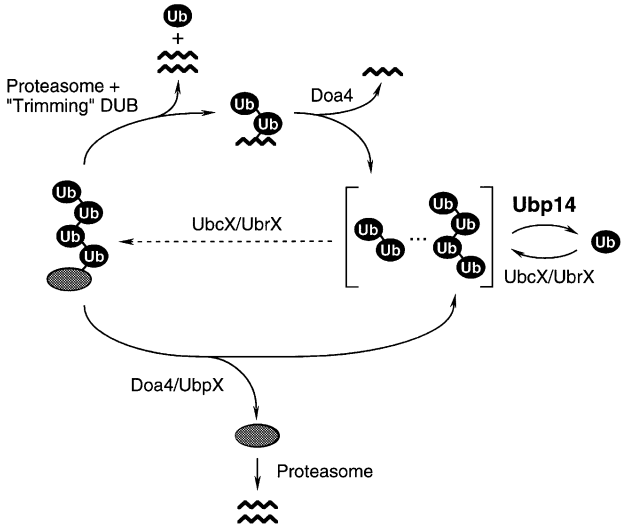

Fig. 8. Model for Ubp14 action and its integration into the ubiquitin-dependent proteolytic system. Release of complete ubiquitin chains from either proteasome-bound protein or peptide is shown, but removal of ubiquitins by 'trimming' DUB enzymes (Lam *et al.*, 1997) is not precluded and may actually 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 get severed from polyubiquitinated substrates that are not bound to proteasomes. The hypothesized ligation of pre-formed ubiquitin chains by ubiquitin-conjugating enzymes (UbcX) and/or ubiquitin–protein ligases (UbrX) to protein is indicated by the dashed arrow. See text for further details.

Our data also suggest a surprising relationship between rates of protein degradation by proteasomes and the steady-state levels of unanchored ubiquitin chains in the cell. Combination of partial loss-of-function mutations in the proteasome with the *ubp14* null allele results in a synergistic increase in unanchored ubiquitin chain levels (Figure 5B). Perturbation of the proteasome alone in *doa3-1* cells also slightly increases ubiquitin chain levels over those seen in wild-type cells (~1.2- to 2.5-fold, based on quantitative immunoblots; see also DeMarini *et al.*, 1995). These results demonstrate that proteasomes play a significant role in ubiquitin chain metabolism *in vivo*.

Interestingly, the levels of ubiquitin tetramers increase far more dramatically than any other unanchored oligomeric species in the proteasome/*ubp14* double mutants. As noted, analyses of UbΔGG-terminated ubiquitin chains *in vivo* suggest that *de novo* synthesis contributes primarily to formation of dimers and trimers but not larger chains (Figure 4A and Arnason and Ellison, 1994). Therefore, release of ubiquitin chains from proteasome-bound poly-

ubiquitinated proteins may account for a substantial portion of at least those cellular chains with four or more ubiquitins. This bias may reflect a binding preference of the proteasome for substrates with longer ubiquitin chains (Hershko *et al.*, 1984; Hough and Rechsteiner, 1986) and must also depend on the population average of ubiquitin chain lengths on polyubiquitinated proteins. Subunit S5a of the 26S proteasome, which binds both ubiquitin chains and polyubiquitinated proteins, shows a sharp increase in affinity for chains with four or more ubiquitins (Deveraux *et al.*, 1994).

In wild-type cells in exponential growth, most polyubiquitinated proteins targeted to proteasomes appear to be substantially degraded before ubiquitin chain removal (Figure 8, top pathway). This inference is based on the fact that *doa4* mutants amass what appear to be ubiquitinated protein remnants (Papa and Hochstrasser, 1993), whereas proteasome mutants accumulate high molecular mass ubiquitinated proteins (Chen and Hochstrasser, 1995; DeMarini *et al.*, 1995) (see Figure 4). The relative shortness of the ubiquitin oligomers observed in *doa4* cells may result from DUB activities that can trim proteasome-bound ubiquitinated proteins or peptides from the distal ends of the ubiquitin chains. An activity in the 19S regulatory component of the 26S proteasome with such a specificity has been described recently (Lam *et al.*, 1997).

It is possible that when rates of proteolysis by the proteasome are reduced by mutation (Figure 5B), a significant fraction of proteasome-bound polyubiquitinated substrates is deubiquitinated before degradation of substrate (or is released from proteasomes and deubiquitinated) (Figure 8, lower pathway). Unanchored ubiquitin chains would accumulate in the proteasome mutants if release of long ubiquitin chains from substrates is more efficient in the mutants than in cells with wild-type proteasomes, e.g. because chain-trimming steps are bypassed. If Ubp14 enzyme is not saturated by the additional ubiquitin chains generated in the proteasome mutants, then a synergistic effect on chain levels by mutation of both proteasomes and Ubp14 would be expected. The model in Figure 8 allows for the order in which a substrate is proteolyzed by the 20S proteasome and deubiquitinated by proteasome-associated DUB enzymes to change depending on the relative rates of these two processes. These rates may change as a result of mutation or alterations in cell state and could vary between substrates.

Ubp14 and the control of intracellular proteolytic rates

How is the enzymatic activity of Ubp14 mechanistically linked 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). 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) that IsoT disassembles polyubiquitin chain end-products and thereby stimulates ubiquitin-dependent proteolysis by the proteasome, although our data indicate that *in vivo*, inhibitory chains may not derive solely from proteasome-generated species.

An important question is whether Ubp14 must associate with proteasomes in order to keep them relatively free of unanchored chains. Remarkably, Ubp14 alone is responsible for most of the soluble cellular deubiquitinating activity against free ubiquitin chains (Figure 3C), so it is not necessary to invoke co-localization of chains and Ubp14 on proteasomes to explain ubiquitin chain accumulation in *ubp14* cells even though they have many other DUB enzymes. However, the dominant-negative effect of the inactive *ubp14-Ala354* allele on ubiquitin-dependent proteolysis (Figure 6) suggests that Ubp14 might function in the context of a protein complex. Alternatively, the inhibitory effect of the *ubp14-Ala354* allele could be due to titration of ubiquitin chains required for efficient protein ubiquitination (see next section). There may be a cellular co-factor(s) for Ubp14 that increases its substrate affinity or catalytic rate, e.g. by converting it from a non-processive to a processive enzyme. The Ubp14/IsoT subfamily is unique among the known DUBs in that its members contain two copies of the so-called UBA motif (Figure 1), a potential protein–protein interaction domain found in several ubiquitin system enzymes and substrates (Hofmann and Bucher, 1996).

Potential role of ubiquitin chains in protein ubiquitination in vivo

The ability of overproduced wild-type Ubp14 to inhibit the proteolysis of a subset of ubiquitin-dependent substrates *in vivo* (Figure 6C) was unexpected. It is possible that excess Ubp14 leads to aberrant complexes between Ubp14 and components of the ubiquitin system, thereby impairing their function. Overproduction of Ubp14 also leads to a reduction of unanchored ubiquitin chains relative to the level seen in wild-type cells (Figure 6A). Hence, the explanation we currently favor for proteolytic inhibition is that ubiquitin chains can be ligated directly to at least some substrates *in vivo* and that such transfer of preformed ubiquitin chains contributes significantly to the rate of degradation of these substrates (Figure 8, dashed arrow). Conceivably, certain substrates, e.g. N-end rule substrates, are deubiquitinated very efficiently by DUBs by progressive trimming of ubiquitin chains, so transfer of whole chains may increase the time such substrates stay in the polyubiquitinated state, increasing the likelihood of their targeting to the proteasome.

These considerations 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 too low a level may reduce the efficiency of polyubiquitinated protein degradation as well, at least for certain substrates. The control of cellular ubiquitin chain levels by regulation of ubiquitin chain assembly and/or disassembly rates (by Ubp14 or its functional homologs) 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* homolog of Ubp14 provides an apparent

Table IV. Yeast strains

Strain	Genotype
MHY501[a]	*MATα his-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1*
MHY606[b]	*MAT***a** *his-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1*
	MATα his-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1
MHY623[c]	*MATα his-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 doa4-Δ1::LEU2*
MHY642[d]	*MAT***a** *his-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 ubi4-Δ2::LEU2*
MHY792[b]	*MATα his-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 doa3-Δ1::HIS3* [YCplac22doa3-1]
MHY794[b]	*MATα his-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 doa5-Δ1::HIS3* [YCplac22doa5-1]
MHY839	*MAT***a** *his-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 ubp14-Δ1::HIS3*
MHY840	*MATα his-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 ubp14-Δ1::HIS3*
MHY844	*MATα his-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 doa4-Δ1::LEU2 ubp14-Δ1::HIS3*
MHY976	*MATα his-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 doa3-Δ1::HIS3 ubp14-Δ1::HIS3* [YCplac22doa3-1]
MHY978	*MATα his-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 doa5-Δ1::HIS3 ubp14-Δ1::HIS3* [YCplac22doa5-1]
MHY981	*MAT***a** *his-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 ubi4-Δ2::LEU2 ubp14-Δ1::HIS3*
MHY1053	*MAT***a** *his-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 ubp14-Δ1::HIS3*
	MATα his-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 ubp14-Δ1::HIS3

[a]Chen *et al.* (1993); [b]Chen and Hochstrasser (1995); [c]Papa and Hochstrasser (1993); [d]Özkaynak *et al.* (1984).

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.

Materials and methods

Strains, media and genetic techniques

The yeast strains used in this work are listed in Table IV. Yeast rich and minimal media were prepared as described, and standard yeast genetic methods were used (Sherman *et al.*, 1986). The *E.coli* strains used were JM101 and MC1061, and standard procedures for recombinant DNA work were used (Ausubel *et al.*, 1989).

Cloning and expression of UBP14 in E.coli

For *UBP14* cloning by PCR amplification, two oligodeoxynucleotides derived from the *UBP14* sequence were synthesized. The first, 5′-TCTTCTAGATATGGCAGTAGAGATGACAGGT-3′, corresponds to the sense strand of *UBP14* and contains the start codon (underlined); an upstream *Xba*I site was introduced for subsequent cloning. The second oligodeoxynucleotide, 5′-ATCTCTCGAGTTAGCATCTTGTATAGAA-ATAAATATAAC-3′, was complementary to the sense strand of *UBP14*. An *Xho*I site was added downstream of the terminator (underlined) to facilitate cloning steps. The two primers were used for PCR amplification of the *UBP14* coding sequence from *S.cerevisiae* genomic DNA. Agarose gel electrophoresis of PCR products showed a single band of the expected size (~2400 bp). The amplified DNA fragment was digested with *Xba*I and *Xho*I, gel-purified, and subcloned into *Xba*I–*Xho*I-digested pGEX-KG (Pharmacia), yielding pGEX-UBP14.

Cloning and expression of human isopeptidase T in yeast

For expression of human IsoT in yeast cells, the IsoT cDNA (Wilkinson *et al.*, 1995) was subcloned into the pVT102-U expression vector (Vernet *et al.*, 1987), between the *S.cerevisiae ADH1* promoter and terminator sequences to make pISOT. IsoT cDNA was amplified with oligodeoxynucleotides 5′-GATTCTAGAATGGCGGAGCTGAGTGAGGAGGCG-CTG-3′ and 5′-GATAAGCTTAGCTGGCCACTCTCTGGTAGAAG-TAG-3′, corresponding to the 5′ and 3′ ends of the IsoT cDNA coding region, respectively (ATG start and TAA stop codons are underlined). *Xba*I and *Hin*dIII restriction sites were built into the 5′ and 3′ primers, respectively. An IsoT cDNA cloned into the pCRII vector (Invitrogen) was used as the template for PCR amplification with Vent polymerase (NEB). Three independent PCR reactions were done. Amplified DNAs were digested with *Xba*I and *Hin*dIII, gel purified and subcloned into *Xba*I/*Hin*dIII-cut pVT102-U.

Site-directed mutagenesis of UBP14 and overexpression of Ubp14^{Ala354} and wild-type enzyme in yeast

The Cys354Ala mutation was introduced into Ubp14 by a two-step PCR-based approach. Oligodeoxynucleotides 5′-CAATTTGGGTAATT-CCGCTTACTTAAATTCGGTTATAC-3′ and 5′-GTATAACCGAATT-TAAGTAAGCGGAATTACCCAAATTG-3′ were used to mutate the Cys354 codon. Oligodeoxynucleotides 5′-CACTCGCATGCTTTGG-ATCATTATCG-3′ and 5′-ATTGTCCGGATATACAACATCCAGTG-3′ served as flanking primers. pGEX-UBP14 was used as a template. The second set of PCRs used the flanking primers and the two initial PCR reaction products. The resulting DNA fragments were digested with *Sph*I and *Bsp*mII (the corresponding restriction sites had been introduced into the first and second flanking primers, respectively) and gel purified. *Sph*I/*Bsp*mII-digested pGEX-UBP14 was used as the backbone for assembly of the full-length *ubp14-C354A* allele. The mutant constructs were verified by dideoxy DNA sequencing.

For overexpression of Ubp14 and Ubp14^{Ala354} in yeast, the pVT102-U plasmid was again used as the expression vector. Mutant pGEX-UBP14-Ala354 or wild-type pGEX-UBP14 was digested with *Xho*I and incubated with Klenow enzyme in the presence of dNTPs to generate blunt ends. Subsequent digestion with *Xba*I and agarose gel purification yielded insert fragments appropriate for subcloning. The pVT102-U vector was digested with *Hin*dIII, treated with Klenow enzyme to fill in the 5′ overhangs and cut with *Xba*I. Two independent isolates of both pUBP14 (wild-type) and pUBP14-Ala354 were used for further analysis. To express *UBP14* under the control of its own promoter in the high-copy YEplac195 vector, a 3 kb DNA fragment including the entire *UBP14* ORF and 550 bp of upstream sequence was amplified using as the upstream primer 5′-ACCAGAGCTCAAGTACTAGAGATAAAGA-GTGTAGAAACC-3′ and the same 3′ primer used above to clone *UBP14* into the pGEX-KG vector. The amplified DNA fragment was digested with *Xho*I, the overhang was filled in using Klenow enzyme and the DNA was then digested with *Sac*I (site underlined in preceding primer). The purified DNA fragment was subcloned into *Sac*I/*Sma*I-cut YEplac195.

Construction of a ubp14 null mutant

To make a null allele of *UBP14*, the *HIS3* gene was amplified with primers whose 5′ sequences corresponded to the region just 5′ of the ATG start codon and to the complement of the sequence just downstream of the termination codon, respectively. The primers used were 5′-ATCAAATTTATCACTTGATGAAATCACAGTGAAAAGCGACTTG-CACTCTTGGCCTCCTCTAG-3′ and 5′-TATATATATGTGTGTGTGT-GTGTGTGTGTGTGTGTAAACGTTCTTTCGTTCAGAATGACACG-3′. The amplified DNA fragments were transformed into MHY606 diploid cells. His$^+$ transformants were checked for the disruption allele by PCR. Heterozygous diploids were sporulated and tetrads dissected. The His$^+$ trait segregated 2:2 in all tetrads. Haploid mutants were verified by PCR and Southern DNA hybridization analysis.

Purification of GST–Ubp14 from E.coli cells

To induce expression of GST–Ubp14, JM101 bacterial cells transformed with pGEX-UBP14 were grown to an OD_{600} of 0.7 in LB + 100 μg/ml

ampicillin at 37°C. After addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and subsequent incubation at 37°C for 3 h, cells (100 ml) were collected by centrifugation and resuspended in 3 ml of phosphate-buffered saline (PBS). The suspension was sonicated with a microtip attachment until clarified; 1/20 volume of 10% Triton X-100 was added, and the suspension was mixed gently by inversions of the tube. Cell debris was removed by centrifugation at 14 000 *g*, for 10 min, 0.25 ml of glutathione–agarose (Sigma) was added to the supernatant and the mixture was adjusted to 0.5 mM EDTA, 10 μg/ml aprotinin, 5 μg/ml pepstatin A. The mixture was incubated at room temperature for 30 min with constant rotation. The resin was pelleted and washed twice with 8 ml of ice-cold PBS. GST–Ubp14 protein was eluted with 10 mM glutathione (3× 0.125 ml). The eluates were pooled and concentrated using a Centricon 10 concentrator (Amicon). The purity of GST–Ubp14 fusion protein was checked by polyacrylamide gel electrophoresis and Coomassie blue staining; all preparations were estimated to be at least 90% pure.

Pulse–chase assays and immunoblot analysis

Pulse–chase analysis of yeast cells grown to logarithmic phase was done as described previously (Chen *et al.*, 1993). Aliquots of cells were disrupted by mixing with an equal volume of 2% SDS, 90 mM HEPES, pH 7.5, 30 mM dithiothreitol (DTT) and heating at 100°C for 10 min. Cell extracts were immunoprecipitated with antibodies against MATα2 (Hochstrasser and Varshavsky, 1990) or *E.coli* β-galactosidase (Cappel). Radiolabeled proteins were visualized by fluorography and/or with a PhosphorImager (Molecular Dynamics). Protein degradation rates were measured from quantitative PhosphorImager data.

For anti-ubiquitin immunoblot analysis, cells were grown at 30°C in minimal media to mid-logarithmic phase, centrifuged, resuspended in Laemmli loading buffer, boiled for 10 min and loaded onto 18% polyacrylamide gels. To enhance resolution of low molecular mass proteins, a Tricine gel system (Schägger and von Jagow, 1987) was used in most of the experiments in this study; in these cases, 12.5% polyacrylamide gels were used. Proteins were transferred to Immobilon-P membranes (Millipore); the blots were then boiled in water and incubated with anti-ubiquitin antibodies. All blots used affinity-purified anti-ubiquitin antibodies provided by C.Pickart, except where noted. Antibody binding was detected by enhanced chemiluminescence (Amersham), except as noted. For quantitative blots, [^{125}I]protein A was used as the secondary antibody, and serial dilutions of yeast extracts were used to determine the linear range for antibody binding. We note that under the conditions used in the current work, reactivity of free ubiquitin and Ub–Ub with the antibody from C.Pickart was very similar based on standard curves with the two proteins.

Preparation of protein extracts from yeast and assay for ubiquitin isopeptidase activity

Yeast extracts were made by disruption with glass beads (Ausubel *et al.*, 1989). Overnight cultures were centrifuged and resuspended in 3 vols of disruption buffer (20 mM Tris–HCl, pH 7.9, 10 mM $MgCl_2$, 1 mM EDTA, 5% glycerol, 1 mM DTT, 0.3 M ammonium sulfate, 200 μg/ml aprotinin, 100 μg/ml pepstatin). Cells were mixed with 4 vols of chilled glass beads and vortexed five times for 45 s each, leaving cells on ice for 1 min between vortexings. The extracts were centrifuged, and supernatants were stored at –20°C as 50% glycerol solutions. Assays of isopeptidase activity were conducted at 37°C in a buffer containing 50 mM Tris–HCl, pH 7.3, 2 mM DTT in a total volume of 20 μl. Reaction mixtures contained 2 μl of yeast extract (4.8 μg of protein) and 1 μg of Ub–Ub or Ub–UbΔGG. Aliquots of 3 μl were removed and analyzed by anti-ubiquitin immunoblotting.

Assays with purified enzymes were conducted at 37°C in a buffer containing 50 mM Tris–HCl, pH 7.6, 10 mM DTT and 0.1 mg/ml ovalbumin as described previously (Wilkinson *et al.*, 1995). Aliquots of the assay mixture (1–2 μg of total ubiquitin) were separated on an HPLC C8 column (isocratic 45% acetonitrile in 50 mM perchlorate, pH 2.0). Peaks were integrated and compared with ubiquitin as a standard for assessing retention time and concentrations of substrates and ubiquitin product.

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