The EMBO Journal Vol.19 No.1 pp.94–102, 2000

Recognition of the polyubiquitin proteolytic signal

Julia S.Thrower, Laura Hoffman[1], Martin Rechsteiner[1] and Cecile M.Pickart[2]

Department of Biochemistry and Molecular Biology, School of Public Health, Johns Hopkins University, 615 North Wolfe Street, Baltimore, MD 21205 and [1]Department of Biochemistry, School of Medicine, University of Utah, Salt Lake City, UT 84132, USA

[2]Corresponding author
e-mail: cpickart@welchlink.welch.jhu.edu

Polyubiquitin chains linked through Lys48 are the principal signal for targeting substrates to the 26S proteasome. Through studies of structurally defined, polyubiquitylated model substrates, we show that tetraubiquitin is the minimum signal for efficient proteasomal targeting. The mechanism of targeting involves a simple increase in substrate affinity that is brought about by autonomous binding of the polyubiquitin chain. Assigning the proteasomal signaling function to a specific polymeric unit explains how a single ubiquitin can act as a functionally distinct signal, for example in endocytosis. The properties of the substrates studied here implicate substrate unfolding as a kinetically dominant step in the proteolysis of properly folded proteins, and suggest that extraproteasomal chaperones are required for efficient degradation of certain proteasome substrates.

Keywords: chaperone/polyubiquitin/26S proteasome/ubiquitin

Introduction

Proteolysis is frequently used to regulate processes that require rapid alterations in protein levels, including cell cycle progression (e.g. Koepp *et al.*, 1999). Most regulated proteolysis in eukaryotes occurs by a mechanism in which conjugation to the conserved protein ubiquitin (Ub) targets substrates for degradation by 26S proteasomes (Hochstrasser, 1996; Hershko and Ciechanover, 1998). Substrates of the Ub–proteasome pathway include soluble proteins of the cytosol and nucleus, and proteins of the endoplasmic reticulum that have been ejected into the cytoplasm (Sommer and Wolf, 1997). Ub also mediates the turnover of certain plasma membrane proteins by targeting them for endocytosis, leading to proteolysis in the lysosome (Hicke, 1997). How the proteasomal and endocytic Ub targeting signals are distinguished is not yet understood.

Substrates of the Ub–proteasome pathway are marked for degradation by covalent ligation to Ub, which then acts as a signal for targeting the modified substrate to the proteasome. Ub is linked to the substrate through an isopeptide bond between the C-terminus of Ub (G76) and a lysine residue of the target protein. Ubiquitylation begins with the ATP-dependent activation of Ub by an activating enzyme (E1). The ligation of ubiquitin to the substrate is then carried out by a specific complex composed of a Ub–protein ligase (E3) and a Ub conjugating enzyme (E2), with the E3 being the primary substrate specificity factor (Hershko and Ciechanover, 1998). During this recognition phase, many Ubs are ligated to the substrate, usually in the form of a polymeric chain (Chau *et al.*, 1989). PolyUb chains linked through K48–G76 isopeptide bonds are the principal signal for proteasomal proteolysis (Chau *et al.*, 1989; Finley *et al.*, 1994).

The 26S proteasome is a 2.1 MDa complex whose ~65 subunits are divided among three subcomplexes (Baumeister *et al.*, 1998; Rechsteiner, 1998). One subcomplex, the 20S proteasome, is a cylindrical stack of four seven-membered rings. Its proteolytic active sites (six in eukaryotes) face an interior chamber that can be entered only through a narrow pore at either end of the cylinder (Löwe *et al.*, 1995; Groll *et al.*, 1997). Because folded proteins cannot reach this chamber, the isolated 20S complex hydrolyzes only small peptides and denatured proteins. The proteasome acquires activity toward folded target proteins following the binding of one 19S complex to each end of the 20S cylinder. In general, a folded target protein is recognized by the 26S proteasome only if it has been conjugated to a K48-linked polyUb chain (see Pickart, 1997). The properties of the 26S proteasome suggest that the 19S complex mediates polyUb recognition and substrate unfolding.

The use of a generalized signal, a polyUb chain, to target proteins for destruction is the defining characteristic of the Ub–proteasome pathway. If the 26S proteasome recognized its target proteins directly, then specificity would be restricted, as seen for the Clp and Lon proteases of *Escherichia coli* (Gottesman *et al.*, 1997). Instead, target proteins are recognized by dedicated E2–E3 complexes. These enzymes generate the covalent polyUb targeting signal, while the proteasome only needs to recognize this signal. The separation of target protein recognition from the catalysis of peptide bond hydrolysis is the key feature that allows the Ub–proteasome pathway to degrade a remarkable array of substrates with high specificity. However, while several specific signals have been identified that lead to the assembly of polyUb chains on substrate proteins (e.g. Koepp *et al.*, 1999; Laney and Hochstrasser, 1999), little is yet known about polyUb signal recognition and transduction. The major polyUb receptor(s) in the 19S complex has not been identified, the signal itself is incompletely characterized, and the molecular mechanism of targeting is poorly understood.

We report an analysis of polyUb recognition by the proteasome that employed, for the first time, a structurally defined polyubiquitylated substrate. The results reveal that tetraubiquitin constitutes the minimum proteasomal

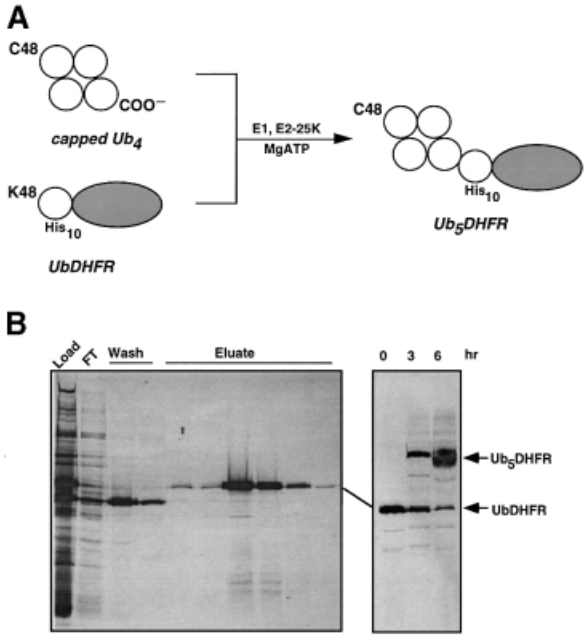


Fig. 1. Synthesis of Ub_5DHFR. (**A**) Scheme. UbDHFR has a polyHis tag at its N-terminus and a hemagglutinin (HA) tag at its C-terminus. (**B**) Purification of [^{35}S]UbDHFR and conjugation to Ub_4 (autoradiographs). Left, successive fractions in purification of [^{35}S]UbDHFR on Ni^{2+}–NTA resin. Right, time course of [^{35}S]UbDHFR conjugation to Ub_4.

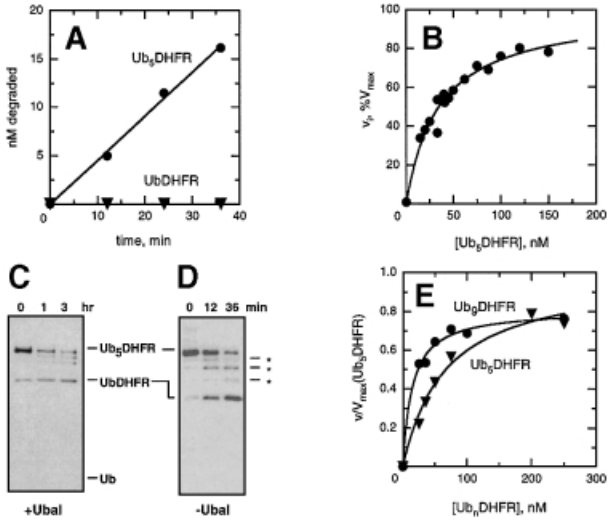


Fig. 2. Properties of Ub_5DHFR. All incubations except that in (D) contained Ubal. (**A**) Branched polyUb chain is essential for proteolytic targeting. Purified 26 proteasomes (~2 nM) were incubated with 150 nM of either [^{35}S]Ub_5DHFR (circles) or [^{35}S]UbDHFR (triangles) as described in Materials and methods. The rate of degradation of Ub_5DHFR doubled when the proteasome concentration was doubled (not shown). (**B**) Dependence of initial degradation rate on substrate concentration. Results of two experiments are combined. Incubations contained 2.5 nM proteasomes; the curve is a least-squares fit of the Michaelis–Menten equation assuming K_M = 35 nM. (**C**) Fused Ub moiety of UbDHFR is degraded (Western blot). Proteasomes (~10 nM) were incubated with unlabeled Ub_5DHFR (75 nM). Aliquots were analyzed by blotting with antibodies against the polyHis tag of UbDHFR. The migration positions of Ub_5DHFR, UbDHFR and Ub are indicated. Ub_5 would migrate just above UbDHFR. Products corresponding to the removal of one or two Ubs from the distal end of the polyUb chain of Ub_5DHFR are faintly visible in the second and third lanes, but represent only a small fraction of the starting substrate. (**D**) Ub_5DHFR disassembly in the absence of Ubal (Western blot). Proteasomes (~3 nM) were incubated with 100 nM Ub_5DHFR (see text). Aliquots were analyzed by blotting with antibodies against the C-terminal HA tag of Ub_5DHFR. Asterisks, deubiquitylated forms of Ub_5DHFR. Note the different sampling times in (C) and (D). (**E**) Influence of polyUb chain length on proteolysis. Degradation of [^{35}S]Ub_5DHFR (triangles) or [^{35}S]Ub_9DHFR (circles) was assayed in incubations with ~2 nM proteasomes. All rates were normalized to the extrapolated V_{max} for Ub_5DHFR. The lines are fits of the Michaelis–Menten equation assuming K_M = 68 nM (Ub_5DHFR, triangles) or K_M = 14.5 nM (Ub_9DHFR, circles). The weaker binding of Ub_5DHFR relative to (B) reflects the use of different substrate and proteasome preparations.

targeting signal, explain the molecular basis of the dependence of signal strength on chain length, and show that only a subset of potential interacting residues on the chain surface is important for recognition. These findings suggest that the higher-order conformation of the chain influences its signaling potential, and explain why a single Ub is an inefficient proteasomal targeting signal. Unexpectedly, the substrates employed here, although recognized with high affinity, were slowly degraded. Several lines of evidence suggest that this slow degradation reflects slow unfolding of the target protein moiety.

Results

Model substrate for 26S proteasomes

The ideal substrate for an *in vitro* analysis of proteasomal signal recognition should carry a homogeneous targeting signal. The model substrate shown in Figure 1A features a single Ub_4 chain that is linked to one lysine residue of the target protein. For the target protein we chose dihydrofolate reductase (DHFR) fused at its N-terminus to Ub. UbDHFR acquires a polyUb chain and is targeted to proteasomes in yeast cells (Johnson *et al.*, 1992, 1995). Although the UbDHFR conjugates seen in yeast feature a K29 linkage in the polyUb chain (Johnson *et al.*, 1995), we reasoned that a homogeneous K48-linked chain would be sufficient to direct UbDHFR proteolysis, and this proved to be correct. UbDHFR was metabolically labeled in *E.coli* and purified via an N-terminal polyHis tag (Figure 1B). We then used the Ub-specific conjugating enzyme E2-25K to link preassembled (K48-linked) Ub_4 to K48 in the Ub moiety of UbDHFR (Haldeman *et al.*, 1997; Piotrowski *et al.*, 1997; Figure 1). The final polyUb-conjugated substrate, designated Ub_5DHFR, was a fully active dihydrofolate reductase (Materials and methods), indicating that its DHFR moiety was properly folded (Stammers *et al.*, 1987). The fused Ub moiety was also correctly folded, since it was recognized by E2-25K.

Ub_5DHFR was a well-behaved substrate for purified mammalian 26S proteasomes. Production of acid-soluble radioactivity from the labeled UbDHFR moiety of Ub_5DHFR was linear with time and depended on the presence of ATP (Figure 2A; data not shown). Degradation was also strictly dependent on the ligation of UbDHFR to Ub_4 (Figure 2A) and was completely inhibited by the well-characterized proteasome inhibitor MG-132 (not shown; Rock *et al.*, 1994). Ub_5DHFR was a high-affinity substrate (K_M = 35 nM; Figure 2B). In a separate experiment involving highly purified proteasomes, the molecular turnover number (k_{cat}) was determined to be 0.05 min^{-1}. Assuming that the ~380-residue UbDHFR protein is hydrolyzed to 10-residue peptides, k_{cat} corresponds to

~2 peptide bond cleavages/min/proteasome. The same preparation of 26S proteasomes hydrolyzed Suc-LLVY-AMC with $k_{obs} = 98\ min^{-1}$, similar to previously reported values (e.g. Dick *et al.*, 1991). The difference in the k_{cat} values for a peptide versus Ub_5DHFR suggests that there is a slow step before peptide bond hydrolysis in the degradation of Ub_5DHFR (below).

The Ub moiety of UbDHFR carries the G76V mutation to prevent its removal by deubiquitylating enzymes (Johnson *et al.*, 1995). To test whether this fused Ub moiety was degraded, Ub_5DHFR was incubated with a high concentration of 26S proteasomes and reaction products were visualized by blotting with antibodies against the N-terminal polyHis tag of UbDHFR. If the fused Ub moiety escaped degradation, it would be converted to a product of ~40 kDa (if linked to Ub_4) or ~8 kDa (if released from Ub_4). Instead, most of the ~68-kDa Ub_5DHFR protein disappeared without the production of smaller immunoreactive products (Figure 2C). These results show that the fused Ub moiety is degraded. However, it may still be recognized as part of the polyUb chain (below).

All degradation assays were carried out in the presence of Ub aldehyde (Ubal), a specific inhibitor of deubiquitylating enzymes (Pickart and Rose, 1986; Hershko and Rose, 1987). When Ubal was omitted, the degradation of Ub_5DHFR was inhibited (not shown). Blotting with antibody against a C-terminal epitope tag of Ub_5DHFR (Figure 2D) showed inhibition was due to disassembly of the substrate's polyUb chain. Deubiquitylation, which was presumably due to the UCH37 subunit of the mammalian 19S complex (Lam *et al.*, 1997b), was efficiently suppressed by Ubal (not shown), allowing us to monitor degradation exclusively. However, these findings suggest that deubiquitylation and degradation could occur at competitive rates on the proteasome *in vivo* (see Discussion).

Ub_4 is the minimum targeting signal

We have suggested that the assembly of Ub into a K48-linked chain creates a unique recognition element that is bound by specific receptors in the 19S complex (Beal *et al.*, 1996, 1998). This model is consistent with the defined conformation seen in the crystal structure of K48-linked Ub_4 (Cook *et al.*, 1994), and with the apparent inability of K63-linked chains to signal proteolysis *in vivo* (Spence *et al.*, 1995). However, it is also possible that the assembly of Ub into a K48-linked chain enhances signaling simply by increasing the concentration of monoUb (Pickart, 1997, 1998). These two models can be distinguished based on the length dependence of polyUb chain signaling. If the chain signals proteolysis by increasing the concentration of Ub, then signaling should increase linearly with chain length; if the chain signals proteolysis by creating a new recognition element, then the dependence is unlikely to be linear.

To investigate how signaling depends on chain length, we first compared the substrate properties of UbDHFR conjugated to polyUb chains of different lengths. In comparison to Ub_5DHFR, Ub_9DHFR had a similar k_{cat} and a 4.7-fold lower K_M (Figure 2E). These results suggest that enhanced signaling is manifested as enhanced substrate affinity, and that affinity depends nonlinearly on chain length (it will be shown below that K_M is equal to the dissociation constant of the substrate). To confirm these hypotheses we studied the binding of different length unanchored chains, as monitored by inhibition of Ub_5DHFR degradation. We first established the validity of unanchored chains as a model for substrate-linked chains by showing that inhibition by Ub_4 could be overcome at a high concentration of Ub_5DHFR (Figure 3A). Such competitive behavior indicates that the unanchored chain binds to the same site as the substrate. Studies with a series of unanchored chains revealed that affinity varied with chain length (Figure 3B; data not shown). The relationship appeared to be hyperbolic: the binding of Ub_2 was too weak to be detected (K_i >15 μM), whereas Ub_{12} and Ub_8 bound with a similar high affinity (K_i ~25 nM; Table I). Because a 6-fold increase in chain length caused an affinity increase of ~600-fold, the chain cannot signal proteolysis by increasing the concentration of monoUb. Instead, Ub_4 appears to be the minimum signal: affinity increased ~100-fold as n increased from 2 to 4, but <10-fold as n increased from 4 to 12 (Table I).

The 6.6-fold difference in the affinities of Ub_8 and Ub_4 (Table I) agrees well with the ~5-fold difference in the K_M values of Ub_9DHFR and Ub_5DHFR (Figure 2E), suggesting that the chain is the principal determinant of substrate binding (below). The relative binding of Ub_8 and Ub_4 seen in Table I also agrees with the 6-fold difference observed in a previous study (Piotrowski *et al.*,

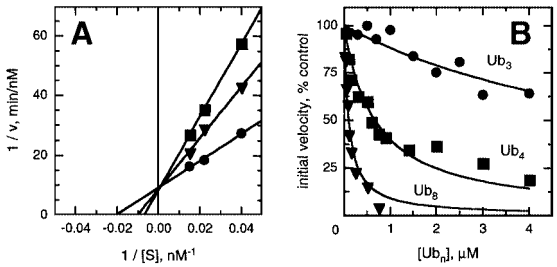


Fig. 3. Length dependence of unanchored polyUb chain binding to 26S proteasomes. (**A**) Unanchored polyUb chains bind competitively with substrate. Incubations contained ~2 nM proteasomes, with 25, 45 or 67 nM [^{35}S]Ub_5DHFR, and no Ub_4 (circles), or 250 nM (triangles) or 500 nM (squares) Ub_4. (**B**) Inhibition versus chain length. Incubations contained ~2 nM proteasomes, 100 nM [^{35}S]Ub_5DHFR and the indicated concentrations of unanchored Ub_3 (circles), Ub_4 (squares) or Ub_8 (triangles). Initial rates are expressed as a percentage of the control reaction without unanchored chains. The curve is a least-squares fit of the equation $v_0 = (v_i K_{0.5})/(K_{0.5} + [Ub_n])$ where $K_{0.5} = (1 + [S]/K_M)K_i$. For K_i values see Table I.

Table I. Length (n) and proximal end effects on polyUb chain binding to 26S proteasomes

n	Proximal end	K_i (nM)	$K_i(Ub_n)/K_i(Ub_8)$
2	Asp77	>15 000	>577
3	Asp77	1933 ± 219	74
4	Asp77	171 ± 16	6.6
6	Asp77	52 ± 4	2.0
8	Asp77	26 ± 4	1.0
12	Asp77	~20	~1.0
4	diol	57 ± 8	
4	NAL	57 ± 4	
5	βGal	35 ± 4	

All values determined from inhibition of Ub_5DHFR degradation. Most values are the mean ± SD, $n = 3$. The value for Ub_4diol is from triplicate determinations at one chain concentration; the value for Ub_5βGal is from Figure 5.

1997), but the K_{app} values determined earlier were ~200-fold weaker than the K_i values measured here. The earlier study employed a widely used proteasome substrate consisting of radiolabeled lysozyme conjugated to heterogeneous length polyUb chains. It is likely that unlabeled conjugates, derived from target proteins contaminating the conjugating enzymes, were also present in this preparation (Piotrowski *et al.*, 1997). The different results obtained in the two studies are consistent with this idea: the presence of such internal competitors would weaken the apparent binding of a given chain, but would not influence the relative binding of different length chains.

The dissociation constant of unanchored Ub_4 (170 nM) is 5-fold larger than the kinetic K_M of Ub_5DHFR (35 nM). This difference could reflect structural differences between the two chains: an unanchored chain has a negatively charged carboxylate at its proximal end, whereas a substrate-linked chain has a neutral isopeptide bond at this position. To test this hypothesis we conjugated Ub_4 to *N*-acetyl lysine methyl ester, to make Ub_4NAL (Materials and methods). Ub_4NAL inhibited Ub_5DHFR degradation with K_i = 57 nM (Table I). The tighter binding of Ub_4NAL (in comparison with Ub_4) is due to the absence of the negatively charged carboxylate, because converting the G76 carboxylate in Ub_4 to an uncharged diol gave a similar increase in affinity (Table I). The affinities of Ub_4NAL and Ub_4diol are very similar to the K_M of Ub_5DHFR (57 versus 35 nM). The slightly higher affinity of Ub_5DHFR probably reflects a contribution of the fused Ub moiety to the recognition of the polyUb chain, i.e. it is as if DHFR is conjugated to Ub_5. Thus, the K_M value of Ub_5DHFR is essentially equal to its dissociation constant. These results show that Ub_4 is a very efficient proteasomal targeting signal.

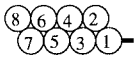
Signal strength depends on the number of Ub_4 units

The results shown in Table I suggest that Ub_4 is the minimum signal for efficient targeting to the proteasome, but they do not explain why longer chains ($n > 4$) bind better than Ub_4. The latter result may be explained in two ways. In one model, longer chains bind better because they contain multiple Ub_4 units. This model predicts that Ub_8 will bind 3-fold more tightly than Ub_4, a factor which is similar to the observed ratios of 6.6-fold for unanchored chains (Table I) and ~5-fold for chains conjugated to UbDHFR (Figure 2E). In a second model, only the proximal Ub_4 unit is recognized, and chains of $n > 4$ bind better because lengthening the chain stabilizes the conformation of the proximal Ub_4 unit. Our finding that the status of the chain's proximal terminus influences binding (Table I) could be explained by this second model. Both models predict that affinity will level off as the chain becomes very long. They are distinguished by the location of the Ub_4 unit responsible for targeting. In the first model, any Ub_4 unit can be recognized, while in the second model only the proximal Ub_4 unit is recognized for productive binding.

Ub harboring the L8A mutation can be assembled into chains, but the mutant chains bind to the proteasome at least 15-fold more weakly than wild-type chains (Beal *et al.*, 1996, 1998). Therefore, to differentiate between the above-described models we compared the binding of chimeric Ub_8 molecules in which four recognition-deficient Ubs were incorporated at the proximal versus the distal end of the chain. As shown in Table IIA, the incorporation of an L8A tetramer into Ub_8 caused a reduction in binding whose magnitude was independent of the location of the mutant tetramer (compare chains 2 and 3 with chain 1). Thus, the recognition of Ub_4 does not depend on its position within the chain. Moreover, each chimera bound to the proteasome with an affinity similar to that of Ub_4 (~6-fold weaker binding than wild-type Ub_8; compare Table IIA with I), providing additional evidence that each Ub_4 unit can be independently recognized. These results show that Ub_4 is the minimum proteolytic signal, and that signal strength (affinity) increases with the number of Ub_4 units.

Table II. Binding of chimeric polyUb chains to 26S proteasomes

A. Octamers

#	chain composition	% inhibition	relative K_i	ΔΔG kcal/mol
1		79.4 ± 4.9	1	0
2		36.1 ± 9.6	6.6	1.16
3		44.4 ± 5.0	4.7	0.96
4		8.5 ± 4.3	40.2	2.28

B. Tetramers

#	chain composition	% inhibition	relative K_i	ΔΔG kcal/mol
5		49.8 ± 3.6	1	0
6		52.4 ± 1.6	1	0
7		36.6 ± 5.3	1.7	0.33
8		40.8 ± 1.6	1.4	0.21
9		21.7 ± 2.5	3.6	0.79
10		25.3 ± 2.4	2.9	0.66
11		24.2 ± 6.3	3.1	0.70
12		9.0 ± 2.5	10.0	1.42

Circles denote Ubs, numbered sequentially from the proximal end (white, wild-type; dark, L8A). Reactions contained 0.13 μM Ub_8 (chains 1–4) or 4 μM Ub_4 (chains 5–12), 150 nM [^{35}S]Ub_5DHFR, and ~2 nM proteasomes. Inhibition of Ub_5DHFR degradation is expressed relative to a control without unanchored chains; values are means ± SD ($n \geq 4$). K_i is expressed relative to K_i of wt Ub_8 (A) or wt Ub_4 (B).

Molecular features of the Ub_4 signal

To define further the molecular properties of the Ub_4 targeting signal, we synthesized a series of chimeric Ub_4 molecules in which two L8A-Ubs were incorporated at

various positions in the chain. In contrast to the data with the chimeric octamers, in which the position of a mutant tetramer did not alter binding (Table IIA), different chimeric tetramers had different affinities (Table IIB). The individual Ubs in Ub_4 are therefore nonequivalent, as expected if Ub_4 is the minimum targeting signal. In the nomenclature used here, the proximal Ub is defined to be the first Ub in the chain. Remarkably, placing L8A-Ub in the second and fourth positions had no effect on binding (compare chains 5 and 6), indicating either that these two L8 residues do not interact with proteasomal receptors, or that the energy derived from their interaction is used to drive an energetically unfavorable transition (see Mildvan *et al.*, 1992). In contrast, the L8 side chains of the first and third Ubs were clearly important for recognition (compare chains 5 and 9). Comparison of the binding properties of chains 7–11 suggests that the L8 side chain of the first (proximal) Ub makes a stronger contribution to recognition than that of the third Ub. As in the case of Ub_8 (chain 4; Table I), the ability of an all-mutant chain (chain 12) to bind, albeit with reduced affinity, suggests that there are recognition determinants besides L8. The results also provide evidence of synergistic effects in the recognition of the elements of the Ub_4 targeting signal. In the absence of such effects, changes in the free energy of binding should be additive (Mildvan *et al.*, 1992). However, the sum of the change in free energy seen when mutating the first and third Ubs (0.8 kcal/mol, chain 9) and the change seen when mutating the second and fourth Ubs (0 kcal/mol, chain 6) underestimates the change due to mutating all four Ubs (1.4 kcal/mol, chain 12). The same conclusion follows from comparing chains 7, 10 and 12. A more detailed knowledge of how chains bind to their cognate receptor(s) will be necessary before this synergy can be interpreted at a mechanistic level. However, the results summarized in Table IIB clearly indicate that the four Ubs in the Ub_4 signal are non-equivalent. In contrast, there was no evidence for synergy in the recognition of individual Ub_4 units in Ub_8 (Table IIA).

Proteasomal binding of a linear polyUb chain

PolyUb chains linked through lysine residues other than K48 have been observed *in vitro* and *in vivo* (see Pickart, 1997, 1998). In particular, K63-linked chains have been implicated in processes that do not appear to depend on targeting to the proteasome, including post-replicative DNA repair (Spence *et al.*, 1995; Hofmann and Pickart, 1999) and endocytosis (Galan and Haguenauer-Tsapis, 1997). K63-linked chains could execute distinct signaling functions if they are conformationally distinct from K48-linked chains, resulting in differential binding to proteasomes or other unidentified receptors. As a first test of this hypothesis, we characterized the proteasomal interaction of linear Ub_5. This chain is the product of the yeast *UBI4* gene (Özkaynak *et al.*, 1987). Its constituent Ubs are joined by G76-M1 peptide bonds. M1 is spatially adjacent to K63 (Vijay-Kumar *et al.*, 1987), suggesting that linear Ub_5 could resemble a K63-linked chain. Linear Ub_5 inhibited Ub_5DHFR degradation with a reduced affinity relative to K48-linked Ub_4 (K_i = 539 versus 170 nM; data not shown). The true affinity difference is somewhat larger, because K48-linked Ub_5 will bind more tightly than Ub_4 (Table I). Inhibition by linear Ub_5 was

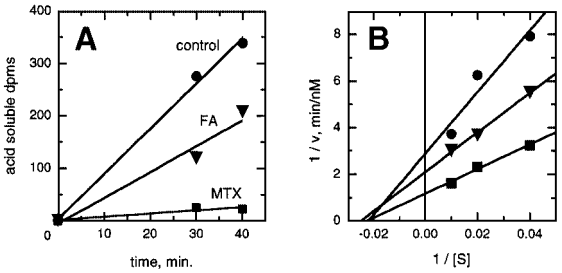


Fig. 4. Ligands of DHFR inhibit degradation. (**A**) Tighter binding ligand is stronger inhibitor. Incubations contained ~2 nM proteasomes, 150 nM [^{35}S]Ub_5DHFR and 100 μM of either folic acid (FA, triangles) or methotrexate (MTX, squares). (**B**) Noncompetitive inhibition by folic acid. Incubations contained ~2 nM proteasomes, with 25, 50 or 100 nM [^{35}S]Ub_5DHFR, and 0 (squares), 20 (triangles) or 40 (circles) μM FA.

competitive (not shown), suggesting that the linear chain occupies the site(s) occupied by the substrate's K48-linked chain. The properties of linear Ub_5 provide the first direct evidence that the linkage in a Ub polymer can influence proteasomal signaling. Linear Ub_5 is highly expressed in stressed cells, but it is co-translationally processed (Finley *et al.*, 1987). Processing provides a high level of Ub for conjugation; our results suggest that it may also prevent inhibition of proteasomes.

Rate-limiting substrate unfolding

The polyUb chain of Ub_5DHFR is a potent targeting signal that fully accounts for this substrate's interaction with proteasomes. However, despite its high affinity, Ub_5DHFR is degraded ~50 times more slowly than a small peptide (above). The most obvious difference between Ub_5DHFR and a peptide is that UbDHFR must be unfolded in order to be degraded. To test whether unfolding of UbDHFR limits the rate of degradation, we determined the effect of stabilizing this moiety through ligand binding. As shown in Figure 4A (squares), a saturating concentration of methotrexate (Appleman *et al.*, 1988) almost completely inhibited Ub_5DHFR degradation, as seen previously in reticulocyte lysate (Johnston *et al.*, 1995). The same concentration of folic acid (FA), which binds DHFR more weakly (Mathews and Huennekens, 1983), inhibited the degradation of Ub_5DHFR to a lesser extent (Figure 4A, triangles). Inhibition by FA was noncompetitive (Figure 4B); the specific k_{cat} effect indicates that the rate of proteolysis of bound Ub_5DHFR decreases, as expected for rate-limiting unfolding.

If k_{cat} monitors unfolding, then its value should vary with substrate identity, because it is unlikely that two different proteins will be unfolded at identical rates. To test this prediction we studied the degradation of Ub_5βGal. Ub_5βGal was enzymatically active, indicating that it is a tetramer of correctly folded 116 kDa subunits; each subunit was conjugated to Ub_4 (Jacobson *et al.*, 1994; Materials and methods). Ub_5βGal was not detectably degraded by 26S proteasomes (Figure 5A), but it bound tightly, as shown by its ability to inhibit Ub_5DHFR degradation (Figure 5B). The K_i value of 35 nM (Figure 5B) shows that the concentration of Ub_5βGal used in Figure 5A was saturating. Therefore, k_{cat} for Ub_5βGal is at least 50-fold smaller than k_{cat} for Ub_5DHFR. This difference is consistent with expectation for rate-limiting unfolding in

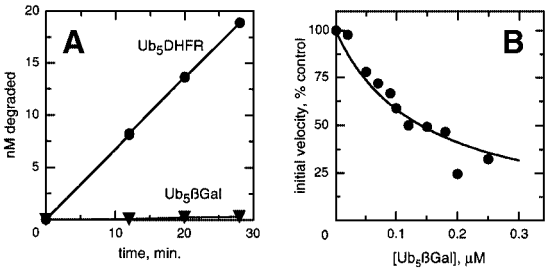


Fig. 5. Substrate properties of Ub_5-βGal. (**A**) Inefficient degradation of Ub_5βGal. Incubations contained ~2 nM proteasomes and 200 nM [^{35}S]Ub_5DHFR (circles) or 400 nM [^{35}S]Ub_5βGal (triangles). The concentration of Ub_5βGal refers to monomeric βGal subunits. (**B**) Efficient binding of Ub_5βGal. Incubations contained ~2 nM proteasomes, 100 nM [^{35}S]Ub_5DHFR, and 0 to 250 nM [^{35}S]Ub_5βGal. The curve is a least-squares fit of the equation in Figure 3B, assuming K_i = 35 nM.

view of the greater structural complexity of βGal. Although we could not detect degradation of Ub_5βGal by purified 26S proteasomes, polyUb-conjugated UbβGal is rapidly degraded by proteasomes in yeast cells (Johnson *et al.*, 1992, 1995; see Discussion).

The identical affinities of Ub_5βGal and Ub_5DHFR confirm that these substrates bind exclusively through their polyUb chains, and that Ub_4 is a high-affinity targeting signal. However, our results provide no indication that the chain performs any function besides targeting. It has been suggested that the polyUb chain helps to unfold the substrate (Ghislain *et al.*, 1996; see Pickart, 1997). However, the unimpaired enzymatic activities of Ub_5DHFR and Ub_5βGal suggest that the chain does not destabilize the equilibrium folding of these target proteins. A similar conclusion applies to polyubiquitylated forms of the plant photoreceptor phytochrome (Shanklin *et al.*, 1989). Nor was there evidence for an effect of the chain on the unfolding kinetics of the proteasome-bound substrate. Since unfolding is rate-limiting for degradation, UbDHFR linked to polyUb chains of different lengths should have been a sensitive reporter of such effects. Instead we found that k_{cat} was independent of chain length (Figure 2E).

Discussion

Function of the polyUb targeting signal

Binding of the polyUb chain signal to its cognate receptor(s) in the 19S complex initiates the proteolysis of most substrates of 26S proteasomes. An understanding of the proteasome's molecular mechanism must therefore begin with an explication of polyUb recognition. Here we used homogeneous K48-linked polyUb chains and novel synthetic substrates to elucidate fundamental properties of the polyUb proteolytic signal. Our results define Ub_4 as the minimum signal for efficient targeting. Ub_4 is the shortest chain that binds with high affinity to proteasomes (K_d <1 μM), and n = 4 defines a transition in the relationship between length and affinity (Table I). In addition, any Ub_4 unit in a chain can bind to proteasomes, in a manner that is independent of its location within the chain (Table IIA). Finally, individual Ubs within Ub_4 interact differently with proteasomal receptors (Table IIB), suggesting that the Ub_4 signal cannot be further subdivided. Assigning the proteasomal signaling function to a specific polymeric unit allows a single Ub to act as a distinct type of signal, for example in endocytosis (Terrell *et al.*, 1998).

Ub_4 is a remarkably efficient proteasomal targeting signal, cf. K_d ~60 nM for Ub_4 conjugated to a blocked lysine residue. The affinity of Ub_4 decreases when its proximal carboxylate is exposed (Table I). Although modest, this affinity difference will be advantageous in a cellular setting. Ub regeneration is thought to begin with release of the chain from a substrate remnant (Papa *et al.*, 1999). This cleavage will facilitate dissociation of the unanchored chain from the proteasome (Table I); it will also strongly stimulate disassembly of the chain by the enzyme known as isopeptidase T or Ubp14p (Wilkinson *et al.*, 1995; Amerik *et al.*, 1997). Together these effects will minimize inhibition of proteasomes by the polyUb chain products of proteolysis.

Our results convincingly demonstrate that a polyUb chain is a universal targeting signal. The interaction of Ub_5DHFR with proteasomes is fully explained by the interaction of its attached polyUb chain, as shown by the nearly identical affinities of Ub_4NAL and Ub_5DHFR and by the mutually exclusive binding of Ub_4 and Ub_5DHFR. In addition, Ub_5DHFR and Ub_5βGal, substrates that feature identical polyUb chains but highly divergent target proteins, bind identically to proteasomes. For these properly folded substrates the molecular mechanism of targeting involves a very large increase in affinity that is brought about by the autonomous binding of the polyUb chain. We found no evidence that the chain affects downstream steps of proteasomal proteolysis. Nor did we observe *trans*-targeting or allosteric effects: a saturating concentration of unanchored Ub_4 did not make UbDHFR susceptible to proteasomal degradation or stimulate peptide hydrolysis by proteasomes (our unpublished data). Johnson *et al.* showed that the polyUb-conjugated subunit(s) of chimeric βGal tetramers were selectively targeted for proteasomal degradation (Johnson *et al.*, 1990). Similarly, proteasomes degrade ubiquitylated cyclins while sparing the associated cyclin-dependent kinase (Feldman *et al.*, 1997; Skowyra *et al.*, 1997; Koepp *et al.*, 1999). The *cis* requirement in polyUb chain signaling is the key factor that allows the proteasome to remodel the compositions of such multisubunit complexes.

An analysis of chimeric wild-type/L8A Ub_4 molecules shows that two of the four L8 residues in Ub_4 contact proteasomal receptors (Table IIB). In the crystal structure of Ub_4 each of these L8 residues is exposed on the same face of Ub_4, while the other two L8 residues are exposed on the opposite face (Cook *et al.*, 1994; Beal *et al.*, 1996). Our results can be explained if these two faces engage in distinct interactions when Ub_4 is bound to its receptors in the proteasome, suggesting that the conformation of Ub_4 is important for its recognition by proteasomal receptors. Thus, different polyUb chains may act as distinct signals in part because they have distinct conformations. The proteasome-binding properties of linear Ub_5 provide support for this model, but also suggest that this (artificial) chain retains significant proteasomal targeting potential. A more rigorous determination of the proteasomal signaling properties of an alternatively linked chain awaits the availability of a substrate linked to such a chain.

The results shown in Table II also place restrictions on

the properties of authentic polyUb receptors in the 19S complex. A 50-kD protein of the mammalian 19S complex, known as S5a, binds polyUb chains with high affinity when assayed outside the complex (Deveraux *et al.*, 1994). Although many of the polyUb binding properties of S5a mimic those of proteasomes (Beal *et al.*, 1996, 1998), no positional effects were seen in an analysis of the binding of chimeric wild-type/L8A Ub_4 molecules to S5a (Beal *et al.*, 1996). These results contrast with those shown in Table IIB for proteasomes, a divergence that is consistent with the conclusion that S5a is not a major polyUb receptor of the proteasome (van Nocker *et al.*, 1996; Fu *et al.*, 1998).

Unfolding as a barrier to proteasomal degradation

The rate-limiting step in the turnover of proteasome-bound Ub_5DHFR was assigned to unfolding based on the ability of DHFR ligands to decrease k_{cat}, and on the precipitous decline in k_{cat} that was seen upon replacing DHFR with the more complex βGal moiety. In addition, peptide hydrolysis by 26S proteasomes was unaffected by a saturating concentration of Ub_5DHFR (our unpublished data), indicating that UbDHFR-derived material is essentially absent from the hydrolytic active sites at Ub_5DHFR saturation. The substrates used in our work featured a fused Ub moiety that contributed the site for Ub_4 conjugation. This moiety was engineered to resist removal by deubiquitylating enzymes, and was degraded during the proteolysis of Ub_5DHFR. Slow unfolding of the fused Ub moiety may contribute to the low k_{cat} values of the synthetic substrates. However, our data suggest that unfolding of the substrate moiety is also kinetically significant. This conclusion follows from the ability of specific DHFR ligands to retard Ub_5DHFR degradation, and especially from the finding that the two synthetic substrates had different k_{cat} values.

We did not detect degradation of Ub_5βGal by purified 26S proteasomes. In marked contrast to this result, pulse–chase measurements have yielded a half-life of $<$10 min for UbβGal in yeast cells (Johnson *et al.*, 1992, 1995). This apparent discrepancy might be reconciled if a large fraction of pulse-labeled Ub_5βGal molecules are degraded before they are completely folded. However, even folded βGal can be degraded in reticulocyte lysate with a half-life as short as 1 h, despite having to undergo ubiquitylation in addition to proteasomal turnover (Gonda *et al.*, 1989).

The properties of Ub_5βGal indicate that a polyUb chain is not a universal degradation signal, even though it is a universal targeting signal. The slow turnover of this substrate by purified proteasomes can be explained in at least two ways. One possibility is that additional factors sometimes assist proteasomes *in vivo*. Given that unfolding is a kinetically dominant step in turnover, these factors may include molecular chaperones. The 19S complex harbors six subunits belonging to the AAA ATPase family (Glickman *et al.*, 1998; Rubin *et al.*, 1998), but these subunits (and other intrinsic subunits of the 19S complex) are evidently unable to unfold the complex βGal molecule efficiently. Ghislain *et al.* showed that conditional mutation of the yeast *CDC48* gene, which encodes a chaperone of the AAA ATPase family, inhibits UbbGal turnover at a post-ubiquitylation step (Ghislain *et al.*, 1996). Valosin-containing protein (VCP), a mammalian homolog of Cdc48p, also functions at a post-ubiquitylation step in the degradation of IκBα (Dai *et al.*, 1998). Cdc48p/VCP thus represents one candidate for an extraproteasomal chaperone that could facilitate the degradation of folded, polyubiquitylated substrates. However, although VCP is found associated with mammalian proteasomes (Dai *et al.*, 1998), it is unlikely that it contributed significantly to the binding or unfolding of Ub_5DHFR in our assays. This conclusion follows from our finding that k_{cat} and K_M values for Ub_5DHFR were independent of substantial variations in the level of proteasome-associated VCP (our unpublished data). A second way in which the proteolysis of UbβGal (and UbDHFR) could be modulated is through changes in the structure of the polyUb chain. Data presented by Johnson *et al.* suggest that K29 in the fused Ub moiety is the initial site of Ub ligation to UbDHFR, while both K29 and K48 are utilized in UbβGal (Johnson *et al.*, 1995). The presence of K29 linkage(s) may recruit Ufd2p, a novel factor that modulates polyUb chain assembly (Koegl *et al.*, 1999) and is required for UbDHFR proteolysis in yeast cells (Johnson *et al.*, 1995). Ufd2p interacts with Cdc48p (Koegl *et al.*, 1999), suggesting that there could even be a relationship between chain structure and chaperone recruitment.

The efficient binding of Ub_4 to proteasomes raises the question of whether longer chains confer a significant advantage in targeting. It is likely that the principal benefit of lengthening the chain is not to increase the substrate's affinity, but rather to increase its residence time on the proteasome (see Lam *et al.*, 1997b). Once bound to the mammalian 19S complex, chains are subject to the action of the UCH37 deubiquitylating enzyme, which sequentially removes Ubs from the distal chain terminus at a rate that is independent of chain length (Lam *et al.*, 1997a,b). If the substrate is conjugated to Ub_4, trimming the chain by just two Ubs will disrupt the minimal signal, causing a drop in affinity of ~100-fold. The substrate may thus escape degradation. If the substrate is conjugated to Ub_8, trimming the chain by two Ubs will not change its affinity significantly. Our results suggest that the fate of a proteasome-bound substrate may be influenced by kinetic partitioning between deubiquitylation and unfolding. Such partitioning could be made to favor destruction in at least two ways: by lengthening the chain so as to increase the substrate's residence time on the proteasome, or by recruiting a chaperone to increase the rate of substrate unfolding.

Materials and methods

Plasmids and antibodies

pET3a-D77-Ub, pET3a-L8A,K48C-Ub and pET3a-L8A,D77-Ub were generated from pre-existing plasmids by standard procedures (Ausubel *et al.*, 1995). pRS-5Ub-D77, encoding linear Ub_5 with a 77th residue (Asp) in the final repeat, was from K.Wilkinson. pET16b-UbDHFR was generated from pUb^{V76}-V-e$^{\Delta K}$-DHFRha (Johnson *et al.*, 1995) in two steps. The complete insert was cloned into pET16b to introduce an N-terminal polyHis tag, and then the lacI-derived e$^{\Delta K}$ domain was deleted by ligating the small fragment of a *Bgl*II–*Eco*RI digest (encoding DHFRha) into the large fragment from a *Bam*HI–*Eco*RI digest (encoding H_{10}-Ub). In the new fusion protein there is a four-residue linker (GSGI) between Ub and mouse DHFR; Ub retains the G76V mutation which confers resistance to deubiquitylating enzymes. Deletion of the e$^{\Delta K}$ domain enhanced the expression of UbDHFR and improved its solubility. Antibodies were from the following sources: Santa Cruz Biotechnology

(anti-HA and anti-polyHis); Affiniti (anti-p45); and C.-C.H.Li (anti-VCP).

Recombinant proteins

Mutant Ubs were expressed and purified as described previously (Haldeman *et al.*, 1997). Linear Ub_5 was purified by subtractive anion exchange followed by gradient cation exchange. [^{35}S]H_{10}UbDHFR was produced in *E.coli* strain BL21(DE3)pLysS at 37°C. Cells (100 ml) were grown to mid-log phase, washed twice with M9 medium, and resuspended in 50 ml of M9 medium containing 1% glucose, 0.063% methionine assay medium (Difco) and 2 mM FA. After 30 min, isopropyl-β-D-galactopyranoside (IPTG) (0.4 mM) was added; after 30 min more, rifampicin (150 μg/ml) was added. After 30 min more, [^{35}S]methionine (2.5 mCi) was added for 5 min, followed by unlabeled methionine (1 mM) for 10 min more. Cells were harvested, frozen and lysed (Haldeman *et al.*, 1997). The clarified lysate was applied to a 1 ml Ni^{2+}–NTA column and the fusion protein was purified by standard procedures, except that FA (2 mM) was included in all buffers, and bovine serum albumin (BSA) was included as a carrier during elution. [^{35}S]UbDHFR was exchanged into 5 mM HEPES pH 7.3, 0.1 mM EDTA, 1 mM dithiothreitol (DTT). Its concentration was estimated by SDS–PAGE (Coomassie Blue staining) using unlabeled UbDHFR as a standard. [^{35}S]UbβGal was expressed similarly, using pKK-UbGal in JM101 cells (Gonda *et al.*, 1989), and purified by affinity chromatography (Ullman, 1984). G76 of the Ub moiety in UbβGal is followed by Pro to inhibit the activity of deubiquitylating enzymes.

PolyUb chains

K48-linked polyUb chains were assembled using E2-25K, which exclusively recognizes K48 in Ub (Piotrowski *et al.*, 1997 and references therein). All chains carried the K48C mutation in the distal Ub; unanchored chains carried D77 at the proximal terminus. These modifications do not affect chain binding to 26S proteasomes (in comparison to chains carrying K48 and G76; Piotrowski *et al.*, 1997). Ub_4diol was made by using E2-25K to ligate Ub_3 to Ubdiol (from R.Cohen; Lam *et al.*, 1997a); in the case of Ub_4NAL, the substrates were Ub_4 and NAL (0.1 M; Sigma).

Proteasome substrates

[^{35}S]UbDHFR (~4 μM, ~4 × 10^4 d.p.m./pmol) was incubated at 37°C overnight with Ub_4 or Ub_8 (90 μM), E1 (0.1 μM), C170S-E2-25K (15 μM) and yeast ubiquitin hydrolase-1 (YUH-1, 10 μg/ml) (Haldeman *et al.*, 1997). (YUH-1 removes the proximal D77 residue, making Ub_4 competent to be conjugated by E2-25K.) FA (2 mM) was included to stabilize UbDHFR. [^{35}S]Ub_5DHFR and [^{35}S]Ub_9DHFR were purified on Ni^{2+}–NTA resin and concentrated into HEPES buffer (above), except that all buffers contained 0.2 mg/ml BSA rather than FA. This step removed FA and unconjugated Ub_4. [^{35}S]Ub_5βGal was synthesized similarly and affinity purified. As in the synthesis of Ub_5DHFR, UbβGal was quantitatively converted to Ub_5βGal. Unlabeled Ub_5DHFR and Ub_5βGal were synthesized similarly, except on a larger scale. The enzymatic activities of UbDHFR and UbβGal, and of their respective Ub_4 conjugates, were measured as described (Mathews and Huennekens, 1983; Richard *et al.*, 1985). Under standard conditions, UbDHFR and Ub_5DHFR (38 nM) each consumed NADPH at a rate of 5.2 μmol/min, and identical βGal activities were seen for UbβGal versus Ub_5βGal.

Proteasomes

26S proteasomes purified from bovine or rabbit erythrocytes, or rabbit reticulocytes, were used interchangeably (Hoffman *et al.*, 1992). Similar results were obtained with proteasomes purified through the gradient anion exchange step (preparation 1) versus proteasomes subjected to further purification on a glycerol gradient (preparation 2). The concentration of proteasomes in preparation 2 was determined from the total protein concentration assuming a molecular mass of 2.1 MDa. The concentration of proteasomes in preparation 1 was estimated from peptidase activity (based on the specific activity of preparation 2), and confirmed by blotting with anti-p45/S8 antibodies. The two preparations had similar kinetic parameters in assays of Ub_5DHFR degradation.

Proteasome assays

Assays of the degradation of Ub_5DHFR and Ub_5βGal (25 ml, 37°C) contained: 50 mM Tris–HCl pH 7.6, 10 mM $MgCl_2$, 2 mM ATP, a phosphocreatine-based ATP-regenerating system, 10% glycerol, 0.4 mM DTT, 1 μM Ubal (except in Figure 2D) and 2 mg/ml BSA. Concentrations of substrates and proteasomes are given in the legends to figures. Ubal was from R.Cohen (Dunten and Cohen, 1989). Reactions were preincubated for 8 min, initiated with proteasomes, and quenched by adding a reaction aliquot to a tube containing 2 vols of 10 mg/ml BSA, followed by 1 vol. of 40% (w/v) trichloroacetic acid. Degradation did not exceed 7% of input substrate in any reaction except that shown in Figure 2C. Initial rates were usually determined from three time points by least-squares linear regression analyses using Sigmaplot (see Figure 2A). Similar results were obtained using different proteasome preparations (types 1 and 2, above) and multiple preparations of Ub_5DHFR (k_{cat}, K_M, and K_i values varied by no more than 2-fold). Hydrolysis of Suc-LLVY-AMC (0.1 mM, Bachem) was assayed at 37°C in incubations containing 50 mM Tris–HCl pH 7.6, 5 mM $MgCl_2$, 4 mM ATP, 10% glycerol and 1 mM DTT.

Acknowledgements

We thank R.Hofmann for assistance in several experiments, and the following individuals for providing reagents: Y.Lam (purified proteasomes), C.-C.H.Li (anti-VCP antiserum), K.Wilkinson (pRS-5Ub) and R.Cohen (Ubal, Ubdiol). For comments on the manuscript we thank R.Cohen, J.Bender and members of our laboratories. Funded by NIH grants DK46984 (C.P.) and GM37009 (M.R.), and by NCI training grant T32CA09110.

References

Amerik,A.Y., Swaminathan,S., Krantz,B.A., Wilkinson,K.D. and Hochstrasser,M. (1997) *In vivo* disassembly of free polyubiquitin chains by yeast Ubp14 modulates rates of protein degradation by the proteasome. *EMBO J.*, **16**, 4826–4838.

Appleman,J.R., Prendergast,N., Delcamp,T.J., Freisheim,J.H. and Blakley,R.L. (1988) Kinetics of the formation and isomerization of methotrexate complexes of recombinant human dihydrofolate reductase. *J. Biol. Chem.*, **263**, 10304–10313.

Ausubel,F.M., Brent,R., Kingston,R.E., Moore,D.D., Smith,J.A. and Struhl,K. (1995) *Current Protocols in Molecular Biology.* John Wiley and Sons, New York, NY.

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

Beal,R., Deveraux,Q., Xia,G., Rechsteiner,M. and Pickart,C. (1996) Surface hydrophobic residues of multiubiquitin chains essential for proteolytic targeting. *Proc. Natl Acad. Sci. USA*, **93**, 861–866.

Beal,R.E., Toscano-Cantaffa,D., Young,P., Rechsteiner,M. and Pickart,C.M. (1998) The hydrophobic effect contributes to polyubiquitin chain recognition. *Biochemistry*, **37**, 2925–2934.

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

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

Dai,R.-M., Chen,E., Longo,D.L., Gorbea,C.M. and Li,C.-C.H. (1998) Involvement of valosin-containing protein, an ATPase co-purified with IκBα and 26S proteasome, in ubiquitin–proteasome-mediated degradation of IκBα. *J. Biol. Chem.*, **273**, 3562–3573.

Deveraux,Q., Ustrell,V., Pickart,C. and Rechsteiner,M. (1994) A 26S protease subunit that binds ubiquitin conjugates. *J. Biol. Chem.*, **269**, 7059–7061.

Dick,L.R., Moomaw,C.R., DeMartino,G.N. and Slaughter,C.A. (1991) Degradation of oxidized insulin B chain by the multiproteinase complex macropain (proteasome). *Biochemistry*, **30**, 2725–2734.

Dunten,R.L. and Cohen,R.E. (1989) Recognition of modified forms of ribonuclease A by the ubiquitin system. *J. Biol. Chem.*, **264**, 16739–16747.

Feldman,R.M.R., Correll,C.C., Kaplan,K.B. and Deshaies,R.J. (1997) A complex of Cdc4$^-$, Skp1p and Cdc53p/cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sic1p. *Cell*, **91**, 221–230.

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

Finley,D., Sadis,S., Monia,B.P., Boucher,P., Ecker,D.J., Crooke,S.T. and Chau,V. (1994) Inhibition of proteolysis and cell cycle progression in a multiubiquitination-deficient yeast mutant. *Mol. Cell. Biol.*, **14**, 5501–5509.

Fu,H., Sadis,S., Rubin,D.M., Glickman,M., van Nocker,S., Finley,D. and Vierstra,R.D. (1998) Multiubiquitin chain binding and protein

degradation are mediated by distinct domains within the 26S proteasome subunit Mcb1. *J. Biol. Chem.*, **273**, 1970–1981.
Galan,J.-M. and Haguenauer-Tsapis,R. (1997) Ubiquitin Lys63 is involved in ubiquitination of a yeast plasma membrane protein. *EMBO J.*, **16**, 5847–5854.
Ghislain,M., Dohmen,R.J., Lévy,F. and Varshavsky,A. (1996) Cdc48p interacts with Ufd3p, a WD repeat protein required for ubiquitin-mediated proteolysis in *Saccharomyces cerevisiae*. *EMBO J.*, **15**, 4884–4899.
Glickman,M.H., Rubin,D.M., Fried,V.A. and Finley,D. (1998) The regulatory particle of the *Saccharomyces cerevisiae* proteasome. *Mol. Cell. Biol.*, **18**, 3149–3162.
Gonda,D.K., Bachmair,A., Wünning,I., Tobias,J.W., Lane,W.S. and Varshavsky,A. (1989) Universality and structure of the N-end rule. *J. Biol. Chem.*, **264**, 16700–16712.
Gottesman,S., Wickner,S. and Maurizi,M.R. (1997) Protein quality control: triage by chaperones and proteases. *Genes Dev.*, **11**, 815–823.
Groll,M., Ditzel,L., Löwe,J., Stock,D., Bochtler,M., Bartunik,H.D. and Huber,R. (1997) Structure of 20S proteasome from yeast at 2.4 Å resolution. *Nature*, **386**, 463–471.
Haldeman,M.T., Xia,G., Kasperek,E.M. and Pickart,C.M. (1997) Structure and function of ubiquitin conjugating enzyme E2-25K: the tail is a core-dependent activity element. *Biochemistry*, **36**, 10526–10537.
Hershko,H. and Rose,I.A. (1987) Ubiquitin-aldehyde: a general inhibitor of ubiquitin-recycling processes. *Proc. Natl Acad. Sci. USA*, **84**, 1829–1833.
Hershko,A. and Ciechanover,A. (1998) The ubiquitin system. *Annu. Rev. Biochem.*, **67**, 425–480.
Hicke,L. (1997) Ubiquitin-dependent internalization of down-regulation of plasma membrane proteins. *FASEB J.*, **11**, 1215–1226.
Hochstrasser,M. (1996) Ubiquitin-dependent protein degradation. *Annu. Rev. Genet.*, **30**, 405–439.
Hoffman,L., Pratt,G. and Rechsteiner,M. (1992) Multiple forms of the 20S multicatalytic and the 26S ubiquitin/ATP-dependent proteases from rabbit reticulocyte lysate. *J. Biol. Chem.*, **267**, 22362–22368.
Hofmann,R.M. and Pickart,C.M. (1999) Noncanonical *MMS2*-encoded ubiquitin conjugating enzyme functions in assembly of novel polyubiquitin chains for DNA repair. *Cell*, **96**, 645–653.
Jacobson,R.H., Zhang,X.J., DuBose,R.F. and Matthews,B.W. (1994) Three-dimensional structure of β-galactosidase from *E.coli*. *Nature*, **369**, 761–766.
Johnson,E.S., Gonda,D.K. and Varshavsky,A. (1990) *Cis–trans* recognition and subunit specific degradation of short-lived proteins. *Nature*, **346**, 287–291.
Johnson,E.S., Bartel,B., Seufert,W. and Varshavsky,A. (1992) Ubiquitin as a degradation signal. *EMBO J.*, **11**, 497–505.
Johnson,E.S., Ma,P.C.M., Ota,I.M. and Varshavsky,A. (1995) A proteolytic pathway that recognizes ubiquitin as a degradation signal. *J. Biol. Chem.*, **270**, 17442–17456.
Johnston,J.A., Johnson,E.S., Waller,P.R. and Varshavsky,A. (1995) Methotrexate inhibits proteolysis of dihydrofolate reductase by the N-end rule pathway. *J. Biol. Chem.*, **270**, 8172–8178.
Koegl,M., Hoppe,T., Schlenker,S., Ulrich,H.D., Mayer,T.U. and Jentsch,S. (1999) A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly. *Cell*, **96**, 635–644.
Koepp,D.M., Harper,J.W. and Elledge,S.J. (1999) How the cyclin became a cyclin: regulated proteolysis in the cell cycle. *Cell*, **97**, 431–434.
Lam,Y.A., DeMartino,G.N., Pickart,C.M. and Cohen,R.E. (1997a) Specificity of the ubiquitin isopeptidase in the PA700 regulatory complex of 26S proteasomes. *J. Biol. Chem.*, **272**, 28348–28446.
Lam,Y.A., Xu.,W., DeMartino,G.N. and Cohen,R.E. (1997b) Editing of ubiquitin conjugates by an isopeptidase in the 26S proteasome. *Nature*, **385**, 737–740.
Laney,J.D. and Hochstrasser,M. (1999) Substrate targeting in the ubiquitin system. *Cell*, **97**, 427–430.
Löwe,J., Stock,D., Jap,B., Zwickl,P., Baumeister,W. and Huber,R. (1995) Crystal structure of the 20S proteasome from the archaeon *T.acidophilum* at 3.4 Å resolution. *Science*, **268**, 533–539.
Mathews,C.K. and Huennekens,F.M. (1983) Further studies on dihydrofolic reductase. *J. Biol. Chem.*, **238**, 3436–3442.
Mildvan,A.S., Weber,D.J. and Kuliopulos,A. (1992) Quantitative interpretations of double mutations of enzymes. *Arch. Biochem. Biophys.*, **294**, 327–340.
Özkaynak,E., Finley,D., Solomon,M.J. and Varshavsky,A. (1987) The yeast ubiquitin genes: a family of natural gene fusions. *EMBO J.*, **6**, 1429–1439.
Papa,F.R., Amerik,A.Y. and Hochstrasser,M. (1999) Interaction of the Doa4 deubiquitinating enzyme with the yeast 26S proteasome. *Mol. Biol. Cell*, **10**, 741–756.
Pickart,C.M. (1997) Targeting of substrates to the 26S proteasome. *FASEB J.*, **11**, 1055–1066.
Pickart,C.M. (1998) Polyubiquitin chains. In Peters,J.-M., Harris,J.R. and Finley,D. (eds), *Ubiquitin and the Biology of the Cell.* Plenum Press, New York, NY, pp. 19–63.
Pickart,C.M. and Rose,I.A. (1986) Mechanism of ubiquitin carboxyl-terminal hydrolase: borohydride and hydroxylamine inactivate in presence of ubiquitin. *J. Biol. Chem.*, **261**, 10210–10217.
Piotrowski,J., Beal,R., Hoffman,L., Wilkinson,K.D., Cohen,R.E. and Pickart,C.M. (1997) Inhibition of the 26S proteasome by polyubiquitin chains synthesized to have defined lengths. *J. Biol. Chem.*, **272**, 23712–23721.
Rechsteiner,M. (1998) The 26S proteasome. In Peters,J.-M., Harris,J.R. and Finley,D. (eds), *Ubiquitin and the Biology of the Cell.* Plenum Press, New York, NY, pp. 147–190.
Richard,J.P., Westerfeld,J.G., Lin,S. and Beard,J. (1985) Structure-reactivity relationships for β-galactosidase (*Escherichia coli*, lacZ). *Biochemistry*, **34**, 11713–11724.
Rock,K.L., Gramm,C., Rothstein,L, Clark,K., Stein,R., Dick,L., Hwang,D. and Goldberg,A.L. (1994) Inhibitors of the proteasome block degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. *Cell*, **78**, 761–771.
Rubin,D.M., Glickman,M.H., Larsen,C.N., Dhruvakaumar,S. and Finley,D. (1998) Active site mutants in the six regulatory particle ATPases reveal multiple roles for ATP in the proteasome. *EMBO J.*, **17**, 4909–4919.
Shanklin,J., Jabben,M. and Vierstra,R.D. (1989) Partial purification and peptide mapping of ubiquitin–phytochrome conjugates from oat. *Proc. Natl Acad. Sci. USA*, **28**, 6028–6034.
Skowyra,D., Craig,K.L., Tyers,M., Elledge,S.J. and Harper,J.W. (1997) F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin–ligase complex. *Cell*, **91**, 209–219.
Sommer,T. and Wolf,D.H. (1997) Endoplasmic reticulum degradation: reverse protein flow of no return. *FASEB J.*, **11**, 1227–1233.
Spence,J., Sadis,S., Haas,A.L. and Finley,D. (1995) A ubiquitin mutant with specific defects in DNA repair and multiubiquitination. *Mol. Cell. Biol.*, **15**, 1265–1273.
Stammers,D.K., Champness,J.N., Beddell,C.R., Dann,J.G., Eliopoulous,E., Geddes,A.J., Ogg,D. and North,A.C. (1987) The structure of mouse L1210 dihydrofolate–drug complexes and the construction of a model human enzyme. *FEBS Lett.*, **218**, 178–184.
Terrell,J., Shih,S., Dunn,R. and Hicke,L. (1998) A function for monoubiquitination in the internalization of a G protein-coupled receptor. *Mol. Cell*, **1**, 193–202.
Ullman,A. (1984) One-step purification of hybrid proteins which have β-galactosidase activity. *Gene*, **29**, 27–31.
van Nocker,S., Sadis,S., Rubin,D.M., Glickman,M., Fu,H., Coux,O., Wefes,I., Finley,D. and Vierstra,R.D. (1996) The multiubiquitin-chain-binding protein Mcb1 is a component of the 26S proteasome in *Saccharomyces cerevisiae* and plays a nonessential, substrate-specific role in protein turnover. *Mol. Cell. Biol.*, **16**, 6020–6028.
Vijay-Kumar,S., Bugg,C.E. and Cook,W.J. (1987) Structure of ubiquitin refined to 1.8 Å resolution. *J. Mol. Biol.*, **194**, 531–544.
Wilkinson,K.D., Tashayev,V.L., O'Connor,L.B., Larsen,C.N., Kasperek,E.M. and Pickart,C.M. (1995) Metabolism of the polyubiquitin degradation signal: structure, mechanism and role of isopeptidase T. *Biochemistry*, **34**, 14535–14546.

Received October 8, 1999; revised November 4, 1999; accepted November 10, 1999