Rad23 Ubiquitin-associated Domains (UBA) Inhibit 26 S Proteasome-catalyzed Proteolysis by Sequestering Lysine 48-linked Polyubiquitin Chains*

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Most substrates of the 26 S proteasome are recognized only following conjugation to a Lys48-linked polyubiquitin chain. Rad23 is one member of a family of proteins that possesses an N-terminal ubiquitin-like domain (UbL) and a C-terminal ubiquitin-associated domain(s) (UBA). Recent studies have shown that UbL-UBA proteins may shuttle polyubiquitinated substrates to proteasomes. Here we show that contrary to prediction from this model, the effect of human Rad23A on the degradation of polyubiquitinated substrates catalyzed by purified proteasomes is exclusively inhibitory. Strong inhibition is dependent on the presence of both UBAs, independent of the UbL, and can be explained by competition between the UBA domains and the proteasome for binding to substrate-linked polyubiquitin chains. The UBA domains bind Lys48-linked polyubiquitin chains in strong preference to Lys63 or Lys29-linked chains, leading to selective inhibition of the assembly and disassembly of Lys48-linked chains. These results place constraints on the mechanism(s) by which UbL-UBA proteins promote proteasome-catalyzed proteolysis and reveal new properties of UBA domains.

The conserved protein Ub1 becomes covalently linked through its C terminus (Gly76) to lysine residues of substrates destined for degradation by the 26 S proteasome, a 2.5 MDA assembly consisting of a central cylindrical 20 S core complex and two distally positioned 19 S regulatory complexes (1). The 19 S complex recognizes the substrate-linked Ub signal, unfolds the substrate polypeptide, and translocates it into a sequestered active site chamber within the 20 S catalytic complex (2). The Ub-proteasome pathway plays a major role in intracellular regulation through its contribution to the homeostasis of important regulatory proteins (1). Ub also functions as a functionally distinct signal in other intracellular processes, including protein trafficking and DNA damage tolerance (reviewed in Refs. 3 and 4).

In the most frequent mode of targeting to proteasomes, multiple Ubs are chained together through Lys48-Gly76 isopeptide bonds, with the proximal Ub linked to a substrate lysine residue (5, 6). The architecture of this Ub signal appears to be specialized for targeting to proteasomes in vivo, whereas other signal structures may be dedicated to distinct processes (reviewed in Refs. 3 and 4). Although a Lys48-linked chain of at least four Ubs in length is an autonomous signal that affords high affinity for purified proteasomes in vitro (Kd of ~50 nm, Ref. 7), recent studies have suggested that factors extrinsic to the 19 S complex might assist in targeting some polyubiquitinated substrates to proteasomes. Leading candidates for such a role are members of the UbL-UBA protein family, including Rad23/Rhp23 and Dsk2/Dph1.

Individual members of the UbL-UBA family were discovered by virtue of their roles in distinct biological processes, including nucleotide excision repair (Rad23, Ref. 8) and spindle pole body duplication (Dsk2, Ref. 9), whose relationship to protein degradation remains uncertain (see for example Refs. 10–13). All family members have an N-terminal Ub-like (UbL) domain that binds to a specific site in the 19 S complex (14, 15), along with one or more Ub-associated (UBA) domains (16) that bind polyUb chains (and mono-Ub) (10, 17–20). These biochemical properties suggest that a substrate bound to a UBA domain through its polyUb chain could be shuttled to the proteasome by means of the UbL-19 S interaction (15, 17, 19, 21, 22).

Existing biological data both support and contradict this model. For example, the turnover of certain model proteasome substrates is retarded in rad23Δ or dsk2Δ yeast (19, 22, 23). Complementation of this phenotype generally requires that both the UbL and UBA domains be present (17, 21–23). UbL-UBA proteins interact physically with S5a/Rpn10 (24), a subunit of the 19 S complex that is also found outside the 19 S (25). UbL-UBA proteins also interact functionally with S5a/Rpn10, in that deletion of individual UbL-UBA genes together with the S5a/Rpn10 gene results in a synergistic stabilization of certain proteasome substrates (17, 20, 21, 23). However, these interactions, although suggestive, do not lead to a defined functional model, in part because the specific role of S5a/Rpn10 in proteasome-catalyzed proteolysis remains uncertain (see Refs. 25 and 26). Still other findings appear to be inconsistent with a critical role for UbL-UBA proteins in targeting substrates to proteasomes. For example, deletion of individual UbL-UBA genes, even together with the gene encoding Rpn10/S5a, does not inhibit the turnover of naturally short-lived proteins (23). Moreover, overexpression of UbL-UBA proteins inhibits, rather

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The abbreviations used are: Ub, ubiquitin; βGal, β-galactosidase; DHFR, dihydrofolate reductase; DUB, deubiquitinating enzyme; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme, E3, ubiquitin-protein ligase; polyUb, polyubiquitin (refers to a branched, isopeptide-linked ubiquitin chain); UBA, ubiquitin-associated domain; Ubal, ubiquitin aldehyde; UbL, ubiquitin-like domain; GST, glutathione S-transferase; FPLC, fast protein liquid chromatography.
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than stimulates, the turnover of natural and model proteasome substrates in yeast cells (19, 21, 27, 28).

The impact of Ubl-UBA proteins on proteasome function has not previously been examined in a defined biochemical system. Here we report results from two different biochemical systems that are inconsistent with a simple trans-targeting function for Rad23 and by analogy, other Ubl-UBA proteins. We also describe new properties of UBA domains that have a high likelihood to be relevant for the biological function(s) of these domains.

EXPERIMENTAL PROCEDURES

Proteins and Antibodies—Bovine lactalbumin and bovine Ub were from Sigma; UbAl was from BostonBiochem; MG-132 was from Peptides International. Poly(His) antibody (H-15) was from Santa Cruz Biotechnology. Polyclonal Rad23 antisera was from P. Howley (Harvard Medical School, Rad23A) or Affiniti (Rad23B). Rabbit polyclonal antibodies against Ub were produced in rabbits by this group and affinity-purified (29). P. Howley provided plasmids pGEXT2-HHR23A and pGEX-6p-1-HHR23B. Related to the published sequence of human Rad23A (30), the sequence of Rad23 used in this work carries T131A and E150K mutations that do not lie in the Ub or UBA domains. Deleted open reading frames (ORFs) were constructed by PCR and cloned between the BamHI and SacII sites of pGEX4T2 (Amersham Biosciences). All ORFs were verified by DNA sequence analysis. Fusion proteins were expressed in BL21pT2 cells (31), absorbed onto GSH beads, and released by thrombin cleavage or eluted with GSH and cleaved subsequently. In all experiments except Fig. 3, below, Rad23 proteins were purified further on an FPLC Mono Q column (Amersham Biosciences) using a salt gradient appropriate to each protein. Bovine erythrocyte 26 S proteasomes and rabbit reticulocyte fractions were expressed and purified by established methods (33, 35): K29C/K48R; K48R/D77R; K48- and K63-Ub4 were produced by the general method used for K48-Ub, except that the KIAA10-CD/UbcH5A (E3/E2) conjugating system was used in place of E2–25K, and all UbS carried the K48R mutation to force the dual-specificity E3 to use K29 in chain assembly (34). Incubations (pH 8) contained KIAA10-CD, UbcH5A, and E1 at 2, 1, and 0.1 mM, respectively. To reduce Ub consumption via auto-ubiquitination of KIAA10-CD, we preincubated the conjugating enzymes for 20 min with 29 mM lysine-less Ub (31) before initiating chain assembly by adding K48R/D77-Ub and K29C/K48R-Ub (1.17 mM each). After 4 h of incubation (37 °C), enzymes were removed by passage through Q-Sepharose (Amersham Biosciences). K29-UbS was purified on an FPLC Mono S column (Amersham Biosciences) as described (38) and divided into two portions. One aliquot was deblocked at its proximal terminus using yeast Ub hydrolase-1 (38) and then irreversibly blocked at its distal terminus by alkylating Cys48 with iodoacetamide. The second aliquot was deblocked at its distal terminus by alkylating Cys48 with ethyleneimine (Chemservice) (38). These two dimers were conjugated to each other and purified as described for K29-Ub.

Polyubiquitin Chain Binding by GST-Rad23 Pull-down—All UbS molecules used in chain binding had an extra amino acid (Asp) at the proximal chain terminus and carried a blocking modification/mutation at the distal terminus (see preceding paragraph). Each type of Ub was radiiodinated using chloramine T (8–20,000 cpm/mmol). GST-fused Rad23 mutants were bound to GSH beads (10 pmol of protein/μl of beads). After washing with binding buffer (20 mM Tris-HCl, pH 7.6, 50 mM NaCl, 0.1% Nonidet P-40, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride), loaded beads (5 μl) were incubated with 1 μM TR25K, His10-Mms2, —14K, equivalent to yeast Ubr1/ Ubc2), conjugated to Lys48-linked polyUb chains and degraded by proteasomes (5, 32, 36, 39). Previous studies have implicated Ubl-UBA proteins in the turnover of N-end rule E3/E2 complex (E3a/E2–14K, equivalent to yeast Ubr1/ Ubc2), and E3/E2 conjugated to Lys48-linked polyUb chains and degraded by proteasomes (5, 32, 36, 39). Previous studies have implicated Ubl-UBA proteins in the turnover of N-end rule substrates. For example, LeuBgal, which is partially stabilized in dsb2A yeast (19), is polyubiquitinated through the N-end rule pathway (40).

Mammals express two closely related isoforms of Rad23, known as Rad23A and B (30). Rad23B complexes with the nucleotide excision repair factor XPC/Rad4 (30), a function that can probably also be performed by Rad23A (41). We employed human Rad23A in the studies described below, which began with purification of the full-length protein and several truncation mutants (Fig. 1, A and B).

Adding a low concentration of full-length Rad23 (7 μM) to fraction II significantly inhibited lactalbumin degradation (~50%, Fig. 2A) and higher concentrations of Rad23 caused stronger inhibition (see Fig. 3, below). Rad23 failed to stimulate degradation at any concentration tested. The lowest concentration used, 0.1 μM (data not shown), represents an excess of only ~3-fold over the estimated concentration of proteasomes in this...
assay system (36). Thus, it is unlikely that we failed to observe stimulation because of a gross excess of Rad23. An earlier study in unfractionated rabbit reticulocyte lysate attributed proteolytic inhibition by Rad23 to a competition between the UbL domain and substrate-linked polyUb chains for access to proteasomes (24). However, we found that deleting the UbL generated a stronger inhibitor, indicating that the UbL is dispensable for inhibition (R23D, Fig. 2A; see also next paragraph). Degradation in the presence of a saturating concentration of R23D was actually slower than in the negative control without added Ub (Fig. 2A), probably because some of the degradation in the negative control relies on a trace of Ub in fraction II and is thus subject to inhibition by R23D. Deletion analysis revealed that strong inhibition (≥50% at 7 μM) was associated with the presence of two UBAs (Rad23, R23D, UBA1–2 in Fig. 2A). The UBAs, rather than the region between them, mediate inhibition, as shown by comparing the effects of UBA1–2 and ID in Fig. 2A.

Titration analysis showed that R23D was a potent inhibitor, displaying half-maximal inhibition at ∼3 μM (Fig. 2B). We tentatively attribute less potent inhibition by full-length Rad23, versus UbL-deleted dual-UBA variants (Fig. 2A), to an interaction that shields the UBA domains of the full-length protein. (As shown below, inhibition of lactalbumin degradation is probably mainly due to binding of lactalbumin-linked polyUb chains to the Rad23 UBA domains.) Consistent with this model, we found that heat treatment increased the inhibitory potency of full-length Rad23 but had little effect on inhibition by R23D. Rad23 was reported to homodimerize through a UBA-UBA interaction (42), but recent NMR results exclude self-interaction of minimal UBA domains (43). NMR results also exclude self-association of minimal UbL domains (44). Stronger proteolytic inhibition by R23D versus Rad23 (Figs. 2A and 3) is therefore most simply explained by an intra- or intermolecular interaction between the UbL and UBA domains that is disrupted upon heat-

![Fig. 1. Rad23 and mutant proteins. A, domain structures and true molecular masses. B, Coomassie Blue-stained gel. Adjacent lanes show ∼2 and 1 μg, respectively, of the indicated purified proteins (Rad23 and some mutants migrate anomalously in SDS-PAGE).](image1)

![Fig. 2. Potent inhibition of 125I-lactalbumin degradation in reticulocyte fraction II requires both UBA domains of Rad23. A, degradation assays (“Experimental Procedures”) were supplemented with the indicated Rad23 protein (7 μM). Rates of Ub-dependent degradation are expressed relative to the control lacking Rad23. B, concentration dependence of inhibition by R23D.](image2)
ing so as to increase accessibility of polyUb chains to the UBA domains. The proposed conformational transition could also increase accessibility of the UbL, as suggested by the results of Rad23-proteasome interaction studies (15). Further work will be necessary to validate this conformational model.

**Rad23 UBA Domains Inhibit the Degradation of a Polyubiquitinated Substrate by Proteasomes**—In the fraction II system, the extract provides both Ub-conjugating and proteasome activities. Rad23 was previously found to inhibit E2-catalyzed histone polyubiquitination (27), suggesting that UbL-UBA proteins could inhibit proteolysis by inhibiting Ub-substrate conjugation. To rigorously determine if Rad23 inhibits 26 S proteasome activity, we studied the degradation of a polyubiquitinated model substrate by purified proteasomes. The high affinity of Ub5DHFR for proteasomes (K_m ~ 35 nM) is due solely to the binding of its polyUb chain to the 19 S complex (7), where the chain contacts the S6/Rpt5 ATPase (26). The structure of Ub5DHFR resembles that of conjugated forms of UbProGal, a Ub fusion degradation (UFD) substrate whose intracellular degradation depends on the extension of a polyUb chain from either Lys48 or Lys29 of a non-cleavable Ub moiety (45). UbProGal is strongly stabilized in rad23Δ or dsk2Δ yeast (22, 23). We therefore anticipated that a stimulatory effect of Rad23 would be manifested with Ub5DHFR. Instead, full-length Rad23 inhibited the degradation of this substrate in a concentration-dependent fashion (Fig. 4A, lanes 5 and 6 versus 4).

Low levels of Rad23 are found associated with affinity-purified proteasomes from budding yeast (46), consistent with the demonstrated interaction of the Rad23 UBL with the 19 S complex (14, 15). Stimulation of degradation by Rad23 could have been missed in the above described experiment (Fig. 4A) if our purified proteasomes were already saturated with Rad23. However, quantitative Western blotting showed that Rad23A, although detectable in the purified proteasomes, was substantially substoichiometric relative to an integral 19 S subunit, S6/Rpt5 (Fig. 5B, estimated content ~0.3 mol of Rad23A/mol19S), while Rad23B was not detected (data not shown). Therefore failure to observe stimulation by Rad23 is not due to prior saturation of proteasomes with Rad23.

Inhibition by Rad23 could be explained if its UBL binds to the chain receptor of the 19 S complex and prevents access of the DHFR-linked polyUb chain (24). Alternatively, the UBA(s) could prevent substrate access by sequestering the DHFR-linked chain. Failure of the isolated UBL domain to inhibit lactalbumin degradation in fraction II and potent inhibition by the UBL-deleted variant R23D in that system (Fig. 2A) argue in favor of the second model. Also in accord with this model, the isolated UBL did not inhibit Ub5DHFR degradation by purified proteasomes (Fig. 4A, lanes 9 and 10 versus 4). Moreover, excess UbL did not inhibit the cross-linking of a reactive polyUb chain to S6/Rpt5, whereas Ub5DHFR does block cross-linking (26). These results show that the UbL domain binds to a distinct site from that which binds polyUb chains as a prelude to proteolysis. They are consistent with findings that different subunits of the 19 S complex contact Lys48-linked chains (S6/Rpt5) and the Rad23 UBL (S2/Rpn1) (15, 26).

We confirmed that the UBA domains are responsible for inhibition by showing that R23D (lacking the UbL) is a more potent proteasome inhibitor than full-length Rad23 (Fig. 4A, lanes 7 and 8 versus 5 and 6). The same relationship holds for lactalbumin degradation in fraction II (Fig. 2A). Inhibition by R23D was more potent (smaller V_max) at a lower Ub5DHFR concentration, indicative of a competitive effect (Fig. 4B, squares versus diamonds). The qualitative agreement of structure/function results in the two experimental systems (Figs. 2A and 4A) suggests that proteasome inhibition is the main underlying cause of the proteolytic inhibition in fraction II. However, it remains possible that other effects of Rad23 (see below) make a contribution to inhibition in fraction II. The more potent inhibition seen with purified proteasomes (Figs. 2B versus 4B) is likely explained by the high concentration of proteasomes and competing polyUb chains in fraction II (36). Overall, our results show that R23D inhibits proteasomes through UBA-mediated sequestration of substrate-linked polyUb chains, an explanation consistent with the demonstrated ability of UBA domains to bind Lys48-linked chains (17, 19, 20).

**Rad23 UBA Domains Modulate Chain Assembly and Disassembly**—Besides inhibiting proteolysis, overexpression of Rad23 (with or without its UBL) raises the intracellular level of ubiquitinated proteins (20, 21, 27, 28, 47). Similarly, 7 μM R23D, a concentration that completely inhibited lactalbumin degradation (Fig. 2B), strongly increased the level of ubiquitinated lactalbumin in fraction II (Fig. 6A, lane 8 versus 2). The major known in vivo consequences of Rad23 overexpression are therefore recapitulated in fraction II (Figs. 2A and 6A). The presence of R23D also altered the size distribution of conjugates: levels of adducts apparently carrying three to five ubiquitins increased strongly (Fig. 6A, lane 8 versus 2). The level of substrate conjugates reflects the balance between substrate polyubiquitination catalyzed by the relevant E3/E2 complex and conjugate degradation and chain disassembly catalyzed by proteasomes and DUBs, respectively. Although inhibiting pro-

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*Y. Lam and C. Pickart, unpublished data.*
teasomes should raise conjugate levels, the effect of R23D greatly exceeded the effect of a saturating concentration of a proteasome catalytic site inhibitor, MG-132 (Fig. 6A, lane 8 versus 6). The striking effect seen in Fig. 6A (lane 8) suggested that R23D might affect additional steps in the Ub-proteasome pathway.

To test if R23D also inhibited DUB activity, we monitored the disassembly of an unanchored Lys48-linked Ub4 chain (K48-Ub4) in fraction II. This reaction is primarily catalyzed by isopeptidase T/Ubp14 (48). At the concentration employed (4 μM), R23D inhibited Ub4 disassembly significantly (Fig. 6B, lane 8 versus 6, Ub4 band), but less strongly than the specific DUB inhibitor Ubal (2.3 μM; lane 7). Because R23D and Ubal show the opposite relative potency in stabilizing ubiquitinated lactalbumin (lanes 4 and 8 in Fig. 6A versus lanes 7 and 8 in Fig. 6B), we suspect that the DUBs that act on ubiquitinated lactalbumin are more sensitive to R23D than is isopeptidase T. Indeed, Ubal did not further increase the level of ubiquitinated lactalbumin in the presence of R23D (Fig. 6A, lane 10 versus 8), suggesting that the relevant DUBs are already fully inhibited by R23D alone. Note that isopeptidase T acts exclusively on unanchored polyUb chains (48, 49) and so should be irrelevant in the stabilization of ubiquitinated lactalbumin. Our results thus suggest that R23D may inhibit deubiquitination gener-
ally. This could be explained if R23D binds to polyUb chains and hinders their accessibility to DUBs. A similar model was proposed above to explain proteasome inhibition by Rad23 UBA domains (above).

Even when combined, MG-132 and Ubal did not cause the accumulation of specific lactalbumin conjugates (data not shown). We therefore suspected that R23D might also affect polyUb chain extension. To test this possibility we used an E3/E2 complex (34) that assembles unanchored Lys48-linked chains. Addition of R23D to chain assembly assays strongly inhibited the production of chains more than three Ubs in length (Fig. 6C, lanes 1–4 versus 5–8). R23D also abolished the formation of extremely high molecular weight products that result from autopolyubiquitination of the E3 (region marked E3-Ubn in Fig. 6C, lanes 5–8).

We attribute the inhibition of free chain assembly to selective binding of K48-Ub3 and longer chains because when the same E3 was constrained to assemble Lys29-linked chains (through the use of K48R-Ub), the assembly of-unanchored chains was unaffected or even stimulated, as seen most clearly for Ub3–6 in Fig. 6C (lanes 9–12 versus 13–16; note that Lys29-linked chains are synthesized more slowly than Lys48-linked chains). However, auto-polyubiquitination was still abolished when this reaction involved Lys29-linked chains (lanes 13–16).

Three conclusions follow from these chain assembly results. First, it is unlikely that R23D inhibits intrinsic E3 activity, because unanchored Lys48- and Lys29-linked chains are assembled at the same active site (34). Second, R23D apparently does not bind to unanchored Lys29-linked chains. This conclusion was verified by two independent methods as discussed below. Third, unlike inhibition of free chain synthesis, inhibition of autopolyubiquitination is independent of chain linkage and is therefore unlikely to be caused by R23D binding to chains.

Selective binding of R23D to K48-Ub3 and longer chains, along with inhibition of deubiquitination, probably contributes to the stabilization of specific lactalbumin conjugates seen in fraction II (Fig. 6A). Although our results are reminiscent of a finding by Madura and co-workers (27) that Rad23 inhibited E2-catalyzed histone polyubiquitination (27), the chains observed in that study were not Lys48-linked. Thus, our results provide the first direct evidence that UBA domains can modulate both positive and negative reactions of proteolytic signal homeostasis.

**Linkage-specific Binding of polyUb Chains to Rad23 UBA Domains**—Rad23 mutants with two UBA domains inhibited...
Analyte Ub4 molecules were mutants were used to pull down 125I-Ub4 as described under afford stronger binding of K48-Ub4. Immobilized, GST-fused Rad23 determined if this effect can fully explain the stronger proteo-

A similar differential was seen in other studies, in which GST-Rad23 and GST-R23D bound 1.5 7. (Fig. 2A). A similar differential was seen in other studies, in which GST-UBA1 and GST-UBA2 (data not shown). We did not detect binding of Ub1 (data not shown, but see Fig. 7C). These results confirm the established ability of a single UBA domain to bind a Lys48-linked chain (17) and suggest for the first time that two UBA domains may afford more robust binding. It remains to be determined if this effect can fully explain the stronger proteolytic inhibition caused by versions of Rad23 with two UBA domains (Fig. 2A).

Although Lys48-linked chains are the principal proteasomal targeting signal (5, 6), Lys29-linked chains have been implicated in the proteasomal degradation of UFD substrates (45). Lys63-linked chains, in contrast, appear to be non-proteolytic signals (3, 50–53). However, the molecular determinants of linkage specificity in polyUb chain signaling remain obscure. To determine if UBA domains have potential to regulate signaling by noncanonical chains, we assembled K29- and K63-Ub4 and tested these chains for binding to immobilized GST-UBA1–2 protein. Remarkably, the canonical chain was strongly favored in its binding (Fig. 7B). Similar results were obtained in assays with GST-fused versions of R23D, UB2, and UbL-UBA1 (data not shown), indicating that this linkage specificity is intrinsic to each UBA domain of Rad23.

Additional lines of evidence confirm this linkage specificity of binding. First, R23D-dependent inhibition of Ub4 disassembly was limited to the Lys48-linked tetramer (Fig. 6B, lanes 4 and 12 versus 8). Since all three chains can be disassembled by isopeptidase T at the same active site,3 linkage-specific inhibition is most simply explained by selective binding of R23D to the K48-Ub4 substrate. Second, R23D strongly inhibited the assembly of Lys48-linked chains of n > 3, but had very little effect on the assembly of chains linked through Lys29 or Lys63 (Fig. 6C, lanes 13–16 and 21–24 versus respective controls). Finally, direct assays of binding by surface plasmon resonance confirmed that K48-Ub4 was strongly preferred by R23D over K63/K29-Ub4, although significant K63-Ub4 binding was detectable by this more sensitive method (Fig. 7C).

**DISCUSSION**

**Effects of Rad23 on Proteasome-catalyzed Proteolysis—UbL-UBA proteins can play a positive role in proteasome-catalyzed degradation (19, 22, 23), but their mechanism of action remains poorly defined. An attractive model postulates that UbL-UBA proteins shuttle polyubiquitinated substrates to proteasomes by using their UbL and UBA domains to interact with the 19 S complex and poly(Ub) chains, respectively. The current work represents the first biochemical test of this hypothesis.**

A principal finding of this work is that the influence of purified Rad23 on proteasome activity *in vitro* is exclusively inhibitory (Figs. 2 and 4), reflecting a competition between the Rad23 UBA domains and the proteasome’s polyUb receptor for substrate-linked chains. Our results show that the binding of poly(Ub) chains to UBA domains can also inhibit other chain recognition events, leading to defects in the assembly and disassembly of substrate-linked chains (Figs. 4 and 6). Since substrate ubiquitination and deubiquitination (54, 55) are both required for degradation, these effects may also contribute to the proteolytic inhibition seen when Rad23 is overexpressed in yeast cells. Indeed, a recent study attributed the Rad23-mediated stabilization of an unstable variant of Rad4/XPC to blockade of ubiquitination (56). However, overexpression effects may also depend on yet to be determined UbL-UBA properties, as suggested by the results of a recent suppression analysis. 

Furomaki et al. (19) found that some mutations which suppressed the growth inhibitory effects of Dsk2 overexpression mapped to the Pre2 catalytic subunit of the 20 S complex and the Rpn1 subunit of the 19 S complex. If the toxicity of Dsk2 overexpression was due solely to proteasome inhibition, then suppressing mutations might be expected to increase proteasome activity. Instead, each of the suppressing mutations crippled Leu4Gal turnover much more severely than did overexpression of Dsk2 (19). Thus, UbL-UBA overexpression probably

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3 C. Tsui and C. Pickart, unpublished data.
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In vitro results are difficult to reconcile with this simple model as well. In particular, it does not easily accommodate functional specificity of different UbL-UBA family members, but Rad23 and Dsk2 are both needed for UbβGal turnover in budding yeast (22, 23). Nor does a simple trans-targeting model explain the strong functional interaction between S5a/Rpn10 and Ubl-UBA proteins (17, 20, 21, 23), particularly given the lack of evidence that S5a/Rpn10 plays a major role in polyUb chain recognition by proteasomes (25, 26).

How do Ubl-UBA proteins promote substrate proteolysis? Further work will be necessary to answer this question. The phenotypes of single-gene knockouts are rather substrate-specific: among model substrates, UbProβGal is strongly affected, LeuβGal is less affected, and naturally short-lived proteins, when assayed in bulk, are unaffected (19, 22, 23). Yet all of these turnover events depend, in whole or in part, on Lys48-linked chains. Functional distinctions among different substrates could be explained if yet unknown determinants (besides the substrate-linked polyUb chain) influence substrate interactions with Ubl-UBA proteins. The possibility of additional interactions is suggested by the diverse set of Rad23-interacting proteins already identified, including XPC/Rad4 (30), the HIV-Vpr protein (57), a cytosolic protein deglycosylase (58), and a DNA glycosylase (59). Alternatively, events that cannot yet be recapitulated in vitro, such as a post-translational modification, may be important for the proteolysis-promoting effects of Ubl-UBA proteins. Nor can it be excluded that these proteins may stimulate proteolysis by modulating ubiquitination or deubiquitination (above). Finally, Ubl-UBA proteins could promote the proteolysis of certain substrates by inhibiting the proteolysis of other substrates. One such model is suggested by the involvement of Lys29 linkages in UFD substrate turnover (3, 45, 60) and the inability of the Rad23 UBA domains to bind Lys48-linked chains. Our findings suggest that Rad23 will neither inhibit the assembly of Lys48-linked chains nor hinder the possible interaction of these chains with proteasomes. Thus, any substrates targeted to proteasomes via Lys48-linked chains should be immune to the inhibitory effects discovered in the present work. Moreover, by sequestering substrates conjugated to Lys48-linked chains, proteasome-bound Rad23 could indirectly promote the proteolysis of substrates conjugated to Lys48-linked chains. However, although certain UFD substrates are targeted by polyUb chains initiating at Lys29 (45), it is not yet known if Lys29 linkages are important for targeting to proteasomes, versus other interactions (60).

Linkage-specific PolyUb Chain Binding by Rad23 UBA Domains—Our results provide the first rigorous evidence that UBA domains can bind polyUb chains in a linkage-dependent manner, despite a previous report that the UBA domain of Dsk2 binds Lys48-linked chains preferentially (19). Funakoshi et al. (19) found that overexpression of K29R- or K63R-Ub in S. cerevisiae did not inhibit the binding of endogenous ubiquitinated proteins to a GST-UBA fusion protein, whereas overexpression of K48R-Ub did inhibit. However, because Lys63-linked chains constitute only a few percent of the chains in budding yeast and endogenous Lys29-linked chains have never been detected (52, 53), dimming reduction of alternatively-linked chains in this experiment would have been impossible to detect against the high background of bound Lys48-linked chains. Thus, the previous results (19) show only that Lys63-linked chains are competent in binding.

Our conclusion that Rad23 does not bind Lys29-linked polyUb chains contradicts another recent report (22). Rao and Sastry (22) detected a yeast two-hybrid interaction between Ub and each UBA domain of Rad23. The interactions were abolished by G76V or K29R/K48R mutations in the Ub bait, effects that were interpreted to indicate that interaction relied on assembly of the bait Ub into a chain through either Lys29 or Lys48. It is possible that interactions in this system may have relied on an unidentified cellular factor(s); or the mutational effects could have reflected structure/function properties of conjugating/deconjugating enzymes as opposed to the chemical structure(s) of chain products. The rigorously established linkages of the Ub molecules used in the present work provide high confidence in our conclusion that Lys29-linked chains have negligible affinity for the Rad23 UBA domains.

The UBA domain is the first protein element found to interact with one type of polyUb chain in preference to another. In view of increasing evidence that different polyUb chains can act as functionally distinct signals (3, 45, 50–53), the molecular basis of this preference is of significant interest. Mueller and Feigon (43) suggested that a hydrophobic surface patch of the UBA interacts with a hydrophobic surface patch of Ub. If this model is correct, then the patches must be differentially accessible in different chains. Alternatively, the selective interaction of Rad23 UBAs with Lys48-linked chains could rely on contacts at the Ub-UBA junctions or with conformationally-induced determinants (not necessarily the hydrophobic patch) that are unique to these chains. A recent NMR analysis provided evidence that Lys48-linked chains can adopt specific conformations in solution (61). Whether or not this property proves relevant to the interaction specificity seen here, selective binding to UBA domains appears likely to contribute to linkage specificity in polyUb chain signaling.

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