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Rad23 and Rpn10 Serve as Alternative Ubiquitin Receptors for the Proteasome*

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The selective recognition of ubiquitin conjugates by proteasomes is a key step in protein degradation. The receptors that mediate this step have yet to be clearly defined although specific candidates exist. Here we show that the proteasome directly recognizes ubiquitin chains through a specific subunit, Rpn10, and also recognizes chains indirectly through Rad23, a reversibly bound proteasome cofactor. Both binding events can be observed in purified biochemical systems. A block substitution in the chain-binding ubiquitin interacting motif of RPN10 when combined with a null mutation in RAD23 results in a synthetic defect in protein degrada-
tion consistent with the view that the direct and indirect recognition modes function to some extent redundantly in vivo. Rad23 and the deubiquitinating enzyme Ubp6 both bind proteasome subunit Rpn1 through N-terminal ubiquitin-like domains. Surprisingly, Rad23 and Ubp6 do not compete with each other for proteasome binding. Thus, Rpn1 may act as a scaffold to assemble on the proteasome multiple proteins that act to either bind or hydrolyze multiubiquitin chains.

The breakdown of ubiquitin-protein conjugates by the proteasome is a major mechanism for biological regulation in eukaryotes (1). The conjugative and degradative machineries are coupled at the ubiquitin chain recognition step. This step, although intensively studied, has remained problematic (1–26). There is currently no general agreement as to the identity or multiplicity of the relevant ubiquitin receptors nor as to whether such receptors are intrinsic proteasome subunits or “shuttling” factors that bind ubiquitinated species and subsequently deliver them to the proteasome.

Among the putative shuttling factors having affinity for both the proteasome and ubiquitin conjugates is a family of proteins exemplified by the Rad23 protein of Saccharomyces cerevisiae (27, 28). Proteasome binding by Rad23-like proteins involves their N-terminal ubiquitin-like (UBL)1 domains (8–19), whereas ubiquitin chain binding is mediated by ubiquitin-associated (UBA) domains (9, 15, 17, 20–23) (see Fig. 1A). The UBL domain of Rad23 binds proteasomes with a higher affinity than does the full-length protein (10), and for the human Rad23 homolog hHR23a binding of ubiquitin via the UBA domain disrupts the intramolecular interactions between the UBL and UBA domains (18). In vivo, modulating the interactions between the UBL and UBA domains might serve to potentiate the binding of the Rad23-conjugate complex to the proteasome.

Despite the logical appeal of the shuttling hypothesis, it remains controversial. A positive role for UBL-UBA proteins in degradation is supported by the observations that some model substrates are stabilized in yeast cells lacking Rad23 and in cells lacking Dsk2, another UBL-UBA family member (9, 17, 29). Additional, depletion of hHR23 by small interfering RNA has been shown to lead to the stabilization of p53 in mammalian cells (30). At variance with a role for Rad23 and similar molecules in promoting degradation is the finding that the influence of Rad23 on the in vitro proteasomal degradation of artificial and native substrates appears to be exclusively inhibitory (25, 30). In addition, Rad4 (a Rad23-binding protein) is destabilized in rad23Δ cells, although this has been attributed to Rad23-dependent inhibition of Rad4 ubiquitination (31, 32). When overexpressed, Rad23 stabilizes Pds1 and model proteasome substrates (33, 34) and causes the accumulation of undefined ubiquitinated species (21). However, such overexpression results do not necessarily imply that Rad23 antagonizes protein degradation when present at wild-type levels.

The capacity of proteasomes to recognize substrates directly through covalently attached ubiquitin chains has long been known (4, 35). S5a was the first proteasome subunit implicated in conjugate binding (2). However, cells lacking Rpn10, the yeast ortholog of S5a, proved to be viable, implying that other ubiquitin receptors must exist (3). rpn10Δ cells are slightly sensitive to canavanine, an arginine analog whose toxicity is typically enhanced in proteolysis-deficient mutants (3). Surprisingly, the canavanine sensitivity does not map to the ubiquitin interacting motif (UIM) but rather to the von Willebrand factor type A domain (36), which is required for proteasome stability under conventional purification conditions (37) (see Fig. 1A). This result and the observation that free Rpn10 cross-links to ubiquitin chains whereas proteasome-associated Rpn10 does not have led to the view that Rpn10 is not a true proteasomal ubiquitin receptor (6).

Recently, rpn10Δ proteasomes were purified in an intact state and found to be partially defective in conjugate recognition (10). However, the participation of the UIM in conjugate recognition has remained an open question especially as it cannot be excluded that rpn10Δ proteasomes (even when in-associated; UIM, ubiquitin interacting motif; GST, glutathione S-transferase; suc, succinyl; AMC, 7-amido-4-methylcoumarin.)
tact) have structural defects that could affect chain recognition by other proteasome subunits. Paralleling the uncertainty over chain binding by the UIM element in the proteasome in vitro is the question of whether it has a positive role in protein degradation in vivo. These issues are addressed below. Using purified systems, we provide evidence that proteasomes recognize ubiquitin chains directly via the UIM element of Rpn10 but also indirectly through association with UBL-UBA proteins. Furthermore, in vitro experiments identify specific genetic interactions between the rpn10-uid mutation and deletions of either RAD23 or DSK2, supporting the model that both direct and indirect pathways exist for chain recognition by the proteasome.

EXPERIMENTAL PROCEDURES

Strain Construction—Yeast strains were constructed according to standard methods and are listed in Table I. For the preparation of the rpn10-uid and control strains, fragments were excised from pEL25e and pEL21h, respectively, and transformed into yeast. To prepare these plasmids, the 1.2-kb EcoRI-BamHI fragment from pAG25 (38) bearing the natMX cassette was cloned into pBluescript SK+ (+). A 640-bp fragment corresponding to the RPN10 open reading frame beginning 85 bp past the start codon and ending 64 bp past the stop codon was cloned upstream of the natMX cassette. To mutate the mutant allele, bases 674–698 were substituted with 5′-GAGCGGCA AAC AAT AAT AAC-3′ corresponding to a change at residues 228–232 from LA-MA to NNNNN and generating a silent BamHI site upstream of the natMX cassette. This fragment overlapped precisely with the 3′ end and stop codon of the adjacent PPX1 gene. Integrants were verified by PCR, and the presence of the mutation was determined by restriction mapping.

For the preparation of rad23::kanMX, dsk2::klTRP1, and ubp6::uraMX deletions, precise substitutions of the open reading frames were made with the markers indicated (39, 40). Integration was accomplished by suc-LLVY-AMC hydrolysis as described previously (10).

Proteasome Purification—For the native gel electrophoretic mobility assays, wild-type and rpn10-uid proteasomes were purified from SY73 and SY74 essentially as described and carried out in the absence of ATP (41). For the competition assays, core particle and proteasome lacking Ubp6 were purified in the presence of ATP from SDL135 and SDL145, respectively (41). For the endogenous conjugate association assay, proteasome was purified from SY290b, SY293e, SY294b, and SY296a. Purifications were carried out in the presence of 1 mm ATP, and salt was omitted from the wash.

Competition Assays—IgG resin was charged with proteasome and core particle as described above and resuspended as a 25% slurry in purification buffer. An aliquot was removed, treated with tobacco etch virus protease, and the protein concentration was measured and used to calculate the quantities present on the resin. Bovine serum albumin was added to the slurries at 0.5 mg/ml. Aliquots of 400 μl were mixed with 25-μl mixtures containing 10 pmol of radiolabeled Rad23 (10,000 cpm/pmol) and cold competitor as indicated. After 15 min of binding at 30 °C, bound material was quantified by scintillation counting.

Canavanine Sensitivity—Strains SY304 through SY307 and SY316 through SY310 were grown overnight, diluted 25- or 50-fold, grown 4 h at 30 °C, diluted to an A650 of 0.2, prepared as 3-fold serial dilutions, and spotted on plates. Plates lacking and containing canavanine sulfate were grown for 3 and 4 days, respectively, at 30 °C.

RESULTS

Docking of Ubiquitin Conjugates to the Proteasome by Rad23—One possible argument against the shuttling hypothesis is that the proteasome has a high intrinsic affinity for conjugates (43), and thus, its affinity does not need to be strengthened by extrinsic factors. We recently developed a direct assay for ubiquitin conjugate binding by the proteasome, employing non denaturing gel electrophoresis followed by proteasome visualization via an in-gel activity stain (10). We have used in vitro synthesized conjugates (Fig. 1B) in this assay to test whether Rad23 can promote the binding to proteasomes of
conjugates that would otherwise not show stable binding. As shown in Fig. 1C, complexes of low electrophoretic mobility can be formed when Rad23, the proteasome, and ubiquitin conjugates are mixed. All three components must be added for the low mobility species to form, indicating that the electrophoretically retarded species is a ternary complex as further verified below. Formation of the retarded ternary complex requires the UBL domain of Rad23 (Fig. 1C) as well as the UBA-containing C-terminal domain (Fig. 1D). Additionally, if the N-terminal UBL domain of Rad23 and the C-terminal UBA domain are added as separate polypeptides to the reaction, the retarded complex does not form (Fig. 1E). This result indicates that ternary complex formation is critically dependent on the ability of Rad23 to bind the proteasome and ubiquitin conjugates simultaneously as expected if conjugates are joined to proteasomes through a Rad23 linker. In the absence of a linked UBL domain, the C-terminal domain effectively competes with proteasomes for ubiquitin conjugate binding (Fig. 1, C–E, and data not shown).

In both S. cerevisiae and Schizosaccharomyces pombe, proteasome subunit Rpn1 docks Rad23 onto proteasomes through recognition of its UBL domain (10, 11, 14). Interestingly, another proteasome-associated protein, deubiquitinating enzyme Ubp6, also binds proteasomes by virtue of an interaction between its UBL domain and Rpn1 (41). Affinity-purified proteasomes are heavily if not stoichiometrically loaded with Ubp6 (41). These data suggest that Rad23 and Ubp6 may compete with each other for binding to proteasomes and that only a minor fraction of proteasomes (those lacking Ubp6) therefore may be available for conjugate docking via Rad23. To test this scenario, the binding of 32P-labeled Rad23 to immobilized proteasome was measured in the presence and absence of unlabeled Ubp6. As seen in Fig. 2, Ubp6 has no influence on Rad23 binding. Thus, although Rad23 and Ubp6 both bind proteasomes via their UBL domains, they do not displace distinct binding sites on Rpn1. To confirm this conclusion, we carried out similar binding assays using GST-Rpn1 as the immobilized ligand. As seen for intact proteasomes, Rad23 and Ubp6 bound Rpn1 independently (data not shown). By simultaneously binding two UBL proteins that are active on ubiquitin chains, Rpn1 may play a critical scaffolding role in the proteasome.

Previously we localized the Rad23 binding site in Rpn1 using a series of deletions constructed from a GST-Rpn1 fusion (10). When analogous experiments were carried out with Ubp6, the Rpn1 segments that had shown specific and essentially wild-type affinity for Rad23 (10) showed little or no specific binding to Ubp6 despite the fact that the binding of Ubp6 to full-length Rpn1 is quite robust as compared with Rad23 (data not shown). These findings are in agreement with the proposal of a distinct, noncompetitive binding mode for Ubp6. However, it has not been possible to localize the Ubp6 binding site on Rpn1, so it remains unclear whether the minimal binding sites for Rad23 and Ubp6 are non-overlapping in sequence.

Docking of Ubiquitin Conjugates to the Proteasome by Rpn10—Rpn10 exists in both proteasome-bound and free forms (3, 44, 45). It is well recognized that free Rpn10 can bind ubiquitin chains and that its UIM motif is essential for this property (5, 46). However, the capacity of proteasomal Rpn10 to bind ubiquitin chains is controversial. In particular, proteasome-associated S5a does not cross-link to ubiquitin chains (6). Although proteasomes purified from rpn10 deletion mutants

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**Table II**

| Conjugates | Figs. | Cdc34 (Type) | Cdc34 | Ubiquitin |
|-----------|------|-------------|-------|-----------|
| Long      | 1, 3B | His<sub>6</sub> | 4     | 50        |
| Short     | 1    | His<sub>6</sub> | 8     | 20        |
| Long      | 3A   | Native      | 4     | 50        |
| Medium    | 3A   | Native      | 8     | 50        |
| Short     | 3A   | Native      | 12    | 50        |

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**Fig. 1.** A ternary complex of Rad23, ubiquitin conjugates, and the proteasome. A, schematics of Rad23 and Rpn10. The Rad23 domains shown include the UBL and UBA domains. The Rpn10 domains shown include the von Willebrand factor type A domain and the UIM. B, Cdc34-ubiquitin conjugates. Long (L) and short (S) conjugates were synthesized as described under “Experimental Procedures,” resolved by 9% SDS-PAGE, and probed with anti-His antibody. C, electrophoretic mobility shift assay detects a proteasome complex dependent on both Rad23 and ubiquitin conjugates. 2 pmol of proteasomes were incubated with either 8 pmol of ubiquitinated His<sub>6</sub>-Cdc34 (long conjugates), Rad23 (full-length or truncated; pmol indicated in figure), or both. Complexes were resolved by native PAGE and visualized by succinyl-L-Leu-Leu-Val-Tyr p-nitroanilide hydrolysis. D, formation of the ternary complex requires both the UBL and UBA domain of Rad23. 2 pmol of proteasomes were incubated with 8 pmol ubiquitinated His<sub>6</sub>-Cdc34 (long conjugates), Rad23 (full-length or truncated; pmol indicated in figure), or both. Complexes were resolved by native PAGE and visualized by succinyl-L-Leu-Leu-Val-Tyr p-nitroanilide hydrolysis. E, ternary complex formation requires linkage between the UBL domain of Rad23 and the UBA domain-containing C-terminal segment. 2 pmol of proteasomes were incubated with either 8 pmol of ubiquitinated His<sub>6</sub>-Cdc34 (long conjugates), various species of Rad23 (as indicated; numbers indicate pmol), or both. Complexes were resolved by native PAGE and visualized by succinyl-L-Leu-Leu-Val-Tyr p-nitroanilide hydrolysis.
are defective in chain binding (10), rpn10 null mutants have a complex phenotype, including multiple defects that do not map to the chain-binding (UIM) motif(5). Therefore we introduced a 5-residue block substitution into the core of the UIM domain (LAMAL → NNNNN) of Rpn10 to test the role of Rpn10 in conjugate recognition in the context of the proteasome. This mutation was integrated into the natural RPN10 chromosomal locus to achieve a physiological expression level in each cell. When proteasomes purified from this strain were assayed for chain binding, they showed a clear deficiency (Fig. 3A). These data indicate that the UIM motif of Rpn10, like UBL-UBA proteins, can function to dock ubiquitin conjugates onto the proteasome.

One potential role of the Rpn10 UIM motif is to cooperate with Rad23 in the docking of conjugates onto the proteasome (see for example Ref. 21). However, Rad23-dependent docking of conjugates at the proteasome was not significantly impaired when rpn10-uim proteasomes were used (Fig. 3B).

Docking of Endogenous Ubiquitin Conjugates to the Proteasome by Rad23 and Dsk2—The shuffling hypothesis predicts that the co-purification of endogenous conjugates with the proteasome should be at least partially dependent on Rad23 and Dsk2. To test this hypothesis, proteasomes were purified using affinity tags and under mild conditions from cells lacking Rad23 and Dsk2 and probed with anti-ubiquitin antibody. Proteasome-associated conjugates were strongly reduced in rad23 dsk2 double mutants (Fig. 4), but this difference is not accounted for by a decreased level of conjugates in the extract (Ref. 15 and data not shown). Because the Rpn10 UIM was implicated in conjugate association in Fig. 3A, proteasomes purified from the rpn10-uim strain were also tested for the association with endogenous ubiquitin conjugates. Mutation of the UIM of Rpn10 did not cause a notable decrease in the level of associated conjugates (Fig. 4). This observation, coupled with the strong dependence of conjugate association on Rad23 and Dsk2, supports a model in which the UBL-UBA proteins dock ubiquitin conjugates onto the proteasome independently of the Rpn10 UIM motif and are more active in conjugate delivery than is Rpn10.

**Rad23, Dsk2, and Rpn10-UIM Are Required for Resistance to Canavanine**—The arginine analog canavanine has been widely used to probe for proteasome defects. Proteins synthesized in the presence of canavanine are prone to misfolding and preferentially ubiquitinated, thereby placing greater demands on the proteasome. Cells lacking Rpn10 have a mild sensitivity to canavanine, but the canavanine sensitivity derives from the loss of the von Willebrand factor type A domain of RPN10, which is required for proper proteasome assembly (36). rpn10-uim mutants show a wild-type level of sensitivity to canava-
rpn10-uim, subtlety of the vivo (3, 5). We have suggested the alternative view that the factors (5, 7). The substantial defect in chain binding by purified proteasomes (Fig. 4) argues strongly in favor of this model. One possible explanation of the failure of proteasomal Rpn10 to cross-link to ubiquitin chains is that Rpn10-bound chains may preferentially cross-link to a neighboring subunit in the particle.

Having confirmed that the UBL-UBA proteins function as proteasomal ubiquitin receptors, we were able to test the possibility that the functions of the Rpn10-UIM element in vivo are essentially masked by the presence of redundant chain-binding factors such as Rad23 and Dsk2. The model predicts strong synthetic interactions between the rpn10-uim mutation and UBL-UBA deletion mutations, which were indeed observed (Fig. 5). There are several previous reports of genetic interactions between rpn10 deletion mutations and UBL-UBA null mutations (15, 21, 29), but these do not address the model persuasively because the major phenotypic effects of rpn10 null mutations (including the canavanine sensitivity phenotype) do not map to the UIM element.

The relationship between the two chain-docking mechanisms described in this study remains largely undefined, although it seems clear that the UBL-UBA mechanisms are more active and that the two docking modes are not acting predominantly in concert. This latter conclusion may seem contradictory to that of Chen and Madura (21), who observed that overexpression of Rad23 leads to the binding of ubiquitin conjugates to the proteasome in wild-type but not in rpn10Δ cells (21). Most likely, in this respect the rpn10Δ mutation does not mimic rpn10-uim, and the earlier data, although appearing to point to a key role for joint chain recognition by Rpn10 and Rad23, actually reflect roles of Rpn10 that are distinct from its chain binding role.

A major question is whether different physiological substrates have varying receptor preferences, and if so what features of substrates might predispose them to a particular docking mode. Thorough analysis of protein turnover in rad23 and rpn10-uim mutants will be necessary to understand the biological significance of multiple ubiquitin receptors for the proteasome. Second, it is unclear whether the detailed fate of ubiquitin conjugates arriving at the proteasome depends on the nature of the receptor that has docked them. A final area in need of additional study is the likely role of Rad23 and Dsk2 in modulating the ubiquitin-proteasome pathway at steps other than the docking of conjugates to the proteasome. The UBL-UBA proteins may serve as inhibitors of both ubiquitination and deubiquitination (24, 25, 30, 34), and how these activities relate to conjugate docking onto proteasomes is so far unexplored.

Yeast cells in which chain recognition by Rpn10, Rad23, and Dsk2 is lacking remain viable and thus competent for ubiquitin conjugate degradation, at least to some extent (this work and Ref. 15). Thus, it is nearly certain that the proteasome employs one or more additional ubiquitin receptors. Candidates include Ddi1 (14), an additional member of the UBL-UBA family, and S6/Prt5, an ATPase subunit of the proteasome that cross-links to ubiquitin chains (6).

Although purified proteasomes are competent to degrade some model substrates, their activity on other ubiquitin conjugates is not as robust as expected from in vivo data (see for example Ref. 43). These and other data suggest that proteins that are not integral subunits of the proteasome may play significant roles in postconjugative steps in the pathway. Many such proteins apparently associate reversibly with the proteasome and are absent from conventionally purified proteasomes because they are removed by routinely performed high-salt washes (41). UBL domains provide one common mechanism for such associations. An example is Ubp6. The majority of protea-

Fig. 5. rpn10-uim confers synthetic sensitivity to canavanine when combined with either rad23Δ or dsk2Δ. Strains were grown to log phase in YPD (1% yeast extract, 2% Bacto Peptone, 2% glucose) and normalized with respect to optical density, and 3-fold serial dilutions were prepared. Cells on synthetic dextrose (SD) and canavanine plates were grown at 30 °C for 3 and 4 days, respectively.

DISCUSSION

Our results establish that at least two mechanisms can be used by proteasomes to recognize ubiquitin chains. Although Rpn10 and Rad23 previously have been shown to bind ubiquitin chains, their capacity to dock chains onto the proteasome has been challenged (25) and has not been demonstrated in a defined in vitro system. One view is that Rad23 and the proteasome can only compete for chain binding, and observations supporting this view have been reported (25). However, our experiments clearly show that Rad23 can enhance chain binding by purified proteasomes. Although previous attempts have not revealed this, our use of nondenaturing gel electrophoresis to assay binding in addition to favorable ratios of Rad23 to proteasome, which preclude inhibition caused by titration effects, are possibly key experimental differences. We also find that ubiquitin chains co-purify with proteasomes, and consistent with the in vitro binding data, co-purification is principally dependent on Rad23 and Dsk2. These results imply that UBL-UBA proteins dock conjugates onto the proteasome not only in vitro but in vivo as well.

The argument against Rpn10 functioning in chain recognition has been based partly on the lack of cross-linking of proteasome-bound chains to Rpn10 (6). This led to the model that the UIM element of Rpn10 is inaccessible within the proteasome (6). In support of this view, the rpn10-uim mutant does not appear to have strong general effects on protein turnover in vivo (3, 5). We have suggested the alternative view that the subtlety of the rpn10-uim phenotype may reflect that the ubiquitin receptor function of Rpn10 overlaps with that of other factors (5, 7). The substantial defect in chain binding by purified rpn10-uim proteasomes (Fig. 4) argues strongly in favor of this model.
somes appear to be loaded with Ubp6 in vivo through an association mediated by recognition of its UBL domain by subunit Rpn1 (41). The ability of UBL-UBA proteins to dock conjugates onto the proteasome could in principle be severely limited by the occupancy of the UBL binding site of Rpn1 by Ubp6. Here we show that Rpn1 has two distinct binding sites for UBL domains, allowing it to dock both Rad23 and Ubp6 simultaneously. The high level of competition between these two proteins appears to be brought into proximity by Rpn1. One implication of this scaffolding function of Rpn1 is that chains docked to the proteasome through Rad23 may be preferential targets of the deubiquitinating activity of Ubp6. Moreover, the spatial organization of factors that recognize and metabolize ubiquitin chains on Rpn1 may help to present the non-ubiquitin components of conjugates to the unfolding machinery of the proteasome, which appears to reside, like Rpn1, in the proteasome base.

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