Proteasome subunit Rpn1 binds ubiquitin-like protein domains

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The yeast protein Rad23 belongs to a diverse family of proteins that contain an amino-terminal ubiquitin-like (UBL) domain. This domain mediates the binding of Rad23 to proteasomes, which in turn promotes DNA repair and modulates protein degradation, possibly by delivering ubiquitinylated cargo to proteasomes. Here we show that Rad23 binds proteasomes by directly interacting with the base subcomplex of the regulatory particle of the proteasome. A component of the base, Rpn1, specifically recognizes the UBL domain of Rad23 through its leucine-rich-repeat-like (LRR-like) domain. A second UBL protein, Dsk2, competes with Rad23 for proteasome binding, which suggests that the LRR-like domain of Rpn1 may participate in the recognition of several ligands of the proteasome. We propose that the LRR domain of Rpn1 may be positioned in the base to allow the cargo proteins carried by Rad23 to be presented to the proteasomal ATPases for unfolding. We also report that, contrary to expectation, the base subunit Rpn10 does not mediate the binding of UBL proteins to the proteasome in yeast, although it can apparently contribute to the binding of ubiquitin chains by intact proteasomes.

Ubiquitin-like domain proteins generally contain an amino-terminal UBL domain, but are heterogeneous in the size, sequence and function of the carboxy-terminal element[1]. A subset of UBL domain proteins, including Rad23 and Dsk2, associate with the proteasome[2–5]. Co-immunoprecipitation of Rad23 with the proteasome requires its UBL domain[2], and this domain promotes efficient DNA repair through interaction with the proteasome[6]. Some model substrates of the proteasome are stabilized in mutants lacking Rad23, which indicates that Rad23 has a positive role in proteolysis[7]. This function of Rad23 requires its ubiquitin-chain binding UBA (ubiquitin-associated) domains[4,8–10]. Given that Rad23 can bind both ubiquitin chains and the proteasome, it has been suggested that Rad23 might serve as an adapter protein that delivers proteins to the proteasome for degradation[4,7].

The earliest events in protein breakdown, including the recognition of multi-ubiquitinylated chains, are thought to be mediated by the 18-subunit regulatory particle of the proteasome (the RP, also known as the 19S complex and PA700)[11,12]. The RP is composed of base and lid subassemblies[13]. The eight-subunit base links the RP to the core particle of the proteasome (the CP, also known as the 20S complex). The cylindrical CP contains the proteolytic sites of the proteasome in its central cavity[14], whereas the RP binds at the cylinder ends and delivers substrates to the CP for degradation.

To test for a direct interaction between Rad23 and the proteasome, we incubated a purified fusion protein of glutathione *S*-transferase and Rad23 (GST–Rad23) with purified yeast proteasomes and resolved the products by nondenaturing gel electrophoresis (PAGE). In the presence of GST–Rad23, proteasome migration through the gel was retarded, whereas GST alone had no effect. These and other data indicate that there is a direct interaction between the proteasome and Rad23 (Fig. 1a and data not shown). Similar gel shifts were observed when only the UBL domain of Rad23 was tested, indicating that this domain is sufficient for binding (Fig. 1a).

The CP was not electrophoretically retarded in the presence of Rad23 (Fig. 1a), which suggests that Rad23 binding to the proteasome may be mediated instead by the RP. Proteasomes from which the eight-subunit lid had been stripped ($base_2$–CP and $base_1$–CP complexes) remained competent to bind Rad23 (Fig. 1a). Similarly, proteasome and base–CP complexes were retained on a GST–Rad23 resin, whereas the lid was not (Fig. 1b). Thus, we concluded that the base of the proteasome is required for Rad23 binding. To determine whether the base is sufficient for binding, we prepared resins loaded with different affinity-tagged subassemblies of the proteasome. The UBL domain of Rad23 was bound by the proteasome holoenzyme, the RP and the base (Fig. 1c). In summary, a direct interaction between Rad23 and the base of the proteasome was observed by three independent methods.

To identify the Rad23-binding component of the base, proteasome subunits were resolved by SDS–PAGE, transferred to nitrocellulose membranes and probed with radiolabelled Rad23. The proteasome contains 32 subunits, of which Rad23 recognized only one, which we subsequently identified by electrophoretic mobility as Rpn1, a component of the base (Fig. 2a). The same activity was shown for purified Rpn1 that had been recombinantly expressed in *Escherichia coli* (Fig. 2a).

As denaturation of Rpn1 by SDS might have affected its binding to Rad23 in the above experiments, we examined the binding of soluble Rpn1 expressed as a GST–fusion protein in *E. coli*. Radiolabelled Rad23 was retained by an affinity column containing GST–Rpn1 (Fig. 2b). By contrast, when control columns were prepared using GST fusions to eight other RP subunits, none showed appreciable binding to Rad23. Thus, the interaction between Rpn1 and Rad23 is specific. Equivalent results were obtained using only the UBL domain of Rad23 (Fig. 2b). Notably, our results indicate a significant functional differentiation between Rpn1 and Rpn2, two subunits that probably evolved from the same ancestral gene[11,15].

To identify the Rad23-binding site in Rpn1, we generated mutant Rpn1 proteins containing deletions in the *RPN1* gene and expressed

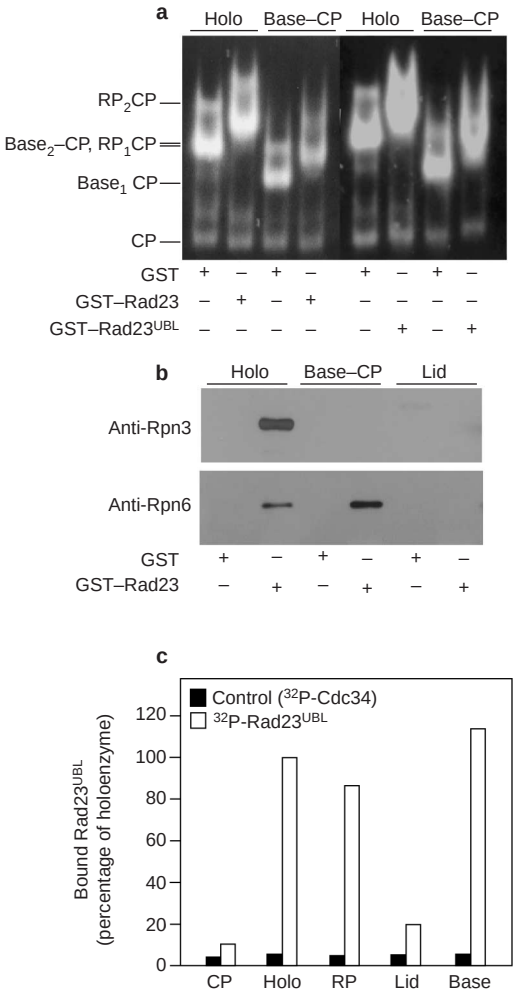

Figure 1 **Rad23 associates with the base of the proteasome. a**, Gel-shift assay of Rad23–proteasome binding. Holoenzyme and base–CP subcomplexes (10 μg of each, as indicated) were incubated with 10 μg of GST, GST–Rad23 or the GST-tagged UBL domain of Rad23 ($Rad23^{UBL}$). Samples were resolved by non-denaturing PAGE and the proteasome was visualized by an in-gel activity assay, using the fluorogenic substrate suc-LLVY-AMC. Binding of GST–Rad23 fusion proteins to proteasomes reduces their mobility in the gel. **b**, Proteasomes bind to resin-bound Rad23. Glutathione–Sepharose was loaded with extracts from *E. coli* cells expressing either GST or GST–Rad23. Proteasomes and proteasome subcomplexes were then applied to these columns. Loading controls (not shown) verified that roughly molar equivalents of holoenzyme and lid were added to the binding reactions. Proteins bound to the column were separated by SDS–PAGE and visualized by immunoblotting, using antibodies against the proteasome subunits as indicated. Rpn3 and Rpt6 are present in the lid and base, respectively. **c**, Binding of $Rad23^{UBL}$ by immobilized proteasome subassemblies. Resins carrying roughly equimolar amounts of various proteasome subassemblies incubated with 70 pmol of radiolabelled Cdc34 or $Rad23^{UBL}$, each at 100 c.p.m. per pmol. Bound protein was measured by scintillation counting.

them in *E. coli* as GST–fusion proteins. We found that a segment containing elements C and D was sufficient for Rad23 binding (Fig. 2c–e). Element C corresponds to the N-terminal block of the bipartite leucine-rich-repeat (LRR)-like domain found in Rpn1 (ref. 15 and Fig. 2d). LRR domains are horseshoe-shaped structures that bind ligands through a β-sheet on their inner, concave surface[15,16]. The carboxy-terminal block of the LRR-like domain was found to be dispensable for binding (Fig. 2c). The LRR-like domain is interrupted by a 134-residue acidic sequence (element D), which was required but not sufficient for binding (Fig. 2c). The location of the Rad23-binding site in Rpn1 was confirmed and localized more precisely using a two-hybrid assay (Fig. 2e). Rad23 binding required only a small, 21-residue portion of the acidic insert, which is contiguous with the first five LRR-like repeats. This is the only conserved segment of the insert.

The functions of Rad23 partially overlap with those of Dsk2 (refs 4, 17, 18). Although distinct in sequence, both of these proteins have N-terminal UBL domains and C-terminal UBA domains. The *Schizosaccharomyces pombe* Dsk2 homologue, Dph1, can be co-immunoprecipitated with the proteasome through its UBL domain[3–5]. We expressed Dsk2 in *E. coli* to determine whether it could directly bind the proteasome. Dsk2 bound proteasomes through its UBL domain (Fig. 3a, b), although less strongly than did Rad23 (Fig. 3b). The UBL domain of Dsk2 bound to the holoenzyme, the RP and the base with relative affinities similar to those of the UBL domain of Rad23, which suggests that the proteasomal receptor for these proteins may be shared (Fig. 3b).

Unlabelled Dsk2 competed with radiolabelled Rad23 for binding to the proteasome, further suggesting that the proteasomal binding sites for these proteins overlap (Fig. 3c). The small UBL domains of these proteins also competed with each other for binding (Fig. 3d, e). As expected—given that the affinity of Rad23 for the proteasome is greater than that of Dsk2—the UBL domain of Rad23 was a more effective competitor than the UBL domain of Dsk2. In addition, the affinity of the Rad23 UBL domain for the proteasome was greater than that of full-length Rad23 (unpublished data), which is consistent with the greater amount of unlabelled Dsk2 competitor needed in the second competition assay (Fig. 3d).

Our finding that Rad23 and Dsk2 bind to the base was unexpected because an interaction between the human orthologues of Rad23 (hHR23) and the proteasome subunit Rpn10 (S5a) has been reported[19]. PLIC, a human homologue of Dsk2, can also bind free S5a[20]. Our findings that Rad23 does not bind free Rpn10 and that base lacking the Rpn10 subunit binds Rad23 suggested that Rpn10 does not mediate the binding of Rad23 by yeast proteasomes. To examine this further, we compared wild-type and *Δrpn10* holoenzymes in binding assays for both Rad23 and Dsk2. We found no detectable difference in binding, confirming that Rpn10 does not contribute substantially to the recognition of UBL proteins by yeast proteasomes (Fig. 4a).

Rpn10 and S5a have also been implicated in the recognition of ubiquitin chains[21,22]; however, those experiments were carried out using purified Rpn10 and S5a, and therefore the contribution of Rpn10 and S5a to ubiquitin chain binding in the context of the proteasome is unclear[22–25]. To address this issue, we assembled ubiquitin chains on recombinant Cdc34, a ubiquitin-conjugating enzyme[26], and exposed the resultant protein to the proteasome (Fig. 4b, c). Although these chains contain canonical K48 linkages (ref. 26 and data not shown), these linkages do not target Cdc34 for degradation[27], presumably owing to structural properties of Cdc34. Thus, these conjugates form complexes with the proteasome that are sufficiently stable to be detected by nondenaturing PAGE (Fig. 4b). Binding of the Cdc34–ubiquitin conjugates to the proteasome was mediated by their ubiquitin chains rather than by Cdc34, because an amino acid substitution in ubiquitin that reduces its ability to target degradation[28] also attenuated the binding interaction (Fig. 4b and data not shown). In addition, Cdc34 alone had no appreciable affinity for proteasomes (Fig. 1c), and the omission of any required component of the conjugation reaction eliminated subsequent retardation of the proteasome complex, indicating that the band shift was produced by products of the conjugation reaction (data not shown).

Using both Cdc34–ubiquitin conjugates and unanchored ubiquitin chains, we tested the contribution of Rpn10 to the recognition of ubiquitin chains. More of the singly capped holoenzyme was shifted in the presence of Rpn10 than in its absence, which suggests

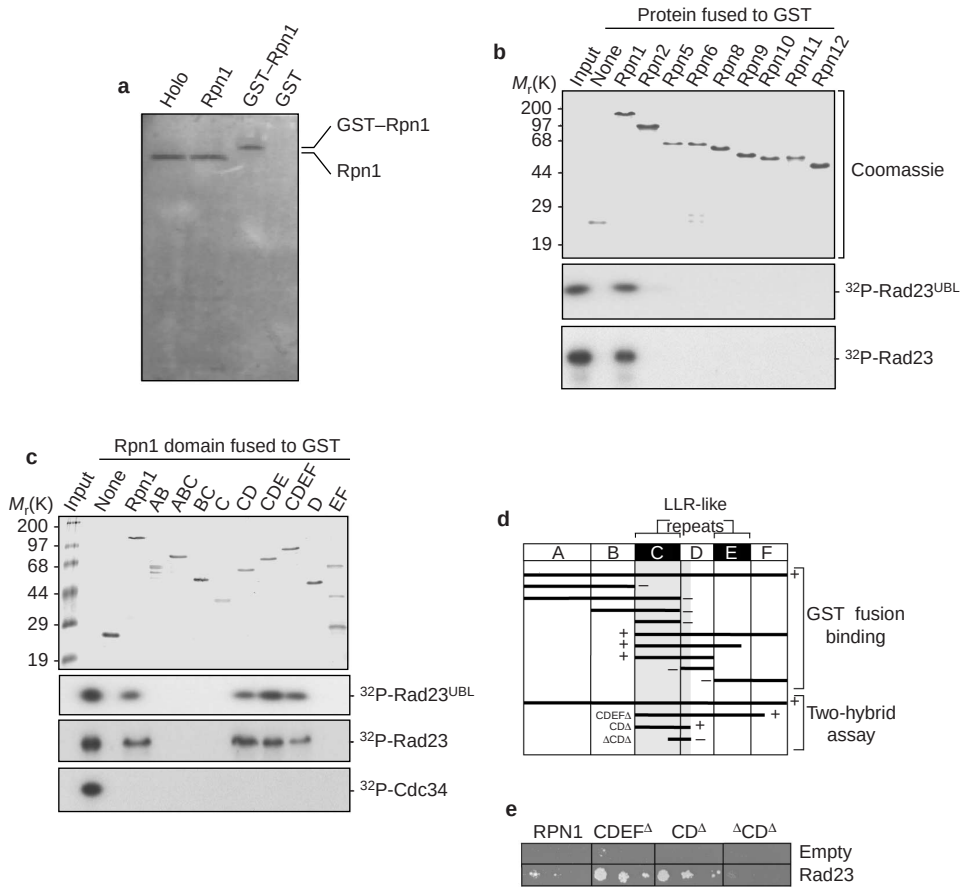

Figure 2 **Rad23 binds proteasome subunit Rpn1 through its LRR-like domain. a**, Filter assay for Rad23 binding. Samples were resolved by SDS–PAGE and the proteins were transferred onto nitrocellulose filters. The filter was then incubated with radiolabelled Rad23, washed and analysed by a phosphoimager. Lane 1, 200 μg of purified proteasome; lane 2, 10 μg of purified Rpn1 expressed in *E. coli*; lane 3, 150 μg of bacterial extract containing GST–Rpn1; lane 4, 150 μg bacterial extract containing GST. **b**, Binding of Rad23 by resin-bound GST–Rpn1. Proteasome subunits were expressed as GST–fusion proteins and bound to 100 μl of glutathione–Sepharose. Thirty picomoles of bacterially expressed, radiolabelled Rad23 or the UBL domain of Rad23 (Rad23^{UBL}) were added to the resin. Bound protein was analysed by SDS–PAGE coupled with Coommassie blue staining (top) or autoradiography (middle and bottom). The relative molecular mass (M_r) of each protein standard is indicated. **c**, Binding of Rad23 by truncated forms of GST–Rpn1, carried out as in **b**. **d**, Representation of constructs and results for **c** and **e**. Rpn1 domains are drawn to scale. LRR-like domains are indicated. Shaded region indicates the minimal domain that is sufficient for binding. **e**, Localization of the Rad23-binding site in Rpn1 by a two-hybrid assay. The bait (right) and prey (top) proteins are indicated. Strains were plated in tenfold serial dilutions. The comparatively low signal for the full-length Rpn1 may reflect reduced expression (unpublished data). A two-hybrid interaction between Rad23 and Rpn1 has also been observed by a whole-genome approach[38].

that Rpn10 can contribute to chain recognition (Fig. 4c, left). The observation that ubiquitin conjugates produce a residual mobility shift in proteasomes lacking Rpn10 is consistent, however, with the existence of at least one additional ubiquitin receptor intrinsic to the proteasome, which crosslinking studies indicate to be the ATPase Rpt5/S6′ (ref. 25). In nondenaturing gels, free ubiquitin chains also produced a mobility shift, although a more modest one as expected from their smaller size (Fig. 4c, right). This shift required a greater molar excess of conjugates, which suggests that the proteasome has a lower affinity for the shorter free chains. In the absence of Rpn10 this shift was abrogated, which again suggests that Rpn10 can contribute to ubiquitin chain recognition in the context of intact proteasomes. It is unclear why the crosslinking of tetraubiquitin chains to S5a was not detected previously[25], although in the present study we have used longer chains that apparently bind proteasomes more tightly than does tetraubiquitin. (Tetraubiquitin cannot be cosedimented with proteasomes[25].) The contribution of Rpn10 to chain binding might be stronger for extended chains.

In summary, we have found that a receptor for Rad23 is located in the proteasome base, and that a subunit of the base, Rpn1, is sufficient for Rad23 binding. The minimal binding site in Rpn1 includes the N-terminal block of LRR-like repeats and a short adjacent sequence. As compared with canonical LRR elements, the solvent-exposed residues of the predicted β-sheets in Rpn1 are highly hydrophobic[15]. Our results indicate that this hydrophobic region may make specific contacts with complementary patches on the surface of several UBL proteins. The UBA domains of Rad23 and Dsk2 bind ubiquitin conjugates[4,10,18], and we propose that Rad23 bound to ubiquitinated proteins may then bind to Rpn1 in the context of the proteasome. In this model, the location of the Rad23/Dsk2 receptor Rpn1 in the base would ensure the delivery of these proteins and their ubiquitinated cargo directly to the proteasomal ATPases where their folding state could be altered, possibly circumventing the requirement for ubiquitin chain recognition by the proteasome. The proteasomal ATPases, which are located in the base, have a potent chaperone-like activity[29,30] and are thought to form a complex for protein unfolding and possibly structural remodelling.

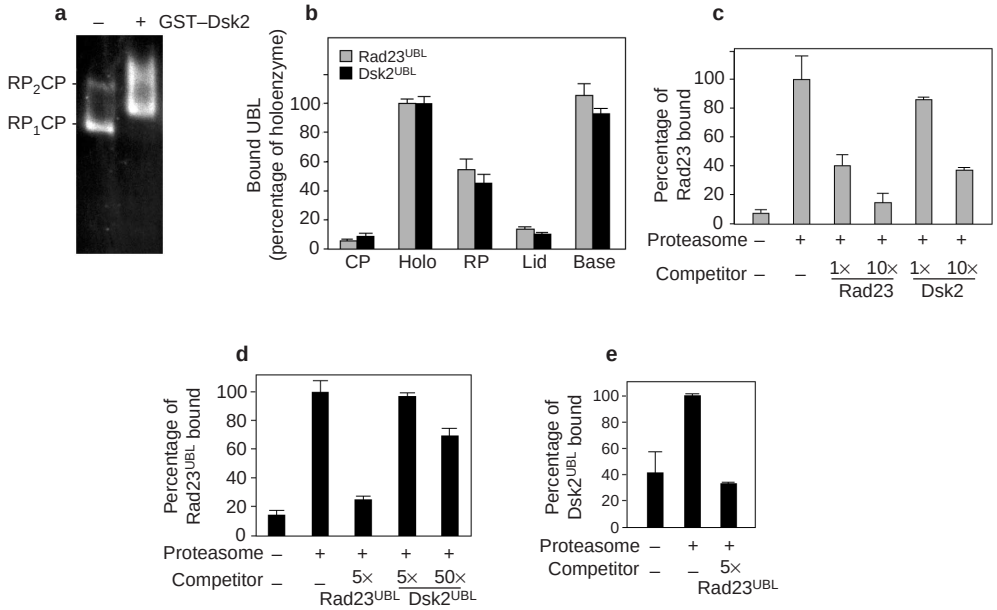

Figure 3 **Dsk2 competes with Rad23 in proteasome binding. a**, Binding of GST–Dsk2 to purified proteasomes. Holoenzyme (1 pmol) was incubated with either GST–Dsk2 (60 pmol) or buffer. Samples were resolved by nondenaturing PAGE and the proteasome was visualized by an in-gel activity assay. **b**, Binding of the UBL domain of Dsk2 ($Dsk2^{UBL}$) by immobilized proteasome subassemblies. Resins carrying equimolar amounts of proteasome subassemblies were incubated with 1 nmol of radiolabelled $Rad23^{UBL}$ or $Dsk2^{UBL}$. Bound protein was measured by scintillation counting. Note that Rad23 binds proteasomes more strongly than does Dsk2, as the maximum binding for $Rad23^{UBL}$ and $Dsk2^{UBL}$ was 106 pmol and 4 pmol, respectively. The Dsk2 and Rad23 data sets were normalized separately by setting the amount of radioactivity (c.p.m.) that bound the holoenzyme to 100%. **c**, Competition binding assay between Rad23 and Dsk2. Resin carrying proteasome holoenzyme was incubated in the presence of radiolabelled Rad23. Cold competitor was added as indicated. Bound protein was detected by scintillation counting. **d**, Competition binding assay for $Rad23^{UBL}$ and $Dsk2^{UBL}$. Resin carrying proteasome holoenzyme was incubated in the presence of 120 pmol of radiolabelled $Rad23^{UBL}$. Unlabelled competitor was added in molar excess as indicated. Binding of the radioactive probes was detected by scintillation counting. **e**, Competition binding assay between $Rad23^{UBL}$ and $Dsk2^{UBL}$ carried out as in **d**, except that $Dsk2^{UBL}$ was used as the radioactive probe. All quantitative experiments were carried out several times with similar results.

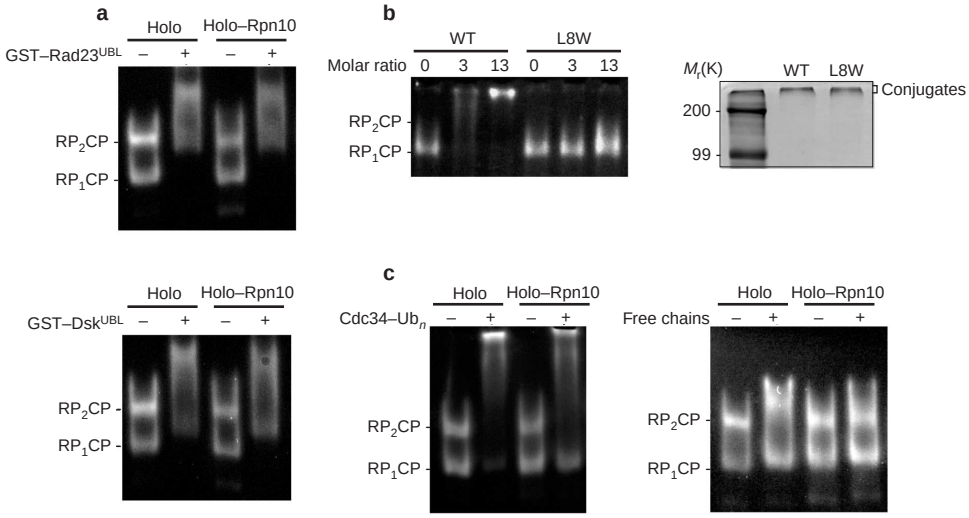

Figure 4 **Recognition of ubiquitin-like proteins and ubiquitin conjugates (Ub_n) by *rpn10Δ* proteasomes. a**, Binding of the UBL domains of Rad23 ($Rad23^{UBL}$) and Dsk2 ($Dsk2^{UBL}$) to proteasomes lacking Rpn10. Holoenzyme (1 pmol) was incubated with 750 pmol of GST–$Rad23^{UBL}$ (+) or GST–$Dsk2^{UBL}$ (+), or with buffer (–). Samples were resolved by nondenaturing PAGE and visualized by suc-LLVY-AMC hydrolysis. **b**, Specificity of Cdc34–Ub_n binding by proteasomes. Left, 1.5 pmol of proteasome was incubated with Cdc34–Ub_n prepared with wild-type or L8W ubiquitin. The molar ratios of ubiquitin to proteasome are indicated. Samples were resolved by nondenaturing PAGE and proteasomes was visualized by suc-LLVY-AMC hydrolysis. Right, Coomassie-stained gel of ubiquitin conjugates. Equal amounts of high molecular mass conjugates are obtained with wild-type and L8W ubiquitin, and are dependent on the presence of E1, Cdc34, ubiquitin and ATP (data not shown). Marker proteins are indicated on the left. **c**, Binding of ubiquitin conjugates to proteasomes lacking Rpn10. Holoenzyme (1 pmol) was incubated with Cdc34–Ub_n (15 pmol), unanchored ubiquitin chains (15 μg) or buffer. Samples were resolved by nondenaturing PAGE and visualized by suc-LLVY-AMC hydrolysis.

Our evidence that budding yeast Rpn10 is not a principal receptor for Rad23 is also supported by previous studies indicating that *rpn10Δ* mutants are (unlike *rad23Δ*) not sensitive to ultraviolet radiation[7,22], and that the efficiency of co-immunoprecipitation of Rad23 with the proteasome is not attenuated in *rpn10Δ* mutants[7]. In humans, however, the free form of S5a (the Rpn10 orthologue) binds both the Rad23 and Dsk2 orthologues. Two similar elements in S5a participate in the recognition of ubiquitin conjugates, only one of which participates in binding the human Rad23 orthologues[19]. Notably, this element in S5a is not found in yeast Rpn10, which possibly explains the lack of interaction between Rpn10 and Rad23. Rpn10 is unlikely to be the only ubiquitin receptor in the proteasome[24,25], and a major issue concerning the site of ubiquitin chain binding in Rpn10 has been whether it is accessible on the surface of the proteasome. We have shown here, in a direct biochemical assay, that Rpn10 can contribute to ubiquitin chain recognition in the context of the proteasome. Sequences related to the ubiquitin-chain-binding element in Rpn10, known as UIM motifs[31], are present in several other proteins that function as ubiquitin receptors in endocytic and endosomal protein sorting pathways[24]. Our results, together with the strong evolutionary conservation of the UIM motif in Rpn10 (ref. 23), suggest that ubiquitin chain binding by Rpn10 is likely to be physiologically significant. □

Methods

Preparation of plasmids

We generated the vectors for bacterial expression of GST fusion proteins by amplifying the desired open reading frame (ORF) from genomic DNA and cloning it into pGEX-2TK. Plasmids for expressing the subdomains of Rpn1 contained the following segments of *RPN1*: AB (codons 1–416), ABC (1–607), BC (267–607), C (417–607), CD (417–737), CDE (417–846), CDEF (413–993), D (603–737), EF (733–993). Plasmids for expressing the GST-tagged UBL domains of Rad23 and Dsk2 each included the first 77 codons of each ORF. We verified all constructs by DNA sequencing.

Preparation of GST fusion proteins

Rad23, Dsk2 and subdomains of these proteins fused to GST were expressed by growing cultures to an absorbance at 600 nm (A_{600}) of 0.8 and inducing expression with 400 μM isopropylthiogalactoside (IPTG). We expressed the fusion proteins either in MC1061 cells with induction for 4 h, or in BL21(DE3) cells with induction for 2 h, with similar results. We expressed GST fusion proteins of the proteasome Rpn1 subunits in BL21(DE3) cells. Cells were grown to $A_{600} = 0.8$, expression was induced with 250 μM IPTG, and cells were collected after 4 h.

Radioactive labelling

Proteins were labelled with [γ-^{32}P]ATP as described[32]. Proteins were cleaved from GST with thrombin.

Purification of ubiquitin and ubiquitinylating enzymes

We purchased wild-type ubiquitin from Sigma. L8W ubiquitin was expressed in *E. coli* and purified conventionally as follows. Expression was carried out in BL21(DE3) cells at 37 °C by induction with 1 mM IPTG for 6 h. Cells were resuspended in 25 mM Tris-acetate (pH 7.5) and 1 mM dithiothreitol (DTT), and lysozyme was added to a concentration of 0.1 mg ml^{-1}. After 30 min at 30 °C, the cells were lysed by sonication, and the extract was cleared by centrifugation at 100,000*g* and 4 °C for 1 h. The supernatant was passed over a DEAE column, which was then washed with one column volume of 25 mM Tris-acetate (pH 7.5). Flow-through and wash were pooled, titrated with acetic acid to pH 4.8 and applied to a CM52 column equilibrated with 25 mM ammonium acetate (pH 4.5). We washed the resin with 25 mM ammonium acetate (pH 4.5) and eluted the protein with 0.2 M ammonium acetate (pH 7.5). Eluent was dialysed against water, titrated to pH 4.6 with acetic acid, and applied to a Mono S column equilibrated with 25 mM ammonium acetate. The column was developed with a linear gradient of 0–0.75 M NaCl. We pooled the peak fractions and precipitated protein with ammonium sulphate. The precipitate was collected by centrifugation, resuspended in H_2O and dialysed against H_2O.

Cdc34 expressed in *E. coli* and purified by conventional chromatography[33]. His-tagged Uba1 (His_6–Uba1) was purified from strain JD77-1A (*MATa leu2-3,-112 lys2-801 his3-Δ200 trp1-Δ63 ura3-52 uba1::HIS3pCUP1-UBA1-6His*) as follows. Cells were inoculated in YPD medium containing 100 μM $CuSO_4$ at $A_{600} = 0.001$, grown to $A_{600} = 4$, collected and then washed in ice-cold water. We prepared the extract as described[34] and further clarified it by centrifugation for 30 min at 4 °C and 100,000*g*. The protein was applied to a DEAE fast-flow resin column. The column was washed with buffer containing 0.1 M KCl, and protein was eluted with buffer containing 0.5 M KCl. We dialysed the eluent against 50 mM sodium phosphate, 10 mM Tris-HCl and 100 mM NaCl (pH 8.0). Dialysate was absorbed in batch to Clontech Talon resin for 20 min at 24 °C. The resin was washed with dialysis buffer, and then with 50 mM sodium phosphate and 100 mM NaCl (pH 7.0). The protein was eluted in buffer containing 100 mM imidazole.

Preparation of ubiquitin conjugates

We prepared ubiquitin-conjugated Cdc34 by incubating 1.5 μM Cdc34, 0.3 μM His_6–Uba1 and 60 μM ubiquitin in a solution of 20 mM Tris-HCl, 10 mM $MgCl_2$, 0.1 mM DTT and 2 mM ATP (pH 7.5) for 15 h at 30 °C. Free ubiquitin chains, ranging from 2 to 15 ubiquitin molecules and prepared as described[35], were supplied by C. Pickart.

Purification of proteasome holoenzyme and subcomplexes

We purified the proteasome, base–CP and lid components by conventional chromatography as described[11,13]. We also preparaed proteasome holoenzyme and subcomplexes by using affinity tags. The strains and purification methods are described in detail elsewhere (ref. 36; see also Supplementary Information). Proteasomes and subcomplexes prepared by this method contain all of the canonical subunits, as described[36].

Nondenaturing PAGE and protease assay

Nondenaturing PAGE and proteasome visualization by the fluorogenic substrate suc-LLVY-AMC were carried out as described[11], with minor modifications. The native gels shown in Figs 1 and 3 were prepared and developed in 90 mM Tris, 90 mM boric acid, 2.5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM ATP and 1 mM DTT (pH 8.35). For those shown in Fig. 4, the buffer was the same except that DTT was omitted, and concentrations of 5 mM $MgCl_2$, 0.5 mM ATP and 3.5% acrylamide were used. For mobility shift assays, we incubated proteins with proteasomes for 20 or 30 min at 30 °C, and then mixed them with native gel loading buffer before electrophoresis.

Binding of proteasome to immobilized GST–Rad23

We expressed GST and GST–Rad23 in bacteria, prepared the cell lysate in buffer (50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 10% glycerol, 1 mM ATP and 1 mM DTT) and then incubated it with glutathione–agarose with tumbling for 1 h. The resin was then washed five times with 20 column volumes of buffer. Purified proteasome components were each mixed with glutathione–agarose carrying GST or GST–Rad23 and incubated with tumbling for 1 h. The resin was loaded into small columns, washed with 20 column volumes of buffer, and eluted with buffer containing 20 mM glutathione. We analysed the eluates by SDS–PAGE followed by immunoblotting.

Binding of proteins to immobilized proteasome subunits

GST fusion proteins, bound to 100 μl of glutathione resin, were washed four times in buffer (25 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM DTT and 0.5 mM EDTA) containing 0.1 mg ml^{-1} bovine serum albumin (BSA) and incubated with 30 pmol of radiolabelled protein in 500 μl of buffer supplemented with 0.1 mg ml^{-1} BSA. The reaction was incubated for 10 min at 24 °C and then for 5 min at 4 °C. The glutathione resin was washed five times with buffer. GST fusion proteins were eluted from the resin by incubation with 30 mM glutathione for 1 h at 4 °C. The proteins were resolved by electrophoresis on an 18% SDS–PAGE gel and identified by staining with Coomassie blue. We detected radioactively labelled proteins by autoradiography.

Binding of proteins to bead-bound proteasome subcomplexes

Proteasome subcomplexes were prepared as described. For the experiment shown in Fig. 1, beads bearing proteasome holoenzyme and subcomplexes were equilibrated in buffer (25 mM Tris-HCl (pH 7.4) 100 mM NaCl, 0.5 mM EDTA and 1 mM DTT), and BSA was added to a final concentration of 0.1 mg ml^{-1}. Radiolabelled proteins were added, incubated with tumbling for 1 h at 4 °C and washed five times with five volumes of buffer per wash. We detected bound radioactivity by scintillation counting. For the experiment shown in Fig. 3b, the buffer was 50 mM Tris-HCl (pH 7.6), 1 mM EDTA, 5 mM $MgCl_2$ and 1 mM ATP, and binding was carried out at 24 °C for 20 min. Bound protein was measured by scintillation counting and verified by autoradiography (data not shown).

Far western assay

Samples were resolved by SDS–PAGE and then transferred to nitrocellulose membranes. Membranes were blocked with 1% BSA for 1 h and 10% powdered nonfat milk for 15 min, and then incubated with radiolabelled Rad23 at 2×10^5 c.p.m. per ml in TBST (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% Tween-20) for 24 h at 4 °C. The membranes were rinsed, washed briefly with TBST, and exposed to phosphoimager screens.

Two-hybrid assay

We prepared the Rad23 bait plasmid in pEG202 (His), and this parent plasmid was used as the empty bait vector. All prey plasmids were prepared in pJG4-5 (TRP). The subdomains of Rpn1 cloned into pJG4-5 (ref. 37) were as follows: $CDEF^{A}$, residues 413–915; CD^{A}, residues 417–628; $^{A}CD^{A}$, residues 563–628. Plasmids were transformed into the reporter strain EGY48 (*MATa his3 leu2-3 lexAop-LEU2 ura3 trp1*). After purifying the transformants, cells were grown overnight in synthetic medium lacking tryptophan and histidine. We diluted overnight cultures to $A_{600} = 0.2$ with water, prepared serial dilutions, and spotted 5 μl of each suspension onto synthetic medium prepared with 2% raffinose and 2% galactose and lacking tryptophan, histidine and leucine. After 3 d incubation at 30 °C, the plates were analysed for growth.

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COMPETING FINANCIAL INTERESTS
The authors declare that they have no competing financial interests.