

# Recognition of Specific Ubiquitin Conjugates Is Important for the Proteolytic Functions of the Ubiquitin-associated Domain Proteins Dsk2 and Rad23\*

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Ubiquitin (Ub) regulates important cellular processes through covalent attachment to its substrates. The fate of a substrate depends on the number of ubiquitin moieties conjugated, as well as the lysine linkage of Ub-Ub conjugation. The major function of Ub is to regulate the *in vivo* half-life of its substrates. Once a multi-Ub chain is attached to a substrate, it must be shielded from deubiquitylating enzymes for the 26 S proteasome to recognize it. Molecular mechanisms of the postubiquitylation processes are poorly understood. Here, we have characterized a family of proteins that preferentially binds ubiquitylated substrates and multi-Ub chains through a motif termed the ubiquitin-associated domain (UBA). Our *in vivo* genetic analysis demonstrates that such interactions require specific lysine residues of Ub that are important for Ub chain formation. We show that *Saccharomyces cerevisiae* cells lacking two of these UBA proteins, Dsk2 and Rad23, are deficient in protein degradation mediated by the UFD pathway and that the intact UBA motif of Dsk2 is essential for its function in proteolysis. Dsk2 and Rad23 can form a complex(es), suggesting that they cooperate to recognize a subset of multi-Ub chains and deliver the Ub-tagged substrates to the proteasome. Our results suggest a molecular mechanism for differentiation of substrate fates, depending on the precise nature of the mono-Ub or multi-Ub lysine linkage, and provide a foundation to further investigate postubiquitylation events.

Ubiquitin (Ub)<sup>1</sup> is a highly conserved 76-residue polypeptide in eukaryotes. In addition to its classical role in protein degradation, Ub is also emerging as a signal for protein transport and processing (1–3). Ubiquitylation, the covalent conjugation of Ub to other intracellular proteins, regulates a broad range of basic cellular processes, including cell growth and differentiation, signal transduction, and stress responses (1–3). The Ub-proteasome system consists of two major parts. In the ubiquitylation part, Ub is activated and conjugated to a substrate through several intermediate steps that involve a Ub-activat-

ing enzyme (E1), a Ub-conjugating enzyme (E2), and a Ub protein ligase (E3). With protein substrates targeted for degradation, successive Ub moieties are conjugated onto a substrate-linked Ub, yielding a substrate-linked multi-Ub chain. The second part of the Ub system involves the delivery of a ubiquitylated substrate to the 26 S proteasome; the substrate is then unfolded and processively degraded to small peptides. The biochemical mechanisms of the postubiquitylation events are poorly understood. Multi-Ub chain-binding proteins are thought to be the key players in this process. Thus far, two distinct proteins, Rpn10 and Cdc48, are known to bind multi-Ub chains and may be involved in this process (4–7). Rpn10 is a proteasomal subunit of the 19 S regulatory complex, but there are no proteolytic defects in cells lacking the Ub-binding domain of Rpn10. Cdc48 is involved in a subset of proteolytic pathways, but its precise roles in proteolysis have yet to be separated from its involvement in cell cycle, homotypic membrane fusion, and apoptosis.

Because Rpn10 and Cdc48 are not essential for the degradation of many proteins in yeast, other Ub-binding proteins likely exist to deliver ubiquitylated substrates to the proteasome. Genetic and biochemical studies have implicated RAD23 in the functioning of the Ub-proteasome system (8–11). Genome-wide two-hybrid analysis previously identified *Saccharomyces cerevisiae* Rad23 as a Ub-interacting protein (12). Rad23 contains a Ub-like domain (UBL) at its N terminus responsible for its interaction with the proteasome (8) and two ubiquitin-associated domains (UBA) (13). Similar structures were found in Dsk2 and Ddi1, although each has only one UBA domain (Fig. 1A). Two groups recently reported the binding of Rad23 in *S. cerevisiae* and its homologs in *Schizosaccharomyces pombe* to single and tetra-Ub chains, respectively, through their UBA domains with conflicting results (see below) (14, 15). Aside from its function in proteolysis, RAD23 also plays roles in DNA repair by using UBL and XPC domains to link the 19 S proteasome cap to the nucleotide excision repair complex (8, 16). The functional significance of UBA motifs in proteolysis is not known. Dsk2 and Ddi1 have not been shown to function in proteolysis, and the functional relationships among Rad23, Dsk2, and Ddi1 in proteolysis are unclear.

Here we have studied the roles of Dsk2 and Rad23 in Ub-mediated proteolysis. Using the two-hybrid and GST binding assays, we found that these UBA-containing proteins bind ubiquitylated proteins, likely through the demonstrated ability of the UBA motif to recognize the multi-Ub chains that are involved in proteolysis. The UFD proteolytic pathway is impaired in cells lacking RAD23 and DSK2. We showed that the UBA motif is required for the functioning of Dsk2 in proteolysis. Because the UBA motif specifically binds Lys-29- and Lys-48-linked Ub chains, but not Lys-63-linked Ub chains, we propose that RAD23-like proteins, which contain both UBA and

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<sup>1</sup> The abbreviations used are: Ub, ubiquitin; UBL, ubiquitin-like element; UBA, ubiquitin-associated domain; GST, glutathione S-transferase;  $\beta$ -gal,  $\beta$ -galactosidase; UFD, ubiquitin fusion degradation.

UBL motifs, are adapter molecules for delivering substrates to the proteasome. Other Ub chain-specific recognition factors are likely to be important for the functions of Ub chains assembled through other lysine linkages.

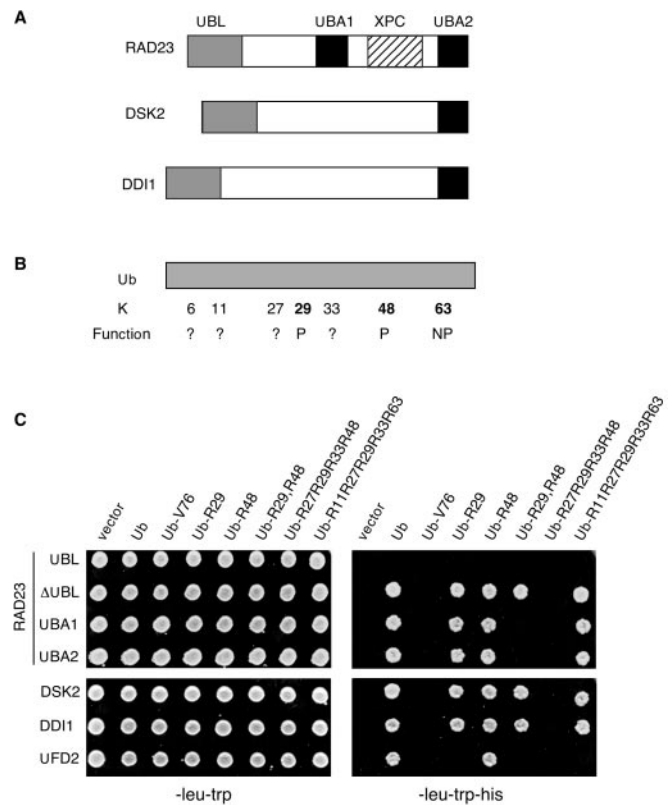
#### MATERIALS AND METHODS

**Strains, Media, and Plasmids**—*S. cerevisiae* PJ69–4A (*MATa gal4Δ gal80Δ GAL2-ADE2 LYS2::GAL1-HIS3 met::GAL7-lacZ*) was used for yeast two-hybrid assays. Strains MY3589, MY3587, MY3588, and MY3592 were gifts from M. Rose (20). *Escherichia coli* BL21 (DE3) was used to express GST fusion proteins. Yeast cultures were grown in rich (YPD containing 1% yeast extract, 2% peptone, and 2% glucose) or in synthetic media containing standard ingredients. Two-hybrid plasmids containing the full length of *RAD23*, *UFD2*, *DSK2*, *DDI1*, *CDC48*, *VAM3*, and *UBP14* were gifts from E. Friedberg, S. Jentsch, K. Frohlich, and T. Ito. The PCR products of *RAD23* derivatives were fused to the 3'-end of the GST coding sequence for *E. coli* overexpression or to the 3'-end of the *GAL4* fragments in pGBD and pGAD vectors for yeast two-hybrid assays. Similarly, Ub and its derivatives were inserted to the 3'-end of the *GAL4* in pGBD and pGAD vectors. Construction details are available upon request.

**GST binding Assays**—GST fusion protein or GST alone (~2 μg) was mixed with the indicated amount of Ub (Sigma) or tetra-ubiquitin chains (Affinity) in 200 μl of binding buffer (150 mM NaCl, 1% Triton X-100, 10% glycerol, 0.1 mg/ml ovalbumin, 50 mM Na-HEPES, pH 7.5) and incubated with 20 μl of glutathione-agarose beads (Sigma) for 2 h. The beads were washed three times with the binding buffer, followed by SDS-PAGE of the retained proteins and immunoblotting with a monoclonal anti-Ub antibody (Chemicon International). For GST pull-down with yeast extracts, ~20 μg of yeast proteins were used for each GST binding reaction.

#### RESULTS

We first confirmed the two-hybrid interaction between *RAD23* and Ub (data not shown). In order to identify other proteins containing similar sequences for Ub binding, we wanted to determine the region of *RAD23* involved in Ub interaction. We constructed two-hybrid fusions containing the UBL domain (*RAD23*<sup>UBL</sup>, amino acids 1–77), the *RAD23* fragment lacking UBL (*RAD23*<sup>ΔUBL</sup>, amino acids 78–398), and the two UBA elements (*RAD23*<sup>UBA1</sup>, amino acids 145–185; *RAD23*<sup>UBA2</sup>, 354–394) separately (Fig. 1). These derivatives of *RAD23* were tested in two-hybrid assays for interactions with wild-type Ub. We found that UBA motifs are sufficient for interaction with Ub (Fig. 1C) and that the two UBA-containing proteins *Dsk2* and *Ddi1* also interact with Ub. The binding could have been specifically to mono-Ub, multi-Ub chains, or ubiquitylated substrates. To distinguish among these possibilities, we first constructed a series of mutations in the Ub coding region that would affect its ability to form various conjugates (1, 2). The design of these constructs was based on the different roles of amino acid residues in Ub. Ub chain formation requires the C-terminal Gly-76 of Ub to attach to a lysine residue of another Ub molecule or a substrate. Ub contains seven lysines, all of which can be utilized for multi-Ub chain assembly (Fig. 1B). Ub chains assembled through Lys-29 and Lys-48 are involved in proteolysis, whereas Lys-63-based chains have non-proteolytic functions (1, 2, 17). We therefore tested the ability of *Rad23* to bind to several Ub variants, including Ub-V76, which contained a Gly to Val mutation that eliminated its ability to conjugate to other proteins although it could still be an acceptor of other Ub molecules. Other constructs were Ub mutants containing various single or multiple Lys to Arg mutations that block Ub conjugation to those lysine residues (Fig. 1C). As a control, we employed *UFD2*, which encodes a multi-Ub chain-binding protein that requires a Lys-29 linkage for its function in Ub chain assembly (18, 19). In the two-hybrid assays, *UFD2* interacted with wild-type Ub and Ub derivatives that could both form Lys-29-linked chains and also be conjugated to the substrates (Fig. 1C). No interaction was observed with the Ub-V76 mutant, which could not be conjugated to other Ubs or

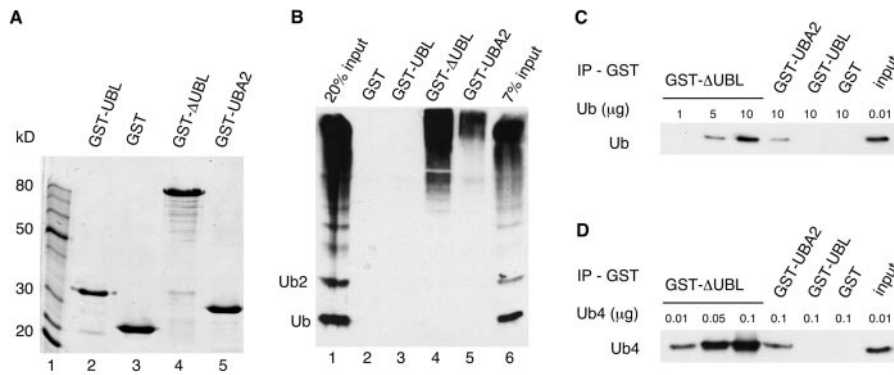


**FIG. 1. Two-hybrid analysis of interactions between Ub- and UBA-containing proteins *in vivo*.** *A*, domain organization of the Rad23-like proteins in *S. cerevisiae*. All three proteins contain a Ub-like domain (UBL) at the N terminus that binds the 19 S proteasome regulatory complex and a C-terminal UBA domain. Rad23 also has an XPC domain that mediates association with Rad4/XPC. *B*, different roles for different ubiquitin linkages. Ub has seven lysines, which could be utilized for multi-Ub chain assembly. Lys-29 and Lys-48-linked chains target proteins to the proteasome, and Lys-63-linked chains have non-proteolytic functions. *C*, two-hybrid interactions between Ub and UBA-containing proteins. Yeast cells were co-transformed with plasmids encoding GAL4 DNA-binding domain fused to the proteins or protein domains indicated on the left with the GAL4 activation domain fused to Ub and its derivatives indicated above each panel. The mutated residues in Ub are indicated. Growth on –His plates is indicative of protein-protein interactions.

proteins. These results suggested that the two-hybrid assay could be used to study the regulators for multi-Ub chain formation and maintenance *in vivo*.

We found that the Ub interaction with UBA-containing proteins specifically required Gly-76 and at least one of the four lysines Lys-29, Lys-27, Lys-33, and Lys-48 but not Lys-63 or Lys-11 (Fig. 1C). The interactions between Ub and UBA domains were weaker, and they required either Lys-29 or Lys-48, both of which are known to be involved in proteolysis. It is worth noting again that Lys-63 has non-proteolytic functions and is not required for these interactions (1, 2, 17). Interestingly, although *Dsk2* and *Ddi1* have only one copy of the UBA motif, their binding specificity toward Ub is similar to *Rad23* but not to the UBA motif alone (Fig. 1C), suggesting that the sequences outside of the UBA motif could contribute to the binding of Ub, possibly through dimer formations (Fig. 3A). Because these interactions with Ub required Gly-76, which is essential for substrate conjugation, and specific lysine residues that are important for Ub chain formation, our data suggest that the UBA-containing proteins likely interact with multi-ubiquitylated substrates containing certain Ub linkages and that the interaction between Rad23-like proteins and Ub conjugates might be important for proteolysis.

The UBA motif is found in many proteins in other organisms



**FIG. 2. The UBA domain binds ubiquitylated substrates and ubiquitin chains.** *A*, purified GST fusions containing Rad23 derivatives. A Coomassie Blue-stained polyacrylamide gel shows the proteins used in the study. *Lane 1* contains a 10-kDa protein standard marker. *Lane 2*, GST-RAD23<sup>UBL</sup>; *lane 3*, GST alone; *lane 4*, GST-RAD23<sup>ΔUBL</sup>; and *lane 5*, GST-RAD23<sup>UBA2</sup> are shown on the gel. *B*, UBA-containing proteins bind ubiquitylated species. The mixtures containing glutathione beads, various GST proteins, and 20- $\mu$ g yeast extracts with overexpressed myc-tagged, Ub-containing Ub conjugates formed *in vivo* were incubated at 4 °C for 2 h. The positions of single and di-Ub are indicated on the left of the gel. *Lanes 1* and *5* contain 20 and 7% input as controls, respectively. The bound proteins were eluted, fractionated by SDS-19% PAGE, and immunoblotted with antibody to myc. *C*, interactions of Ub with UBA-containing proteins. Free Ub was tested for interactions with GST or GST fusion proteins, which are labeled on top of the gel. The reactions are similar to *B*, except different amounts of Ub were used instead of yeast extracts. In the reactions for the first 3 lanes, increasing amounts of Ub (1, 5, and 10  $\mu$ g) were added to 2  $\mu$ g of GST-RAD23<sup>ΔUBL</sup>. The last lane contains 0.01  $\mu$ g of Ub as a control. The immunoblot was probed with an antibody to Ub. The identity of the proteins is indicated on the left of the gel. *D*, interactions of tetra-ubiquitin chains with UBA-containing proteins. The reactions are similar to those in *C*, except that different amounts of tetra-ubiquitin chains were substituted for Ub.

(13). Using the yeast two-hybrid assay, we found that Ub could not interact with two of these UBA-containing yeast proteins, Ubp14 and Vam3 (data not shown), suggesting that not all UBA-bearing proteins bind to Ub stably. We also failed to observe two-hybrid interactions between Ub and *RPN10* or *CDC48*, both of which have been demonstrated to bind multi-Ub chains using other biochemical assays (5, 6). This suggests that Rpn10 and Cdc48 may adopt altered conformations when fused to the Gal4 DNA-binding domain or have lower affinities for Ub chains compared with the RAD23-like proteins in our assays.

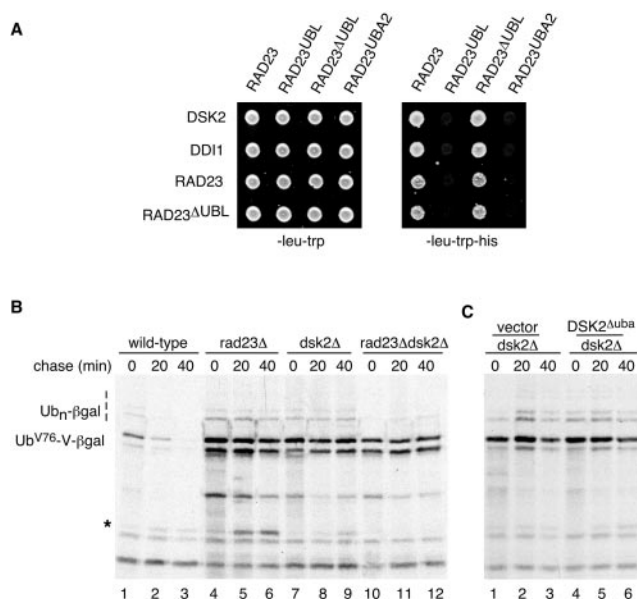
Although the two-hybrid assay is powerful, this genetic approach has its limitations, and the results obtained often need to be confirmed by other biochemical means. Hence we employed the GST pull-down assay to test the binding between ubiquitylated substrates and *S. cerevisiae* Rad23-like proteins. RAD23<sup>ΔUBL</sup>, RAD23<sup>UBL</sup>, and RAD23<sup>UBA2</sup> were separately fused to the C terminus of GST. These proteins were overexpressed in *E. coli* and subsequently purified using glutathione beads (Fig. 2A). Purified proteins were then mixed with yeast extracts overexpressing myc-tagged Ub in the presence of glutathione beads. Ub-Rad23 bindings were assayed by immunoblotting with monoclonal anti-myc antibody to determine the amount of Ub retained in the GST beads. Ubiquitylated species, but not free Ub, were precipitated along with UBA-containing proteins (Fig. 2B), supporting the conclusion that the UBA motif binds preferentially to ubiquitylated substrates. The binding affinity of the UBA domain alone (RAD23<sup>UBA2</sup>) for ubiquitylated substrate is weaker than that of RAD23<sup>ΔUBL</sup>, which contains two UBA motifs.

Does the UBA domain recognize a single Ub moiety or the multi-Ub chain attached to ubiquitylated substrates? Two groups recently reported the binding of Rad23 in *S. cerevisiae* and its homologs in *S. pombe* to single and tetra-Ub chains, respectively, with some conflicting results. Bertolaet *et al.* (14) focused mainly on the ability of *S. cerevisiae* Rad23 and Ddi1 to interact with single Ub. Wilkinson *et al.* (15) found that *S. pombe* Rad23 homologs interact with Ub chains specifically but not with single Ub, suggesting that Rad23 homologs in *S. pombe* may behave differently from those in *S. cerevisiae*. We wanted to compare the affinities of *S. cerevisiae* Rad23 to single and multi-Ub chains. To accomplish this, we used purified GST

fusion proteins mixed with either single Ub or Lys-48-linked tetra-Ub chains (Ub4) in the presence of glutathione beads. Whereas about 0.1% Ub was recovered when 10  $\mu$ g of Ub was mixed with GST-RAD23<sup>ΔUBL</sup>, ~50% Ub4 was recovered when 0.01  $\mu$ g of Ub4 was mixed with GST-RAD23<sup>ΔUBL</sup> (Fig. 2, C and D). The results indicated that the UBA-containing proteins preferentially bind Lys-48-linked tetra-Ub chains, the shortest multi-Ub chains that are recognized by the proteasome. Consistent with *in vivo* two-hybrid analysis, we found that the UBA motif is sufficient for binding to the Ub molecules, albeit with weaker affinity (Fig. 2, C and D). Our results show that the *S. cerevisiae* Rad23 protein, like its *S. pombe* counterpart, binds Ub chains preferentially, further suggesting that the failure in detecting an interaction between *S. pombe* Rad23 and single Ub could be due to the lower affinity for free Ub (Fig. 2C). Results of these GST assays further validated the two-hybrid approach as an effective means to detect Ub and Ub chain binding activities *in vivo*.

Since these Rad23-like proteins share similar structural domains and have similar specificities toward ubiquitin (Figs. 1 and 2), do these proteins function together or separately? We used the two-hybrid assay to examine the interactions among Rad23-like proteins. Interestingly, we found that Rad23 interacts with itself, Dsk2, and Ddi1 *in vivo* (Fig. 3A). Although the interactions are mediated by the fragment lacking the UBL domain, the UBA motif is not sufficient for the interactions (Fig. 3A). Our results suggest that these Rad23-like proteins may form complex(es) and cooperate with each other. Conceivably, different combinations of Rad23-like proteins may bind multi-Ub chains with different lysine linkages and thus provide chain selectivity or specificity.

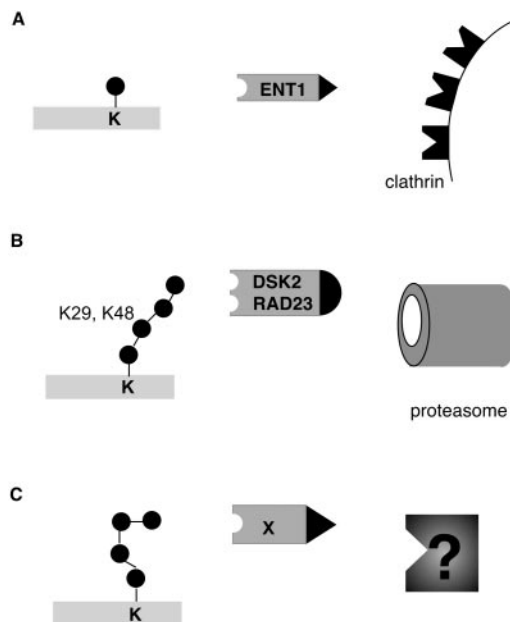
Do these RAD23-like proteins cooperate to function in proteolysis? Proteolysis defects were previously reported for the *rad23Δ* mutant (9). Stronger stabilization of substrates was found in cells lacking both RAD23 and RPN10, which encodes a proteasome subunit that binds multi-Ub chains, suggesting overlapping proteolytic functions between Rad23 and Rpn10 (9, 15). However, it was not known whether DSK2 and DDI1 were involved in proteolysis. DSK2 was shown to be involved in duplication of the spindle pole body, and no proteolytic defects were found in cells lacking DSK2 (20). To determine the roles of Dsk2 in proteolysis and the functional relationship between



**FIG. 3. The *RAD23*-like molecules interact among themselves and function in the UFD proteolytic pathway.** *A*, interactions among the Rad23-like proteins. Two-hybrid analysis was performed as in Fig. 1C. The identity of the baits is shown on the left with the identity of the prey indicated above each panel. *B*, the UFD pathway is impaired in cells lacking *DSK2* and *RAD23*. Pulse-chase analysis of Ub<sup>V76</sup>-V-βgal, a UFD substrate in yeast strains MY3589 (wild-type), MY3587F (*rad23Δ*), MY3588 (*dsk2Δ*), and MY3592 (*rad23Δ dsk2Δ*) was performed as described (7, 18). A dashed line indicates the “ladder” of multiubiquitylated Ub<sup>V76</sup>-V-βgal (18, 19). An asterisk indicates the 90-kDa cleavage product, an intermediate breakdown product of β-gal degradation. *C*, the UBA motif is essential for the proteolytic functions of *DSK2*. Pulse-chase analysis with *S. cerevisiae* MY3588 (*dsk2Δ*) cells co-expressing Ub<sup>V76</sup>-V-βgal with either vector alone (lanes 1–3) or *DSK2*<sup>ΔUBA</sup> expressed from its own promoter (lanes 4–6).

*Dsk2* and *Rad23*, we examined the degradation of substrates of the N-end rule and UFD pathways in cells lacking either or both *RAD23* and *DSK2*. We carried out pulse-chase assays with Ub<sup>V76</sup>-V-βgal, a model substrate targeted by the UFD pathway (18). Deletion of *RAD23* compromised the UFD pathway, as ~35% of Ub<sup>V76</sup>-V-βgal was degraded after a 40-min chase (Fig. 3B, and data not shown) (9). Interestingly, deletion of *DSK2* caused stronger stabilization of the UFD substrate, as ~15% of Ub<sup>V76</sup>-V-βgal was degraded after a 40-min chase (Fig. 3B). The double mutant *dsk2Δrad23Δ*, which is temperature sensitive for growth (20), completely stabilized the substrate at permissive temperature (Fig. 3B). It was noticed previously that a 90-kDa fragment is often produced during degradation of the β-gal protein (9). This 90-kDa β-gal fragment was present in the pulse-chase of wild-type, *rad23Δ*, and *dsk2Δ* cells but absent in *rad23Δdsk2Δ* cells, further supporting that β-gal degradation is totally inhibited in the double mutant (Fig. 3B).

Because the substrate Ub<sup>V76</sup>-V-βgal was ubiquitylated in *rad23Δ* and *dsk2Δ* single mutants, our results suggested that *Rad23* and *Dsk2* act primarily after ubiquitylation. Failure to form complexes may partially account for the compromised UFD pathway in cells lacking *RAD23* and *DSK2* (Fig. 3, *A* and *B*). Our results show that *DSK2* and *RAD23* function in the same proteolytic pathway and are both required for the efficient degradation of the UFD substrate and suggest that *Dsk2* and *Rad23* likely cooperate to mediate Ub-dependent proteolysis. The reduction of multiubiquitylated forms in the *rad23Δdsk2Δ* cells (Fig. 3B) may be due to the lack of protection of ubiquitylated substrates from deubiquitylating enzymes in these mutants, although more experiments are required to further explore this possibility. The N-end rule pathway was unaffected by mutations in *DSK2* and *RAD23* (data not shown).



**FIG. 4. Model for the mechanisms underlying distinct fates of ubiquitylated substrates.** *A*, endocytosis of monoubiquitylated substrates. *Ent1* may use separate domains to bind clathrin and ubiquitin, thereby acting as an adapter in linking a monoubiquitylated target to the endocytic machinery (2, 3). *B*, proteolysis of multiubiquitylated substrates with Lys-29- or Lys-48-based Ub linkage. *Dsk2*, *Rad23*, and *Ddi1* interact and use the UBA domain to bind directly to the Lys-29- or Lys-48-linked Ub chain and the UBL domain to bind to the 19 S proteasome cap. Thus they escort ubiquitylated substrates to the proteasome. Based on this model, we speculate that Lys-27- and Lys-33-linked Ub chains, which are recognized by *Rad23*-like proteins, have proteolytic functions. *C*, other fates of ubiquitylated substrates. We speculate that other adapters could either stabilize the conformation of Ub-tagged substrates or bring substrates modified with Ub chains assembled through different lysines to other proteolytic or non-proteolytic destinations.

Note, however, that the Ub chains of N-end rule substrates utilize a Ub linkage different from those used by UFD substrates. It would be of interest to determine the half-life of N-end rule substrates in cells lacking *DDI1* alone or *DDI1* with the additional mutations in *DSK2* and *RAD23*.

Is the UBA domain important for proteolysis? Previous studies showed that the UBA motif is not important for the function of *Rad23*-like proteins in DNA repair, a role separate from proteolysis (14, 16). In *dsk2Δ* cells, the normally short lived Ub<sup>V76</sup>-V-βgal was long lived (Fig. 3, *B* and *C*). Transformation of *dsk2Δ* cells with a low copy plasmid expressing *DSK2*<sup>ΔUBA</sup> from its own promoter did not rescue the degradation of Ub<sup>V76</sup>-V-βgal (Fig. 3C). In contrast, expression of full-length *DSK2* restored the degradation of Ub<sup>V76</sup>-V-βgal (data not shown). The data suggest that the UBA domain, and likely its ability to bind multi-Ub chains, is essential for the function of UBA-bearing proteins in proteolysis. Furthermore, the expression of *DSK2*<sup>ΔUBA</sup> from its own promoter did not restore the viability of cells lacking *RAD23* and *DSK2* at 37 °C (data not shown), suggesting that its function in proteolysis may be important for cell growth.

#### DISCUSSION

We found that *S. cerevisiae* *Rad23*-like proteins bearing the UBA motif preferentially bind a subset of ubiquitin conjugates and multi-Ub chains that perform proteolytic functions. We have demonstrated that the intact UBA motif is essential for *Dsk2* functioning in proteolysis and likely is important for the proteolytic functions of *Rad23* and *Ddi1*. We found that these proteins bearing the UBA and UBL motifs could interact with

themselves and each other, which may be the basis for the cooperation between Rad23 and Dsk2 in proteolysis.

Ub chains linked through Lys-63 and Lys-48 can both signal proteolysis by the proteasome in an *in vitro* system. Interestingly, *in vivo*, Lys-48-linked chains can still signal proteolysis, but Lys-63-linked chains are recognized as non-proteolytic signals (17). This suggested that specific chain-recognition molecules may exist *in vivo* to determine the functions of these Ub chains. The UBA motif is the first to be shown to exhibit the specificity to Ub conjugates with Lys-29 and Lys-48 linkages. The ability of Rad23-like proteins to interact with both ubiquitylated targets and the proteasome makes them likely adapter molecules for delivering substrates to the proteasome (Fig. 4). Other adapters likely exist to mediate the transport of ubiquitylated substrates to other non-proteolytic or proteolytic destinations (Fig. 4). Because Ent1 was shown to bind clathrin and the presence of the UIM motif suggests the possibility that Ent1 may bind Ub through the UIM motif (2, 3), it can be speculated that Ent may link monoubiquitylated substrates to the endocytic machinery (Fig. 4A). The employment of these adapters offers more selection for substrates and regulation of the Ub system. Proteasomal subunits that are capable of multiubiquitin chain binding may cooperate with RAD23-like molecules in transferring, unfolding, and degrading the substrates, a possibility consistent with the synthetic phenotype enhancement in the double mutant of *rpn10Δrad23Δ* in *S. cerevisiae* and *S. pombe* (9, 15).

The UBL and UBA motifs do not always co-exist in the same protein (13). In an UBA-independent role, Rad23 also has non-proteolytic functions in DNA repair (8, 14, 16). The XPC domain within Rad23 (Fig. 1A) interacts with DNA repair machineries, and the UBL domain within Rad23 interacts with the 19 S proteasome cap (8, 16). In this case, Rad23 acts as an adapter linking the 19 S proteasome regulatory complex to the Nucleotide Excision Repair complex. It is possible that Dsk2 and Ddi1 may similarly link the 19 S proteasome particle to complexes involved in other cellular processes, such as cell cycle progression or transcription elongation. Therefore, understanding the functions of *DSK2* and *DDI1* may lend insights to the non-proteolytic functions of the 19 S proteasome cap. Finally, the UBA motif exists in many proteins that do not

contain the UBL domain. The UBA motifs found in E2, E3, and UBP enzymes may facilitate their functions in the assembly and disassembly of multi-Ub chains. Interestingly, the UBA domain also occurs in proteins that seemingly play no direct role in proteolysis, such as kinases, transcription factors, and splicing factors. However, these proteins or their binding partners may be ubiquitylated, and it is possible that these proteins utilize the UBA motifs to determine the fate of ubiquitylated substrates in ways similar to those depicted in Fig. 4C.

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