The Png1–Rad23 complex regulates glycoprotein turnover

Ikjin Kim,[1] Jungmi Ahn,[1] Chang Liu,[1] Kaori Tanabe,[2] Jennifer Apodaca,[1] Tadashi Suzuki,[2] and Hai Rao[1]

[1]Department of Molecular Medicine, Institute of Biotechnology, University of Texas Health Science Center at San Antonio, San Antonio, TX 78245
[2]Department of Biochemistry and 21st Century Center of Excellence Program, Graduate School of Medicine, Osaka University, Suita, Osaka 565-0871, Japan

Misfolded proteins in the endoplasmic reticulum (ER) are destroyed by a pathway termed ER-associated protein degradation (ERAD). Glycans are often removed from glycosylated ERAD substrates in the cytosol before substrate degradation, which maintains the efficiency of the proteasome. Png1, a deglycosylating enzyme, has long been suspected, but not proven, to be crucial in this process. We demonstrate that the efficient degradation of glycosylated ricin A chain requires the Png1–Rad23 complex, suggesting that this complex couples protein deglycosylation and degradation. Rad23 is a ubiquitin (Ub) binding protein involved in the transfer of ubiquitylated substrates to the proteasome. How Rad23 achieves its substrate specificity is unknown. We show that Rad23 binds various regulators of proteolysis to facilitate the degradation of distinct substrates. We propose that the substrate specificity of Rad23 and other Ub binding proteins is determined by their interactions with various cofactors involved in specific degradation pathways.

Introduction

In eukaryotes, the 26S proteasome handles the majority of regulated proteolysis and is pivotal for the proper functioning of the cell (DeMartino and Slaughter, 1999; Pickart and Cohen, 2004). One important function of selective proteolysis is to remove misfolded proteins. For example, in the ER, misfolded proteins are eliminated by a stringent quality-control process termed ER-associated protein degradation (ERAD; Ahner and Brodsky, 2004; Hirsch et al., 2004a). Only properly folded proteins are allowed to proceed to their destination to carry out their physiological functions.

Most proteins that are targeted to the proteasome for degradation are first modified by the ubiquitin (Ub) system (Schwartz and Hochstrasser, 2003; Pickart and Cohen, 2004). Specifically, successive Ub molecules join to form a Ub chain on the substrates through the concerted actions of several enzymes, including a Ub-activating enzyme (E1), a Ub-conjugating enzyme (E2), and a Ub protein ligase (E3). The ubiquitylated substrate is then delivered to and degraded by the 26S proteasome. Many components involved in the recognition and Ub conjugation of ERAD substrates have been identified, such as E2s and E3s (Ahner and Brodsky, 2004; Hirsch et al., 2004a). How the ubiquitylated proteins are transferred to the proteasome remains elusive (Elsasser and Finley, 2005).

Two interacting proteins, Png1 and Rad23, are suspected to play important roles in the degradation of ERAD substrates (for review see Suzuki et al., 2002). Png1 is a highly conserved protein that resides mainly in the cytosol but also in the nucleus (Suzuki et al., 2000; Hirsch et al., 2003). Functional studies suggest that Png1 is the primary, if not the only, deglycosylating enzyme in the cytosol (Suzuki et al., 2000; Blom et al., 2004; for review see Suzuki et al., 2002). Many ERAD substrates are *N*-glycosylated in the ER (for reviews see Yoshida, 2003; Helenius and Aebi, 2004). Computer modeling suggests that the bulky N-linked oligosaccharide must be cleaved off the substrate before its degradation to maintain the efficiency of the proteasome (Hirsch et al., 2003). Coincidentally, ERAD substrates (e.g., class I myosin heavy chains [MHCs]) are found to be deglycosylated in the cytosol upon the inhibition of proteasome activity (Blom et al., 2004). Png1 preferentially deglycosylates misfolded proteins in vitro (Hirsch et al., 2004b; Joshi et al., 2005) and in cell extracts upon the overexpression of Png1 or glycoproteins (Hirsch et al., 2003). However, a definitive ERAD substrate that requires Png1 for its degradation in vivo has not been identified. The turnover of glycosylated ERAD substrates, including a mutant carboxypeptidase Y (CPY), T cell receptor α chain, and class I MHC, was not drastically affected in yeast *png1*Δ mutant, the Png1 small interfering RNA cell lines, or cells treated with the Png1 inhibitor (Suzuki et al., 2000;

Correspondence to Hai Rao: raoh@uthscsa.edu

Abbreviations used in this paper: CPY, carboxypeptidase Y; EndoH, endoglycosidase H; ERAD, ER-associated protein degradation; MHC, myosin heavy chain; RTA, ricin A chain; Ub, ubiquitin; UBA, Ub-associated; UBL, Ub-like; XPCB, XPC binding; UFD, Ub fusion degradation.

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Blom et al., 2004; Misaghi et al., 2004). These results raise significant doubts about the requirement of Png1 in proteolysis in vivo.

Rad23 belongs to a family of proteins that contains both the Ub-associated (UBA) domain and a Ub-like (UBL) motif (Madura, 2004; Elsasser and Finley, 2005; Fig. 1 A). Notably, the UBA motif binds specifically to Ub chain/conjugates in vivo and in vitro (Bertolaet et al., 2001; Wilkinson et al., 2001; Rao and Sastry, 2002; Raasi and Pickart, 2003). The UBL motif directly binds the proteasome (Schauber et al., 1998; Elsasser et al., 2002). The loss of *Saccharomyces cerevisiae* Rad23 leads to the stabilization of a Ub fusion degradation (UFD) substrate (Lambertson et al., 1999; Rao and Sastry, 2002) and the cell cycle inhibitors Sic1 and Far1 (Verma et al., 2004), and the homologues of Rad23 are involved in the degradation of the Cdk inhibitor Rum1 (Wilkinson et al., 2001) and the tumor suppressor p53 (Glockzin et al., 2003). The stabilized substrates in the *rad23* mutant cells are fully ubiquitylated, suggesting that Rad23 functions at a postubiquitylation but preproteasome step (Rao and Sastry, 2002; Medicherla et al., 2004). Importantly, Rad23 is required for the formation of the proteasome–Ub conjugates complex (Elsasser et al., 2004; Verma et al., 2004). Therefore, Rad23 has been proposed to facilitate the substrate transfer to the proteasome. The mechanism underlying the substrate specificity of Rad23 remains poorly defined. In degrading two ERAD substrates (Deg1-Sec62 and Hmg2), Rad23 binds Ufd2, which is a Ub chain elongation factor, and together they couple substrate ubiquitylation and degradation (Kim et al., 2004; Richly et al., 2005). However, the role of the Png1–Rad23 pathway in ERAD is far from clear (for review see Suzuki et al., 2002).

In this paper, we identify the first in vivo substrate of the Png1- and Rad23-dependent pathway as glycosylated protein ricin A chain (RTA), an Hrd3–Hrd1–dependent ERAD substrate.

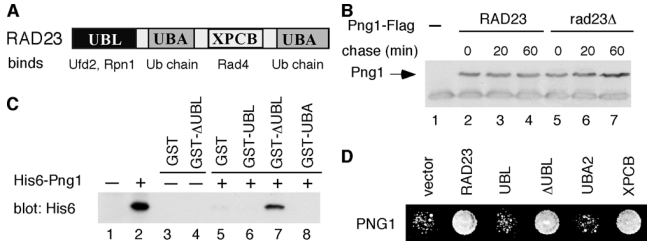

Figure 1. **Interactions between Rad23 and Png1.** (A) Domain organization of Rad23. Proteins that bind to each domain are indicated. (B) Png1 is stable in wild-type and *rad23*Δ cells. Png1 is fused to the Flag epitope, which does not interfere with its function. The stability of Png1 was determined by promoter shutoff assay. Equal sample loading was confirmed by blotting the extracts with anti-Rpt5 antibody (not depicted). The identity of Png1 is indicated on the left. As a negative control, the extract containing no Png1-Flag was processed similarly (lane 1). (C) GST pull-down assays. The mixtures containing glutathione beads, various GST fusion proteins, and yeast extracts with overexpressed His6-tagged Png1 were incubated at 4°C for 2 h. Equal amounts of GST fusion proteins were used (Rao and Sastry, 2002). The bound proteins were eluted, fractioned by SDS-PAGE, and immunoblotted with antibody to His6. Lanes 1 and 2 contain 10% input extracts without or with tagged Png1, respectively. (D) Two-hybrid interactions. Yeast cells were cotransformed with plasmids encoding Gal4 activation domain fused to Png1 indicated on the left with Gal4 DNA binding domain linked to Rad23 or portions of Rad23, as indicated above the panel. Growth on this plate is indicative of protein–protein interaction.

We show that the XPC binding (XPCB) domain–mediated Png1–Rad23 interaction is important for the degradation of glycosylated RTA. Furthermore, we find that both the Ub chain binding activity of Rad23 and the deglycosylation activity of Png1 are required for efficient degradation of glycosylated RTA, suggesting that the Png1–Rad23 complex couples substrate deglycosylation and degradation. Interestingly, Ufd2, another Rad23 binding protein, is required for the degradation of Deg1-Sec62 but not RTA. Our results suggest that Rad23 binds various cofactors (e.g., Ufd2 and Png1) to regulate distinct proteolytic substrates. Finally, we more generally propose that the substrate selectivity of Ub binding proteins (e.g., Rad23, Rpn10, and Cdc48) may be determined by various protein–protein interactions.

Results

The XPCB domain of Rad23 binds Png1

Rad23 interacts with Png1 in yeast and mouse (Park et al., 2001; Suzuki et al., 2001). Mutation analysis indicated that the COOH-terminal fragment of Rad23 containing its substrate-recognition domain (i.e., UBA) binds Png1 (Suzuki et al., 2001). If the Ub chain binding (UBA) domain directly binds Png1, Png1 may be a proteolytic substrate of Rad23. To test for this possibility, we examined the stability of Png1 in wild type and *rad23* mutant. We found that Png1 is stable in both wild-type and *rad23* mutant cells (Fig. 1 B), suggesting that Png1 and Rad23 may form a stable complex in regulating ERAD.

To define the role of the Png1–Rad23 complex, it is critical to determine the domain of Rad23 responsible for Png1 binding. Derivatives of Rad23 containing various functional domains (Fig. 1 A) were tested in the GST pull-down assay for interaction with Png1 (Fig. 1 C). Specifically, $Rad23^{\Delta UBL}$, $Rad23^{UBL}$, and $Rad23^{UBA2}$ were separately fused to the COOH terminus of GST and purified from *Escherichia coli* (Rao and Sastry, 2002). Consistent with a previous study (Suzuki et al., 2001), the $Rad23^{\Delta UBL}$ fragment binds Png1. However, the COOH-terminal UBA domain alone is not sufficient for Png1 binding (Fig. 1 C).

To further examine the binding between Png1 and Rad23 in vivo, derivatives of Rad23 (Rao and Sastry, 2002) were tested for interaction with Png1 by the two-hybrid assay (Fig. 1 D). Interestingly, our findings reveal that the XPCB domain (amino acids 250–307) is sufficient for the interaction with Png1 (Fig. 1 D), which is consistent with a recent structural analysis of the Png1–Rad23 complex (Lee et al., 2005). The XPCB domain binds Rad4 (called XPC in mouse; Fig. 1 A) and is essential for the functioning of Rad23 in DNA repair (Masutani et al., 1997). Note that Rad4 is not part of the Png1–Rad23 complex (Suzuki et al., 2001). The results suggest a novel function of the XPCB domain outside of DNA repair.

Glycosylated RTA is degraded by the Rad23- and Png1-dependent ERAD pathway

Because Png1 is not a substrate of Rad23, we hypothesized that the Png1–Rad23 complex may regulate substrate proteolysis.

What substrates do Rad23 and Png1 share? The UBA/UBL proteins Rad23 and Dsk2 were recently shown to be required for the efficient turnover of the ERAD substrates CPY (Medicherla et al., 2004) and Deg1-Sec62 (Richly et al., 2005). However, we could not detect obvious stabilization of CPY in *png1*Δ cells and cells lacking *RAD23* and *DSK2* under the assay condition (unpublished data), and Deg1-Sec62 does not require Png1 for its degradation (see Fig. 5).

The ERAD pathway can be exploited by viruses (e.g., human cytomegalovirus) and toxins (Hirsch et al., 2004a; Roberts and Smith, 2004). We found that RTA is a substrate of Png1. Ricin is a plant protein toxin consisting of A and B subunits (Simpson et al., 1999; Roberts and Smith, 2004). RTA is the catalytic subunit of ricin, which inhibits protein synthesis. Ricin uses multiple endocytic routes to enter cells and is then transported into the ER. Separated from the B subunit, RTA is degraded by the cytosolic proteasome in yeast, plants, and mammals (Simpson et al., 1999; Roberts and Smith, 2004). RTA degradation in yeast requires the Sec61 translocon and the proteasome, suggesting that RTA is an ERAD substrate (Simpson et al., 1999).

Because RTA is glycosylated (Simpson et al., 1999), we wanted to test whether glycosylated RTA is a substrate of the Png1–Rad23 complex in yeast. We appended Flag epitope to a misfolded RTA with a short deletion surrounding its active site to eliminate RTA-induced toxicity. RTA was fused to the yeast Kar2 signal sequence to be targeted to the ER. In wild-type yeast cells, we detected two immunoreactive bands corresponding to RTA modified with no (g0 form) or one (g1 form) sugar chain (Fig. 2 A, lanes 1 and 2). To ascertain that the g0 form does not contain untranslocated RTA, RTA was expressed in the *sec65-1* mutant that is defective in the cotranslational translocation of ER proteins (Ng et al., 1996). We detected an endoglycosidase H (EndoH)–resistant form of RTA located between the g1 and g0 bands (Fig. 2 A, lanes 3 and 4), which was likely the product of defective translocation, suggesting that RTA is efficiently translocated in wild-type cells (Fig. 2 A, lanes 1 and 2).

To identify the components critical for the degradation of RTA, we evaluated RTA degradation in cells lacking *DOA10* or *HRD3*, which define two major ERAD pathways in yeast (Ahner and Brodsky, 2004; Hirsch et al., 2004a). Although Doa10 is a single-component E3, Hrd3 and -1 (a RING [really interesting new gene] finger–containing protein) form an E3 complex. We found that glycosylated (g1) but not nonglycosylated (g0) RTA was stabilized in the *hrd3* mutant, suggesting that the g1 and g0 forms of RTA are degraded by different pathways (Fig. 2 B). Glycosylated RTA was also stabilized in cells lacking *HRD1* (unpublished data), indicating that glycosylated RTA

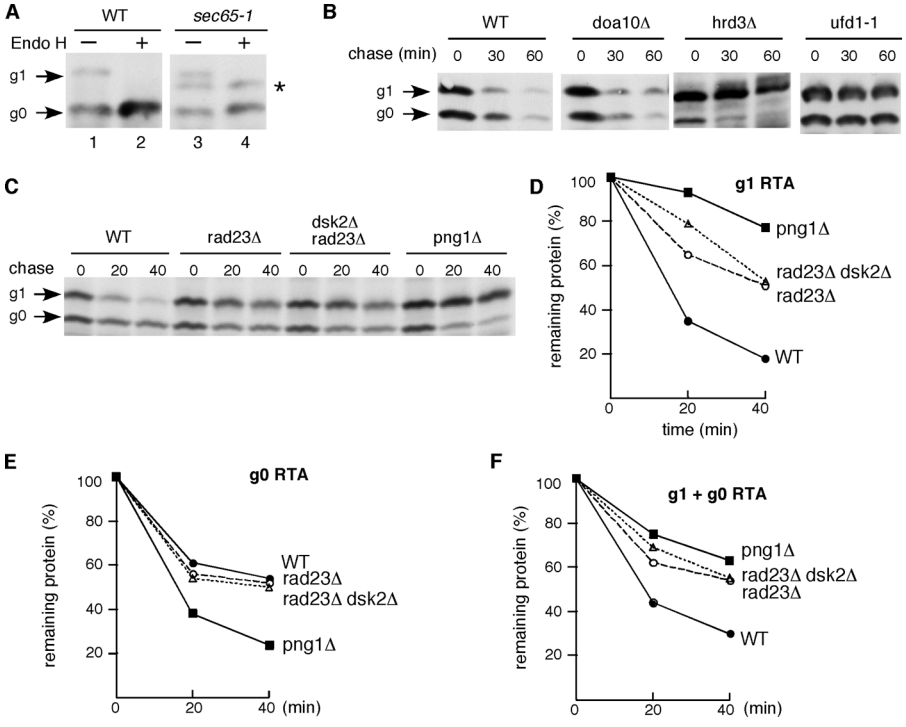

Figure 2. **Degradation of RTA in various yeast mutants.** (A) Expressed RTA proteins exist in both g1 and g0 forms in yeast. Flag-tagged RTA was overexpressed in wild-type or *sec65-1* mutant yeast cells and recovered by immunoprecipitation. RTA immunoprecipitates were mock treated (−) or digested (+) with EndoH, resolved by SDS-PAGE, and visualized by immunoblotting. The arrows indicate RTA proteins modified with one (g1) or no (g0) glycan. In *sec65-1* cells (lanes 3 and 4), untranslocated RTA containing its ER signal sequence was also detected and is indicated by an asterisk. (B) Involvement of known ERAD components in RTA degradation. Wild-type (BY4741) and mutant cells containing a *GAL1* promoter–regulated Flag-RTA were first grown in raffinose-containing media. Expression of RTA was induced by the addition of galactose. Samples were taken after promoter shutoff at intervals and analyzed by anti-Flag Western blots. Equal amounts of protein extracts were used and confirmed by blotting with anti-Rpt5 antibody in all the promoter shutoff experiments (not depicted). (C) Efficient degradation of glycosylated RTA requires Rad23 and Png1. Pulse-chase analysis of RTA in wild-type and mutant cells was performed as described in Material and methods. (D–F) Quantitation of the data in C for glycosylated (g1) RTA, g0 RTA, and the combined intensity of both g1 and g0 RTA. The amount of proteins was determined by phosphorimager analysis.

is a substrate of the Hrd1–Hrd3 E3 complex. Furthermore, the Ufd1–Cdc48–Npl4 complex has been proposed to extract ubiquitylated proteins out of the ER (Hampton, 2002). We found that both the g1 and g0 forms of RTA were stabilized in *ufd1-1* mutant cells (Fig. 2 B).

We next investigated RTA turnover in yeast cells lacking *PNG1*, *RAD23*, and/or *DSK2*. Significantly more (g1 + g0) RTA proteins accumulate in cells lacking *RAD23* or *PNG1* (Fig. 2, C and F), suggesting that RTA is a proteolytic substrate of Png1 and Rad23. The half-life of glycosylated RTA is ∼15 min in wild-type cells, compared with ∼45 min in *rad23*Δ and >90 min in *png1*Δ (Fig. 2, C and D). Importantly, the degradation kinetics of the g0 form is similar in wild-type, *rad23*Δ, and *rad23*Δ *dsk2*Δ cells (Fig. 2 E), suggesting that the degradation of the g1, but not the g0, form of RTA is impaired in *rad23*Δ and *rad23*Δ *dsk2*Δ cells. The role of Rad23 in glycosylated RTA turnover is also supported by the compromised degradation of the g1 form of RTA in *rad23* mutants defective in binding to Png1 or Ub (Fig. 3 D and Fig. 4 G).

Glycosylated RTA clearly requires Png1 for its degradation (Fig. 2, C, D, and F). We note that the disappearance of glycosylated RTA is faster in *rad23*Δ than that in the *png1*Δ mutant (Fig. 2, C and D), suggesting that Rad23-independent Png1 function may exist. Further, the g0 form is degraded faster in *png1*Δ than in wild-type or *rad23*Δ cells (Fig. 2, C and E). Therefore, some of the g1 RTA may be converted to g0 instead of being degraded by the Png1–Rad23 pathway. The parsimonious interpretation of the available data is that there are two pools of Png1, Rad23 bound and Rad23 free, which define two parallel pathways for the disappearance of g1 RTA. More specifically, the degradation pathway of g1 RTA is mediated by the Png1–Rad23 complex, and the deglycosylation pathway is performed by Rad23-free Png1. As a result, some of the g0 RTA in wild-type and *rad23*Δ cells is derived from g1 RTA. This would explain the faster disappearance of g0 RTA in *png1*Δ than in wild-type or *rad23*Δ cells (Fig. 2, C and E).

It is important to note that the profile of g0 RTA is similar in wild-type and *rad23*Δ cells (Fig. 2 E), suggesting that an increased amount of Rad23-free Png1 in *rad23*Δ cells does not enhance the deglycosylation of g1 RTA. (In Fig. 1 B, we show that Png1 stability is not altered in *rad23*Δ cells.) Therefore, the pool of Rad23-free Png1 that deglycosylates g1 RTA in wild-type cells is likely in complex with other proteins or at a location different from the cytosolic Png1–Rad23 complex (Suzuki et al., 2001). This model of two parallel Png1 pathways could also explain the slower disappearance of only g1 RTA in *rad23*Δ cells than in wild-type cells (Fig. 2, D and E) as the degradation pathway, but not the deglycosylation route, for g1 RTA is abolished in *rad23*Δ cells.

RTA degradation proceeds normally in cells lacking *DSK2* (unpublished data). Deletion of *DSK2* did not further stabilize RTA in *rad23*Δ cells (Fig. 2 C). It is worth noting that other previously tested Rad23 substrates (e.g., Ub^{V76}-V-βgal, Hmg2, and Deg1-Sec62) are more stable in *rad23 dsk2* double-mutant cells than in either single mutant, suggesting that Rad23 and Dsk2 have redundant roles (Rao and Sastry, 2002; Medicherla et al., 2004). Our results show that Rad23 and Dsk2 can play nonoverlapping functions in substrate proteolysis.

To further understand the requirement of Png1 and Rad23 for glycosylated RTA degradation, we constructed a cytosolic version of RTA (cyRTA), which lacks the ER-targeting signal sequence and likely resides in the cytoplasm. The cyRTA is not glycosylated (unpublished data). We found that cyRTA is degraded by a Png1–Rad23–independent pathway (unpublished data), suggesting that the involvement of Png1 and Rad23 in RTA turnover requires prior glycosylation in the ER.

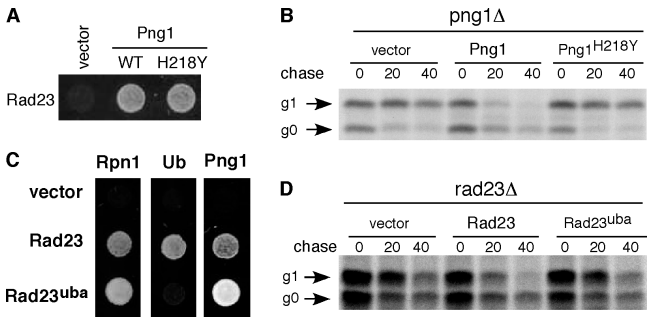

Figure 3. **The abilities to remove glycans and bind Ub chains are required for the functioning of the Png1–Rad23 complex.** (A) The $Png1^{H218Y}$ mutant binds Rad23. This shows a two-hybrid analysis of interactions between Png1 derivatives and Rad23. The experiments were performed as in Fig. 1 D. (B) Degradation of glycosylated RTA is compromised in the $Png1^{H218Y}$ mutant. We cotransformed the plasmids harboring the *PNG1* wild type or H218Y mutation with a plasmid expressing Flag-RTA to *png1*Δ cells. Pulse-chase experiments were done as described in Fig. 2 C. (C) $Rad23^{uba}$, which contains the double mutation L183A and L392A, binds Rpn1 and Png1 but not Ub. A two-hybrid assay was used to examine the interactions between Rad23 derivatives and the Rad23 binding partners Png1, Rpn1, and Ub. (D) Glycosylated RTA degradation is impaired in Rad23 mutants defective in Ub binding. Effect of Rad23 mutations on RTA degradation was determined by pulse-chase analysis.

Ub binding and deglycosylation activity are required for the functioning of the Png1–Rad23 complex

The Png1–Rad23 complex can directly couple protein deglycosylation and degradation, two important postubiquitylation events. What are the activities required for the functioning of the Png1–Rad23 complex in ERAD.

Although computer modeling suggests that bulky sugar chains may clog up the proteasome (Hirsch et al., 2003), and ERAD substrates are found to be deglycosylated in cytosol upon the inhibition of the proteasome activity (Blom et al., 2004), it remains to be demonstrated that deglycosylation is indeed a prerequisite of proteasome-mediated degradation. In fact, an inhibitor of Png1 activity blocks deglycosylation but not degradation of class I MHCs (Misaghi et al., 2004). To examine the role of Png1-mediated deglycosylation in ERAD, we used a His218Tyr mutation that abolishes the enzymatic activity of Png1 (Suzuki et al., 2000). The Png1 His218Tyr mutant interacts with Rad23 (Fig. 3 A). We found that RTA degradation in *png1*Δ cells is restored by wild-type but not mutant Png1 (Fig. 3 B), indicating that the enzymatic activityof Png1 is essential for its function in ERAD.

Is Ub chain binding activity required for the functioning of Rad23? It is conceivable that Rad23 can bring substrates

processed by Png1 to the proteasome through the UBL domain without the help of the UBA domain (Fig. 1 A). In this case, Ub chains attached to RTA are not required for proteasome targeting but rather are important for other functions, such as facilitating substrate translocation (Hampton, 2002). To establish the role of the UBA domains of Rad23 in glycoprotein turnover, we introduced the double mutation L183A and L392A into the UBA domains of Rad23 to inactivate its Ub binding ability (Bertolaet et al., 2001; Fig. 3 C). The Rad23$^{L183A,\ L392A}$ mutant still binds Png1 and Rpn1 (Fig. 3 C), suggesting that the mutation specifically eliminates its Ub binding activity.

The plasmid bearing the *RAD23* wild type or mutant was cotransformed with a plasmid expressing Flag-RTA to *rad23*Δ cells. Wild-type but not mutant Rad23 fully restored the degradation of glycosylated RTA in *rad23*Δ cells (Fig. 3 D), suggesting that the Ub chain binding activity of Rad23 is critical for its functioning in degradation. Our results support the essential role of Ub chain binding activity of Rad23 for substrate proteolysis in vivo.

Mutation in the XPCB domain of Rad23 specifically alters its binding to Png1

To establish the significance of the Rad23–Png1 complex, we looked for mutations in Rad23 that would alter its binding to Png1. We compared sequences of XPCB domains of various Rad23 to identify highly conserved residues among them (Fig. 4 A). We constructed eight variants of the XPCB domain of Rad23, each containing a mutation in one of the conserved residues (Fig. 4 A). These mutations were introduced into full-length Rad23 linked to the Gal4 DNA binding domain. The two-hybrid assay was used to determine their interactions with Png1 (XPCB domain dependent), Rad4 (XPCB domain dependent), Rpn1 (UBL domain dependent), Ufd2 (UBL domain dependent), Ub (UBA dependent), and Rad23 (UBA-dependent self-dimerization; Fig. 1 A). Four mutations—Q267V, Q267Y, N272A, and N297G—did not affect the bindings to Png1 and Rad4 (unpublished data). Three mutations—D261A, P273G, and L280A—reduced but did not eliminate the interactions with both Png1 and Rad4. These three mutations may significantly

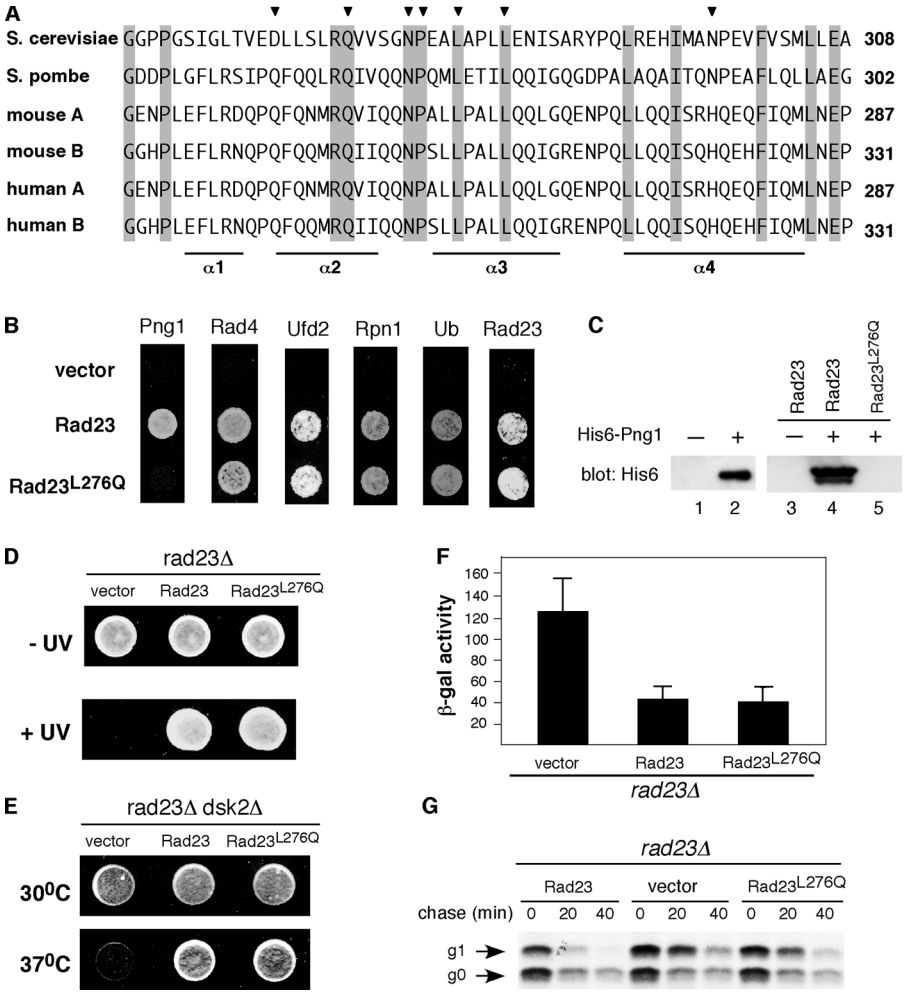

Figure 4. **Mutation L276Q in Rad23 specifically affects its binding to Png1 and its function in glycosylated RTA degradation.** (A) Sequence alignment of the XPCB domain of Rad23 from yeast, mouse, and human. Conserved residues are indicated by the gray boxes. Mutations constructed are indicated above the sequences. Four α helices are indicated below the sequences. (B) Two-hybrid analysis of interactions between Rad23 derivatives and the Rad23 binding proteins Png1, Rad4, Rpn1, Ufd2, Ub, and Rad23. The baits are indicated on the left of the panels. The experiments were performed as in Fig. 1 D. (C) Purified Rad23^{L276Q} mutant does not bind Png1. Lanes 1 and 2 contain 5% input extracts without or with tagged Png1, respectively. Flag-tagged, wild-type, and mutant Rad23 were expressed in *E. coli* and purified to homogeneity (not depicted) by previously described procedures (Kim et al., 2004). The mixtures containing Flag beads, equal amounts of Rad23 or Rad23^{L276Q} proteins, and yeast extracts with His6-tagged Png1 were incubated at 4°C for 2 h. The bound proteins were eluted, fractioned by SDS-PAGE, and immunoblotted with antibody to His6 (lanes 3–5). (D) The Rad23 mutant maintains a fully functional nucleotide excision repair pathway. Yeast cultures were grown to an OD A600 of ~0.5 and were spotted onto YPD media. Cells were left untreated or exposed to various doses of UV radiation from 20 to 150 J/m^2. The plates were incubated at 30°C for 2–4 d. *rad23*Δ cells were transformed with a low-copy plasmid with or without expressing *RAD23* derivatives from the *RAD23* promoter as indicated. The RAD23 alleles on the plasmids are labeled above the panel. No difference was observed between wild-type and mutant Rad23 in these experiments. The plate exposed to 20 J/m^2 UV radiation is shown. (E) Temperature sensitivity of cells lacking *RAD23* and *DSK2* is restored by the L276Q mutant. Yeast strains were grown to an OD A600 of ~1.2 and were spotted onto YPD media. The plates were incubated at 30 and 37°C for 2–5 d. The plasmids transformed are labeled above the panel. (F) The UFD pathway is not affected by the L276Q mutation. Levels of β-gal activity in *rad23*Δ cells carried a plasmid bearing Ub^{V76}-V-βgal, a UFD substrate, and a low-copy plasmid with or without expressing *RAD23* derivatives as indicated. The experiments were done as previously described (Kim et al., 2004), and the average values of three experiments with standard deviation are shown. (G) Degradation of glycosylated RTA is impaired in *rad23* mutant cells. Pulse-chase analysis was performed with yeast *rad23*Δ cells coexpressing Flag-RTA with either vector alone or Rad23 derivatives as indicated.

alter the overall structure of Rad23 and were not further analyzed. Interestingly, the L276Q mutation specifically abolished its interaction with Png1 but maintained the bindings with other partners—Rad4, Rpn1, Ufd2, Ub, and Rad23 (Fig. 4 B)—indicating that the Png1 binding is separable from these other interactions. The defective binding of the Png1 and Rad23 L276Q mutant was also confirmed by coimmunoprecipitations (Fig. 4 C). Therefore, the L276Q mutant was further characterized to determine the specific role of the Png1–Rad23 complex in various Rad23-dependent pathways.

Mutation in Rad23 impairs the degradation of RTA but maintains its roles in DNA repair, cell survival at 37°C, and the UFD proteolytic pathway

To understand the function of the Png1–Rad23 complex in vivo, the mutation L276Q was introduced into full-length Rad23 under the control of its own promoter on a low-copy plasmid to avoid potential artifacts caused by overexpression. Rad23 is known to function as a DNA damage recognition factor in nucleotide excision repair through the binding of Rad4 (Masutani et al., 1997; Ortolan et al., 2004). We examined the UV sensitivity of *rad23*Δ cells expressing the *RAD23* allele. Consistent with the unaltered binding between Rad4 and the Rad23^{L276Q} mutant (Fig. 4 B), UV resistance was restored in *rad23*Δ cells expressing the Rad23^{L276Q} mutant (Fig. 4 D), suggesting that the Rad23 mutant is sufficient in fulfilling the function of Rad23 in the DNA repair.

The UBA/UBL proteins Rad23 and Dsk2 are important for yeast survival under several stress conditions. Cells lacking both *RAD23* and *DSK2* are known to be unviable at 37°C (Biggins et al., 1996). We found that the L276Q mutant restored the growth of *rad23*Δ *dsk2*Δ cells at 37°C (Fig. 4 E), suggesting that the Png1–Rad23 interaction is not required for cell growth at higher temperature.

Rad23 is also known to regulate the UFD pathway in yeast through its UBL domain–mediated association with Ufd2 (Kim et al., 2004). Is the XPCB domain required for a functional UFD pathway? The plasmids bearing *RAD23* variants were cotransformed with a plasmid expressing the UFD substrate Ub^{V76}-V-βgal (Johnson et al., 1995) to *rad23*Δ cells. We used the LacZ assay to gauge the effects of the XPCB mutation on the intracellular concentration of the UFD substrate (Kim et al., 2004). *rad23*Δ cells had much higher levels of β-gal activity than wild-type cells (Kim et al., 2004). The expression of *RAD23* in the *rad23*Δ cells restored the low levels of β-gal activity (Fig. 4 F). Interestingly, lower levels of β-gal activity were detected in *rad23*Δ cells expressing the *RAD23* mutant (Fig. 4 F), suggesting that Png1 binding is not important for the degradation of the UFD substrate.

Next, we examined the effect of the L276Q mutation on the degradation of RTA by measuring the stability of RTA in yeast cells with pulse-chase assay. Glycosylated RTA is stabilized in *rad23*Δ cells (Fig. 4 G). The expression of wild-type but not L276Q mutant *RAD23* in the *rad23*Δ cells restored rapid degradation of glycosylated RTA (Fig. 4 G), suggesting that the amino acid residue L276 is essential for the proteolytic function of *RAD23* in ERAD. Differential effects of L276Q on two distinct proteasomal substrates, RTA and UFD, support that Rad23 uses multiple means in regulating various substrates (see the following paragraph). Our combined results suggest that the XPCB domain–mediated Png1 interaction is essential for the function of Rad23 in the degradation of glycosylated RTA but not in DNA repair, cell survival at 37°C, and the UFD proteolytic pathway. Combined (Figs. 3 and 4), our results suggest that the Png1–Rad23 complex can couple substrate deglycosylation and degradation.

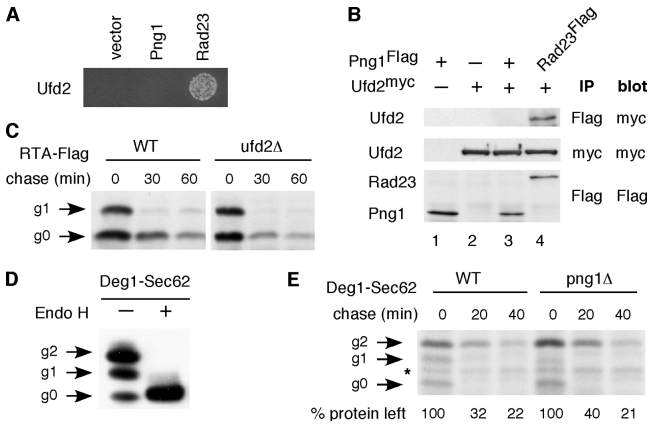

Figure 5. **Ufd2 and Png1 define two distinct Rad23-dependent pathways.** (A) Ufd2 does not interact with Png1. This shows a two-hybrid analysis of interactions between Ufd2 and Png1 or Rad23. The bait and preys used are indicated. (B) Coimmunoprecipitation analysis of interactions between Ufd2 and Png1 or Rad23. Proteins were extracted from yeast cells expressing various epitope-tagged proteins as indicated and immunoprecipitated with beads coupled to various antibodies. Immunoprecipitates were separated on SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and probed with antibodies. The identity of the bands is indicated on the left. The antibodies used for immunoprecipitation (IP) and Western blot (blot) are indicated to the right of the panels. (C) Degradation of RTA is not regulated by Ufd2. Flag-RTA was expressed in wild-type (BY4741) and *ufd2*Δ cells. The experiments were performed as in Fig. 2 B. (D) Deg1-Sec62 is glycosylated. Flag-tagged Deg1-Sec62 was recovered by immunoprecipitation with Flag beads and incubated with or without EndoH. Proteins were resolved by SDS-PAGE and immunoblotted with anti-Flag antibody. (E) Degradation of Deg1-Sec62 is Png1 independent. Flag-tagged Deg1-Sec62 was expressed in wild-type (W303-1A) and *png1*Δ cells. Pulse-chase experiments were performed as described in Material and methods. The asterisk denotes a protein cross-reacting with anti-Flag antibody. The amount of Deg1-Sec62 left is indicated at the bottom of each lane.

Ufd2 and Png1 regulate different Rad23 substrates

Recently, Ufd2, a Ub chain elongation factor, was shown to be required for the efficient turnover of two membrane-associated ERAD substrates, Hmg2 and Deg1-Sec62 (Richly et al., 2005). We previously demonstrated that Ufd2 binds Rad23 and the resulting complex is important for the functioning of Rad23 in the UFD pathway (Kim et al., 2004). Does Png1 bind Ufd2? We did not detect the interaction between Ufd2 and Png1 (Fig. 5, A and B). Furthermore, Ufd2 is not critical for the degradation of glycosylated RTA (Fig. 5 C).

We found that Deg1-Sec62 is also glycosylated in vivo (Fig. 5 D). We evaluated Deg1-Sec62 degradation in cells lacking *PNG1*, and we found that the degradation of Deg1-Sec62

was unaltered in *png1*Δ cells (Fig. 5 E), indicating that not all glycoproteins are degraded by the Png1 pathway. Our results suggest that Rad23 regulates the degradation of distinct substrates through its interactions with various cofactors that are involved in specific proteolytic pathways.

Discussion

In this paper, we show that glycosylated RTA is an in vivo substrate of the Png1–Rad23 degradation pathway. Furthermore, we demonstrate that the efficient degradation of glycosylated RTA also requires the association between Png1 and Rad23, which in turn binds ubiquitylated substrate and removes *N*-glycans from the substrate. The physiological significance of Png1 in ERAD is not clear because the in vivo degradation of misfolded glycoproteins was not significantly altered in cells defective of Png1 activity (Blom et al., 2004; Misaghi et al., 2004). Because many ERAD substrates are deglycosylated before their degradation (Hirsch et al., 2003; Blom et al., 2004; Joshi et al., 2005), Png1 has long been suspected to play a critical role in this process (for reviews see Suzuki et al., 2002; Yoshida, 2003). Png1 can remove *N*-glycans from several glycosylated proteins in vitro (Hirsch et al., 2004b; Joshi et al., 2005) and in vivo when Png1 is overexpressed (Hirsch et al., 2003). In a recent review, Png1 was proposed to regulate glycoprotein turnover universally (Yoshida, 2003). However, unaltered degradation of glycosylated class I MHCs in cells treated with a Png1 inhibitor and in Png1 small interfering RNA cell lines challenged the requirement of Png1 in glycoprotein turnover (Blom et al., 2004; Misaghi et al., 2004). Our results demonstrate that Png1 plays a key role in the degradation of a subset of glycosylated ERAD substrates. We also show that not all glycoproteins are degraded by the Png1 pathway (Fig. 5), suggesting that Png1 is not universally required for glycoprotein turnover. It will be of interest to determine how these Png1-independent glycoproteins are degraded, such as the regulators involved and whether other deglycosylating activities are required.

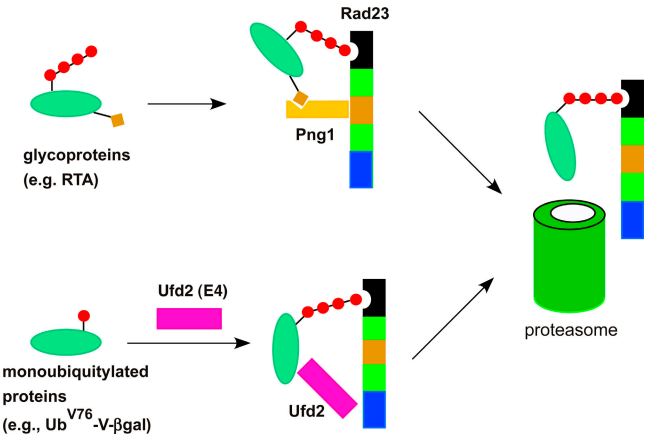

Figure 6. **Schematic model for Rad23-mediated substrate proteolysis.** Rad23 uses different cofactors to escort distinct substrates to the proteasome. Domains of Rad23 are colored as follows: blue for the UBL motif, brown for the XPCB element, and black for the COOH-terminal UBA domain. Depicted in the top pathway, glycosylated ER proteins (e.g., RTA) are ubiquitylated and transported back to the cytosol. Png1 removes N-linked glycan. The XPCB domain of Rad23 binds Png1, which in turn facilitates the substrate recognition of Rad23. Through interactions with Ub chains and the proteasome mediated by the UBA and UBL domains in Rad23, Rad23 facilitates substrate transfer to the proteasome. The red dots depict Ub, and the brown diamonds depict sugar moiety. In the bottom pathway, Ufd2 (E4) catalyzes Ub chain elongation to substrates attached with one or two Ub (e.g., Spt23^{p90} and Ub^{V76}-V-βgal, possibly Deg1-Sec62). Through interactions mediated by the UBL and UBA domains in Rad23 with Ufd2 and Ub chains, Rad23 recognizes the substrate and facilitates substrate transfer to the proteasome. See Discussion for details.

The Png1–Rad23 complex directly couples protein deglycosylation and degradation (Figs. 3 and 4), thereby ensuring rapid turnover of misfolded glycoproteins and maintaining more efficient proteasomes. At the molecular level, what is the biological function of the Png1–Rad23 interaction? Nuclear magnetic resonance studies indicate that Rad23 contains four structured regions: UBL, XPCB, and two UBA domains (Fig. 1 A; Walters et al., 2003). Further, the intramolecular interactions between the UBL element and the UBA domains keep Rad23 in a closed conformation. Interestingly, the interaction with the proteasome induces Rad23 to adopt an open conformation (Walters et al., 2003) that is active for proteolysis. Rad23 exists in a stable complex with Png1. It is tempting to speculate that binding of Png1 may also open up the conformation of Rad23, which in turn facilitates its bindings to the proteasome and/or ERAD substrates. We propose that the XPCB domain–mediated Png1–Rad23 interaction facilitates not only substrate recognition of Rad23 and/or Png1 but also the direct transfer of deglycosylated ERAD substrates to the proteasome, which binds the UBL domain of Rad23 (Fig. 6).

Rad23 also binds Ufd2, a Ub chain elongation factor. The Ufd2–Rad23 association is important for the degradation of UFD substrates (Kim et al., 2004), the transcription factor Spt23^{p90}, and two ERAD substrates (i.e., Hmg2 and Deg1-Sec62; Richly et al., 2005). The Ufd2–Rad23 interaction likely couples substrate ubiquitylation and degradation (Fig. 6; Kim et al., 2004). At present, it remains to be determined why some ERAD substrates (e.g., RTA) require Png1 for their degradation, whereas other Rad23-regulated ERAD substrates (e.g., Deg1-Sec62 and Hmg2) are degraded by the Ufd2 pathway. It is possible that the nature of glycosylation (e.g., the attachment site, structure, and number of glycans attached) may influence the substrate degradation. There exists a wide array of ERAD substrates, and it will be important to understand the factors that divert various glycoproteins to distinct ERAD pathways (Ahner and Brodsky, 2004; Vashist and Ng, 2004). One obvious contributing factor is the localization of the substrates because RTA is soluble and Deg1-Sec62 is embedded in the ER membrane. Recent findings suggest that at least two checkpoints are used to sort out ERAD substrates to different degradation pathways based on the location of the misfolded domain (e.g., membrane, lumen, or cytosol) and the topology of the protein (Taxis et al., 2003; Vashist and Ng, 2004). We are systematically determining the involvements of Rad23 and Png1 in the turnover of these different types of ERAD substrates.

Our results not only reveal how Rad23 regulates the degradation of various substrates but also may affect the studies on

other Rad23-like proteins. An increasing number of proteins have been shown to bind Ub and/or Ub chains in yeast and human. Many of these proteins play important regulatory functions in diverse cellular pathways because cells without them exhibit different phenotypes, suggesting distinct substrate selectivity (Verma et al., 2004; Elsasser and Finley, 2005). How do these Ub binding proteins achieve their substrate specificity? It is worth noting that in regulating two distinct Rad23-dependent proteolytic substrates (i.e., RTA and Deg1-Sec62), Rad23 uses the XPCB domain and the UBL motif to interact with two key players, Png1 and Ufd2, in these pathways (Fig. 6). Our results indicate that these interactions are essential for the functioning of Rad23 in these processes (Fig. 4; Kim et al., 2004). More generally, various protein–protein interactions may be used to facilitate the functions of Rad23 and other Ub binding proteins (e.g., Rpn10 and Cdc48) in Ub-mediated processes.

Rad23 binds Rad4/XPC in nucleotide excision repair (Watkins et al., 1993; Masutani et al., 1997; Schauber et al., 1998; Russell et al., 1999). We have also uncovered a novel function of the XPCB domain of Rad23 in Ub-mediated proteolysis. Rad23 and Png1 are highly conserved from yeast to human. Two Rad23 homologues, hHR23A and -B, exist in humans. Interestingly, XPC is mainly found in complex with hHR23B instead of -A (Ng et al., 2003). It is possible that Png1 and/or Ufd2 mainly associate with one of the two homologues.

Materials and methods

Yeast strains and plasmids

S. cerevisiae PJ69-4A (*MATa lys2-801 ura3-52 gal4Δ gal80Δ GAL2-ADE2 LYS2::GAL1-HIS3 met::GAL7-lacZ*) was used for two-hybrid assays. Isogenic strains W303-1A, *rad23Δ*, and *png1Δ* (*png1::S.pombe his5*$^{+}$) were previously published (Suzuki et al., 2000). Yeast strains YHR114 (*dsk2::LEU2*) and YHR132 (*rad23 dsk2::LEU2*) were constructed by replacing *DSK2* with *LEU2* in strains W303-1A and *rad23Δ*, respectively. Cultures were grown in rich (yeast extract/peptone/dextrose [YPD]) or synthetic media containing standard ingredients and 2% glucose (SD medium), 2% raffinose (SR medium), or 2% raffinose + 2% galactose (SRG medium). Yeast strains lacking *UFD2*, *HRD1*, *DOA10*, or *HRD3* were obtained from Open Biosystems. *ufd1-1* and *sec65-1* strains were obtained from E. Johnson (Thomas Jefferson University, Philadelphia, PA) and D. Ng (National University of Singapore, Singapore, Singapore).

The plasmids containing *RAD23* wild type and its derivatives or His6-tagged Png1 and Png1-1 (H218Y) mutant were previously described (Suzuki et al., 2000; Kim et al., 2004). *PNG1* was amplified by PCR to incorporate the Flag epitope and cloned to the 3′-end of the *GAL1* promoter in pRS414Gal1 for its expression. A nontoxic allele of RTA (Simpson et al., 1999) was amplified by PCR to incorporate the Flag epitope at its COOH terminus and fused downstream to the yeast Kar2 signal sequence to pRS-416Gal1 vector to construct an ER version of RTA. Mutations in the XPCB domain or UBA domains of Rad23 were obtained using the Quick Change mutagenesis kit (Stratagene).

Expression shutoff assay

Yeast cells carrying plasmids that expressed Flag-tagged Png1 or RTA from the P_{GAL1} promoter were grown at 30°C to an OD_{600} of ∼1 in SR-ura medium with auxotrophic supplements and 2% raffinose as the carbon source. Expression of Flag-Png1 or Flag-RTA was induced with galactose for 1 h and then repressed by the addition of 2% glucose. Samples were withdrawn at the indicated time points and harvested by centrifugation. Proteins were extracted and processed for immunoprecipitation with Flag antibody (Sigma-Aldrich), followed by SDS–9% PAGE, as described previously (Kim et al., 2004). Immunoblots were probed with anti-Flag antibody (Sigma-Aldrich) and goat anti–mouse HRP conjugate and were developed using ECL reagents (GE Healthcare). The stable protein Rpt5 was used as a loading control to ensure that an equal amount of extracts was used in expression shutoff experiments.

Pulse-chase analysis

Pulse-chase analysis was done as described previously (Rao and Sastry, 2002). Yeast cells carrying plasmids that expressed Flag-tagged RTA or Flag-tagged Deg1-Sec62 from the P_{GAL1} promoter were grown at 30°C to an OD_{600} of ∼1 in SRG medium with auxotrophic supplements and 2% raffinose and galactose as the carbon sources. Cells were harvested, washed with 0.8 ml SRG, resuspended in 0.4 ml SRG, and labeled for 8 min with 0.16 mCi of ^{35}S-Express (PerkinElmer), followed by centrifugation and resuspension of cells in SD medium with 4 mM methionine and 2 mM cysteine. 0.1-ml samples were taken at the indicated time points and processed for immunoprecipitation with Flag beads (Sigma-Aldrich), followed by SDS-PAGE and autoradiography. The amount of proteins was quantified by phosphorimager analysis.

GST binding assays

GST fusion proteins were purified as previously described (Rao and Sastry, 2002). GST fusion protein or GST alone (∼2 μg) was mixed with yeast extracts containing His6-Png1 in 200 μl of binding buffer (Rao and Sastry, 2002) and incubated with 10 μl (bed volume) of glutathione-agarose beads (Sigma-Aldrich) for 2 h at 4°C. The beads were washed three times with the binding buffer, followed by SDS-PAGE of the retained proteins and immunoblotting with anti-His6 antibody (GE Healthcare).

Coimmunoprecipitation/immunoblotting assay

W303-1A (wild-type) cells carrying either pRS414Gal-Png1^{Flag} (expressing Flag-tagged Png1) and pYes2 vector, pYes2-Ufd2^{myc} (expressing myc-tagged Ufd2) and p414Gal vector, or pYes2-Ufd2^{myc} and pRS425Gal-Rad23^{Flag} (expressing Flag-tagged Rad23) were grown in the galactose-containing SG medium to an OD_{600} of ∼1, followed by preparation of extracts, immunoprecipitation with beads linked to specific antibodies indicated, SDS–8% PAGE, and immunoblotting, separately, with anti-Flag (Sigma-Aldrich) and anti-myc (Covance).

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References

Ahner, A., and J.L. Brodsky. 2004. Checkpoints in ER-associated degradation: excuse me, which way to the proteasome? *Trends Cell Biol.* 14:474–478.

Bertolaet, B.L., D.J. Clarke, M. Wolff, M.H. Watson, M. Henze, G. Divita, and S.I. Reed. 2001. UBA domains of DNA damage-inducible proteins interact with ubiquitin. *Nat. Struct. Biol.* 8:417–422.

Biggins, S., I. Ivanovska, and M.D. Rose. 1996. Yeast ubiquitin-like genes are involved in duplication of the microtubule organizing center. *J. Cell Biol.* 133:1331–1346.

Blom, D., C. Hirsch, P. Stern, D. Tortorella, and H.L. Ploegh. 2004. A glycosylated type I membrane protein becomes cytosolic when peptide: *N*-glycanase is compromised. *EMBO J.* 23:650–658.

DeMartino, G.N., and C.A. Slaughter. 1999. The proteasome, a novel protease regulated by multiple mechanisms. *J. Biol. Chem.* 274:22123–22126.

Elsasser, S., and D. Finley. 2005. Delivery of ubiquitinated substrates to protein-unfolding machines. *Nat. Cell Biol.* 7:742–749.

Elsasser, S., R.R. Gali, M. Schwickart, C.N. Larsen, D.S. Leggett, B. Muller, M.T. Feng, F. Tubing, G.A. Dittmar, and D. Finley. 2002. Proteasome subunit Rpn1 binds ubiquitin-like protein domains. *Nat. Cell Biol.* 4:725–730.

Elsasser, S., D. Chandler-Militello, B. Muller, J. Hanna, and D. Finley. 2004. Rad23 and Rpn10 serve as alternative ubiquitin receptors for the proteasome. *J. Biol. Chem.* 279:26817–26822.

Glockzin, S., F.X. Ogi, A. Hengstermann, M. Scheffner, and C. Blattner. 2003. Involvement of the DNA repair protein hHR23 in p53 degradation. *Mol. Cell. Biol.* 23:8960–8969.

Hampton, R.Y. 2002. ER-associated degradation in protein quality control and cellular regulation. *Curr. Opin. Cell Biol.* 14:476–482.

Helenius, A., and M. Aebi. 2004. Roles of N-linked glycans in the endoplasmic reticulum. *Annu. Rev. Biochem.* 73:1019–1049.

Hirsch, C., D. Blom, and H.L. Ploegh. 2003. A role for *N*-glycanase in the cytosolic turnover of glycoproteins. *EMBO J.* 22:1036–1046.

Hirsch, C., E. Jarosch, T. Sommer, and D.H. Wolf. 2004a. Endoplasmic reticulum-associated protein degradation—one model fits all? *Biochim. Biophys. Acta.* 1695:215–223.

Hirsch, C., S. Misaghi, D. Blom, M.E. Pacold, and H.L. Ploegh. 2004b. Yeast *N*-glycanase distinguishes between native and non-native glycoproteins. *EMBO Rep.* 5:201–206.

Johnson, E.S., P.C.M. Ma, I.M. Ota, and A. Varshavsky. 1995. A proteolytic pathway that recognizes ubiquitin as a degradation signal. *J. Biol. Chem.* 270:17442–17456.

Joshi, S., S. Katiyar, and W.J. Lennarz. 2005. Misfolding of glycoproteins is a prerequisite for peptide: *N*-glycanase mediated deglycosylation. *FEBS Lett.* 579:823–826.

Kim, I., K. Mi, and H. Rao. 2004. Multiple interactions of rad23 suggest a mechanism for ubiquitylated substrate delivery important in proteolysis. *Mol. Biol. Cell.* 15:3357–3365.

Lambertson, D., L. Chen, and K. Madura. 1999. Pleiotropic defects caused by loss of the proteasome-interacting factors Rad23 and Rpn10 of *Saccharomyces cerevisiae*. *Genetics.* 153:69–79.

Lee, J.H., J.M. Choi, C. Lee, K.J. Yi, and Y. Cho. 2005. Structure of a peptide: *N*-glycanase-Rad23 complex: insight into the deglycosylation for denatured glycoproteins. *Proc. Natl. Acad. Sci. USA.* 102:9144–9149.

Madura, K. 2004. Rad23 and Rpn10: perennial wallflowers join the melee. *Trends Biochem. Sci.* 29:637–640.

Masutani, C., M. Araki, K. Sugasawa, P.J. van der Spek, A. Yamada, A. Uchida, T. Maekawa, D. Bootsma, J.H. Hoeijmakers, and F. Hanaoka. 1997. Identification and characterization of XPC-binding domain of hHR23B. *Mol. Cell. Biol.* 17:6915–6923.

Medicherla, B., Z. Kostova, A. Schaefer, and D.H. Wolf. 2004. A genomic screen identifies Dsk2p and Rad23p as essential components of ER-associated degradation. *EMBO Rep.* 5:692–697.

Misaghi, S., M.E. Pacold, D. Blom, H.L. Ploegh, and G.A. Korbel. 2004. Using a small molecule inhibitor of peptide: *N*-glycanase to probe its role in glycoprotein turnover. *Chem. Biol.* 11:1677–1687.

Ng, D.T.W., J.D. Brown, and P. Walter. 1996. Signal sequences specify the targeting route to the endoplasmic reticulum membrane. *J. Cell Biol.* 134:269–278.

Ng, J.M., W. Vermeulen, G.T. van der Horst, S. Bergink, K. Sugasawa, H. Vrieling, and J.H. Hoeijmakers. 2003. A novel regulation mechanism of DNA repair by damage-induced and RAD23-dependent stabilization of xeroderma pigmentosum group C protein. *Genes Dev.* 17:1630–1645.

Ortolan, T.G., L. Chen, P. Tongaonkar, and K. Madura. 2004. Rad23 stabilizes Rad4 from degradation by the Ub/proteasome pathway. *Nucleic Acids Res.* 32:6490–6500.

Park, H., T. Suzuki, and W.J. Lennarz. 2001. Identification of proteins that interact with mammalian peptide:*N*-glycanase and implicate this hydrolase in the proteasome-dependent pathway for protein degradation. *Proc. Natl. Acad. Sci. USA.* 98:11163–11168.

Pickart, C.M., and R.E. Cohen. 2004. Proteasomes and their kin: proteases in the machine age. *Nat. Rev. Mol. Cell Biol.* 5:177–187.

Raasi, S., and C.M. Pickart. 2003. Rad23 ubiquitin-associated domains (UBA) inhibit 26 S proteasome-catalyzed proteolysis by sequestering lysine 48-linked polyubiquitin chains. *J. Biol. Chem.* 278:8951–8959.

Rao, H., and A. Sastry. 2002. Recognition of specific ubiquitin conjugates is important for the proteolytic functions of the ubiquitin-associated domain proteins Dsk2 and Rad23. *J. Biol. Chem.* 277:11691–11695.

Richly, H., M. Rape, S. Braun, S. Rumpf, C. Hoege, and S. Jentsch. 2005. A series of ubiquitin binding factors connects CDC48/p97 to substrate multiubiquitylation and proteasomal targeting. *Cell.* 120:73–84.

Roberts, L.M., and D.C. Smith. 2004. Ricin: the endoplasmic reticulum connection. *Toxicon.* 44:469–472.

Russell, S.J., S.H. Reed, W.Y. Huang, E.C. Friedberg, and S.A. Johnston. 1999. The 19S regulatory complex of the proteasome functions independently of proteolysis in nucleotide excision repair. *Mol. Cell.* 3:687–695.

Schauber, C., L. Chen, P. Tongaonkar, I. Vega, D. Lambertson, W. Potts, and K. Madura. 1998. Rad23 links DNA repair to the ubiquitin/proteasome pathway. *Nature.* 391:715–718.

Schwartz, D.C., and M. Hochstrasser. 2003. A superfamily of protein tags: ubiquitin, SUMO and related modifiers. *Trends Biochem. Sci.* 28:321–328.

Simpson, J.C., L.M. Roberts, K. Romisch, J. Davey, D.H. Wolf, and J.M. Lord. 1999. Ricin A chain utilises the endoplasmic reticulum-associated protein degradation pathway to enter the cytosol of yeast. *FEBS Lett.* 459:80–84.

Suzuki, T., H. Park, N.M. Hollingsworth, R. Sternglanz, and W.J. Lennarz. 2000. *PNG1*, a yeast gene encoding a highly conserved peptide:*N*-glycanase. *J. Cell Biol.* 149:1039–1052.

Suzuki, T., H. Park, M.A. Kwofie, and W.J. Lennarz. 2001. Rad23 provides a link between the Png1 deglycosylating enzyme and the 26 S proteasome in yeast. *J. Biol. Chem.* 276:21601–21607.

Suzuki, T., H. Park, and W.J. Lennarz. 2002. Cytoplasmic peptide:*N*-glycanase (PNGase) in eukaryotic cells: occurrence, primary structure, and potential functions. *FASEB J.* 16:635–641.

Taxis, C., R. Hitt, S.H. Park, P.M. Deak, Z. Kostova, and D.H. Wolf. 2003. Use of modular substrates demonstrates mechanistic diversity and reveals differences in chaperone requirement of ERAD. *J. Biol. Chem.* 278:35903–35913.

Vashist, S., and D.T. Ng. 2004. Misfolded proteins are sorted by a sequential checkpoint mechanism of ER quality control. *J. Cell Biol.* 165:41–52.

Verma, R., R. Oania, J. Graumann, and R. Deshaies. 2004. Multiubiquitin chain receptors define a layer of substrate selectivity in the ubiquitin-proteasome system. *Cell.* 118:99–110.

Walters, K.J., P.J. Lech, A.M. Goh, Q. Wang, and P.M. Howley. 2003. DNA-repair protein hHR23a alters its protein structure upon binding proteasomal subunit S5a. *Proc. Natl. Acad. Sci. USA.* 100:12694–12699.

Watkins, J.F., P. Sung, L. Prakash, and S. Prakash. 1993. The *Saccharomyces cerevisiae* DNA repair gene Rad23 encodes a nuclear protein containing a ubiquitin-like domain required for biological function. *Mol. Cell. Biol.* 13:7757–7765.

Wilkinson, C.R.M., M. Seeger, R. Hartmann-Petersen, M. Stone, M. Wallace, C. Semple, and C. Gordon. 2001. Proteins containing the UBA domain are able to bind to multi-ubiquitin chains. *Nat. Cell Biol.* 3:939–943.

Yoshida, Y. 2003. A novel role for *N*-glycans in the ERAD system. *J. Biochem. (Tokyo).* 134:183–190.