Yeast Pth2 is a UBL domain-binding protein that participates in the ubiquitin–proteasome pathway

Takashi Ishii^{1,2}, Minoru Funakoshi^{1,3}
and Hideki Kobayashi^{1,2,*}

¹Department of Molecular Biology, Graduate School of Medical Sciences, Kyushu University, Higashi-ku, Fukuoka, Japan and ²CREST, Japanese Science and Technology Agency, Kawaguchi, Saitama, Japan

Ubiquitin-like (UBL)-ubiquitin-associated (UBA) proteins such as Rad23 and Dsk2 mediate the delivery of polyubiquitinated proteins to the proteasome in the ubiquitin–proteasome pathway. We show here that budding yeast peptidyl-tRNA hydrolase 2 (Pth2), which was previously recognized as a peptidyl-tRNA hydrolase, is a UBL domain-binding protein that participates in the ubiquitin–proteasome pathway. Pth2 bound to the UBL domain of both Rad23 and Dsk2. Pth2 also interacted with polyubiquitinated proteins through the UBA domains of Rad23 and Dsk2. Pth2 overexpression caused an accumulation of polyubiquitinated proteins and inhibited the growth of yeast. Ubiquitin-dependent degradation was accelerated in the *pth2A* mutant and was retarded by overexpression of Pth2. Pth2 inhibited the interaction of Rad23 and Dsk2 with the polyubiquitin receptors Rpn1 and Rpn10 on the proteasome. Furthermore, Pth2 function involving UBL-UBA proteins was independent of its peptidyl-tRNA hydrolase activity. These results suggest that Pth2 negatively regulates the UBL-UBA protein-mediated shuttling pathway in the ubiquitin–proteasome system.

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Introduction

Recruitment of ubiquitinated proteins to the proteasome is a crucial step in the ubiquitin–proteasome pathway. The integrity of this pathway is ensured by a sequential mode of action: capture of ubiquitin conjugates, delivery to the proteasome and release from the proteasome ubiquitin receptors. Ubiquitinated proteins can be recognized directly by the 19S proteasomal subunit Rpn10 (van Nocker *et al*, 1996; Saeki *et al*, 2002; Elsasser *et al*, 2004; Verma *et al*, 2004). In addition to this pathway, UBL-UBA proteins help to deliver proteins to be degraded to the proteasome. UBL-UBA proteins belong to a family of proteins that contain a UBL domain at

the N terminus and a UBA domain at the C terminus. Through the ability of the UBL domain to interact with polyubiquitin receptors on the 19S proteasome regulatory particle and the UBA domain to bind to polyubiquitin, UBL-UBA proteins such as Rad23 and Dsk2 in *Saccharomyces cerevisiae* serve as shuttle factors delivering ubiquitinated substrates to the proteasome (Schauber *et al*, 1998; Lambertson *et al*, 1999; Wilkinson *et al*, 2001; Chen and Madura, 2002; Funakoshi *et al*, 2002; Rao and Sastry, 2002). These shuttle factors interact with the 19S proteasomal subunit Rpn1, which acts as a scaffold on the proteasome (Elsasser *et al*, 2002). A recently developed cell-free system has demonstrated that UBL-UBA shuttle proteins promote protein degradation by the proteasome (Verma *et al*, 2004). These accumulated findings have advanced our understanding of the molecular basis of recognition of polyubiquitinated proteins by the proteasome (reviewed by Hartmann-Petersen *et al*, 2003; Madura, 2004; Elsasser and Finley, 2005). However, how the shuttle proteins regulate the flux of polyubiquitinated proteins to the proteasome receptors is not well understood.

To investigate the regulation of UBL-UBA proteins in shuttling pathways, we searched for interacting factors of UBL-UBA proteins in a two-hybrid screen, and identified peptidyl-tRNA hydrolase 2 (Pth2) from *S. cerevisiae* as an interacting protein of Rad23 and Dsk2. Pth is an enzyme that cleaves peptidyl-tRNA to tRNA and peptides during translation elongation (Menninger, 1976) and is essential for growth in bacteria. In archaea, a second Pth enzyme named Pth2 has been identified, whose amino-acid sequence is completely different from bacterial Pth (Rosas-Sandoval *et al*, 2002). Eukaryotic cells encode both Pth and Pth2, and curiously, the two eukaryotic enzymes are dispensable for yeast viability (Menez *et al*, 2002; Rosas-Sandoval *et al*, 2002; Fromant *et al*, 2003). Pth2 has two domains: a conserved C-terminal half is the catalytic domain (de Pereda *et al*, 2004), but no function has been ascribed to the highly divergent N-terminal half, suggesting that this region may confer species specificity to Pth2. As we show in this report, budding yeast Pth2 is a UBL domain-binding protein that inhibits ubiquitin-mediated protein degradation. In addition, the C-terminal domain of Pth2 is required for the interaction with Rad23 and Dsk2, but this Pth2 function is independent of its C-terminal peptidyl-tRNA hydrolase activity. Based on our findings, we discuss a regulatory role of Pth2 in substrate delivery to the proteasome via an interaction with the UBL domain of UBL-UBA proteins. Budding yeast Pth2 may have a role in the ubiquitin–proteasome pathway and as a peptidyl-tRNA hydrolase.

Results

Yeast Pth2 interacts with Dsk2 and Rad23 in vivo and in vitro

We screened for proteins that interact with a UBL-UBA protein in a two-hybrid screen using Dsk2 as bait. Among 2×10^5 transformants from an *S. cerevisiae* cDNA library,

*Corresponding author. Department of Molecular Biology, Graduate School of Medical Sciences, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812-8582, Japan. Tel.: +81 92 642 6179; Fax: +81 92 642 6183; E-mail: hkobaya@molbiol.med.kyushu-u.ac.jp
³Present address: Department of Molecular Biophysics & Biochemistry, Yale University, 266 Whitney Avenue, New Haven, CT 06520-8114, USA

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Figure 1 Pth2 binds to Dsk2 and Rad23 but not to Ddi1. (A) Identification of *PTH2* in a two-hybrid assay. Pth2 was obtained as a Dsk2-interacting clone. Interaction of Pth2 with Dsk2 was tested by histidine-prototrophic growth, using *DSK2* as bait and *PTH2* as prey (upper panel) or vice versa (lower panel). (B) Binding of Pth2 to Dsk2 and Rad23 *in vitro*. His₆-T7-Pth2 (1 μg) was mixed with 1 μg of GST-Dsk2, GST-Rad23, GST-Ddi1 or GST alone in lysis buffer and incubated with 20 μl glutathione-Sepharose (GSH beads) for 1 h. Material bound to GSH beads was immunoblotted with the indicated antibodies. (C) Binding of Pth2 to Dsk2 and Rad23 *in vivo*. T7-Pth2 was coexpressed with GST-Dsk2, GST-Rad23, GST-Ddi1 or GST alone in wild-type yeast or in *dsk2Δrad23Δ*. The extracts (lanes 1–6) and the GST precipitates from wild-type (YPH499) (lanes 7–12) and *dsk2Δrad23Δ* (lanes 13–16) were immunoblotted as indicated. (D) Interaction of Pth2 with Dsk2 at endogenous expression level in yeast. FLAG-Pth2 was expressed from *PTH2* own promoter in *pth2Δ*, and was immunoprecipitated with anti-FLAG M2 beads. The extracts (top and middle panels) and the FLAG-precipitates from the extracts (bottom panel) were immunoblotted with anti-Dsk2 or anti-Pth2 antibodies as indicated. An asterisk indicates FLAG-Pth2. Because the sensitivity of our anti-Rad23 is lower than that of anti-Dsk2 (ca. 1/10, see Materials and methods), we could not show a Rad23 signal under this condition. Also, we could not test co-immunoprecipitation of Dsk2/Rad23 with anti-Pth2 antibody, probably because our Pth2 antibody was not effective for immunoprecipitations.

three clones interacted with Dsk2 strongly, and each of these clones was identified as *PTH2* (Figure 1A), which encodes a full-length peptidyl-tRNA hydrolase. First, we tested for a direct interaction *in vitro* between Pth2 and the UBL-UBA proteins (Figure 1B). His₆-T7-tagged Pth2 bound directly to

GST-Dsk2 (lane 5) and GST-Rad23 (lane 6), but not to the DNA damage-inducible UBL-UBA protein, Ddi1 (lane 7). Next, we coexpressed the T7-Pth2 with GST-Dsk2 or GST-Rad23 in yeast, and *in vivo* binding was tested using a GST pull-down assay followed by immunoblotting with anti-T7

(Figure 1C). T7-Pth2 co-precipitated with GST-Dsk2 (lane 10) and GST-Rad23 (lane 11) and, to a lesser extent, with GST-Ddi1 (lane 12). The weak binding to Ddi1 was abolished in the *dsk2Δrad23Δ* mutant (lane 16), suggesting that the interaction of Pth2 is mediated by Dsk2 and Rad23. Therefore, Pth2 binds selectively to the UBL-UBA proteins Rad23 and Dsk2 *in vivo*. Next, we confirmed *in vivo* interaction of Pth2 with UBL-UBA protein at endogenous expression level (Figure 1D). FLAG-tagged Pth2 was expressed from *PTH2* own promoter in *pth2Δ* cells (middle panel, lane 4) and the extracts were immunoprecipitated with anti-FLAG beads. FLAG-Pth2 expressing at endogenous levels or even less in *pth2Δ* co-precipitated with endogenous Dsk2 (bottom panel, lane 4).

Pth2 interacts with polyubiquitinated proteins indirectly via Dsk2 and Rad23 *in vivo*

To further investigate the interaction of Pth2 with UBL-UBA proteins, yeast cell extracts were fractionated by gel filtration, and each fraction was immunoblotted (Figure 2A). Pth2 reproducibly eluted in fractions containing high molecular weight proteins (bottom panel) but not at the expected monomer size (~25 kDa). The Pth2-containing fractions contained Dsk2, Rad23 and polyubiquitinated proteins in cells. Also, GST-Pth2 expressed in yeast co-precipitated with polyubiquitinated proteins (Figure 2B, lane 7) together with Dsk2 and Rad23 in wild-type cells but did not co-precipitate with polyubiquitinated proteins in *dsk2Δrad23Δ* (lane 10). Like the wild-type strain, GST-Pth2 co-precipitated polyubiquitin in either *dsk2Δ* (lane 8) or *rad23Δ* cells (lane 9). It thus seems that the Pth2–polyubiquitin interaction seen in wild-type cells depends on Dsk2 and Rad23. Pth2 alone did not bind directly to polyubiquitin *in vitro* (Figure 2C).

Overexpression of Pth2 caused accumulation of polyubiquitinated proteins (Figure 2D, lane 5; and B, lane 2); therefore, we also tested the growth of cells overexpressing Pth2 (Figure 2E). Growth was inhibited in wild-type cells overexpressing Pth2 (top left panel), whereas Pth2-induced growth inhibition was not observed in the double-deletion mutant *dsk2Δrad23Δ* (bottom left panel). However, growth of the single-deletion mutants, *dsk2Δ* and *rad23Δ*, was inhibited when *PTH2* was overexpressed (right top and bottom panels). These genetic analyses suggest that the Pth2-induced growth inhibition we observed is also mediated by Dsk2 and Rad23.

Pth2 inhibits ubiquitin-mediated degradation

We next investigated whether Pth2 affects ubiquitin-mediated protein degradation. Degradation of the N-end rule substrate Leu-β-gal was accelerated in *pth2Δ* (Figure 3A) compared with wild-type cells. As a control, degradation of Ala-β-gal was not affected by *PTH2* deletion. The *in vivo* substrate Sic1, a cyclin-dependent kinase inhibitor, was also examined (Figure 3B; Supplementary Figure 1). Sic1-HA was expressed in cells arrested by α-factor and released synchronously into the cell cycle. Degradation of Sic1-HA was slightly accelerated in *pth2Δ* (Figure 3B, left panels). To confirm this Pth2 effect, we also tested Sic1 degradation in cells overexpressing *PTH2*. Supporting the acceleration by the *PTH2* deletion, overexpression of *PTH2* retarded degradation of Sic1-HA (right panels). Consistent with these effects of Pth2 on degradation, *PTH2* disruption suppressed the growth defect of the

temperature-sensitive proteasome mutant *pre9Δ* (Figure 3C). Furthermore, the cells' response to amino-acid analogs was affected by *PTH2* disruption (Figure 3D); that is, *pth2Δ* became resistant to the amino-acid analogs azetidine and canavanine. Contrary to our expectations, however, the triple mutant *dsk2Δrad23Δpth2Δ* was more sensitive to azetidine than *dsk2Δrad23Δ*, suggesting that Pth2 functions in a distinct pathway in the ubiquitin–proteasome system (see Discussion). In contrast to the drug response results, *pth2Δ* did not affect survival after UV irradiation (Figure 3D). Collectively, these data indicate that Pth2 is involved negatively in the ubiquitin-mediated degradation pathway.

Pth2 inhibits interactions of Rad23/Dsk2 with polyubiquitin receptors on the proteasome

To further delineate Pth2's role in delivery of ubiquitinated proteins to the proteasome, we examined the effects of Pth2 on the interaction between the proteasome ubiquitin receptors Rpn1 and Rpn10 and UBL-UBA proteins Rad23 and Dsk2. First, we confirmed that Rpn1 interacts with Rad23 (Figure 4A, lane 6) but not with Dsk2 (lane 5) *in vitro* (cf; Elsasser *et al*, 2002). Then, to test whether Pth2 alters the Rad23–Rpn1 interaction, GST pull-down experiments were carried out using purified GST-Rpn1 and His₆-T7-Rad23 in the presence of His₆-T7-Pth2. Figure 4B shows that increasing amounts of Pth2 decreased binding of Rad23 to Rpn1 *in vitro* (lanes 4–6). Similarly, we confirmed that Rpn10 interacts with Dsk2 (Figure 4A, lane 12) but not with Rad23 (lane 13) *in vitro*. Again, increasing amounts of Pth2 competed with Dsk2 for Rpn10 binding (Figure 4C).

Next, we tested the effect of Pth2 on the interaction between the proteasome and UBL-UBA proteins (Figure 5A). To address this issue, an intact proteasome 19S regulatory particle containing FLAG-Rpt1 and polyubiquitin receptors Rpn1 and Rpn10 (data not shown, Elsasser *et al*, 2002) was immunoprecipitated from yeast cells with anti-FLAG beads and then incubated with the UBL domain fragment (1–77) of Rad23-GST or Dsk2-GST in the presence of His₆-T7-Pth2. We used the 19S particle instead of the 26S proteasome because the FLAG-Pre1-tagged 26S proteasome was prone to dissociate in our buffer conditions in the GST pull-down assay (data not shown). Figure 5A shows that increasing amounts of Pth2 decreased the binding of 19S regulatory particle to Dsk2 (lanes 3–5) and to Rad23 (lanes 6–8), indicating that Pth2 inhibits the interaction of Dsk2 and Rad23 with the 19S particle *in vitro*. In the case of Rad23, a higher dose of Pth2 was required to compete with the proteasome–Rad23 interaction *in vitro* (lanes 6–8) (Supplementary Figure 2).

We also examined whether Pth2 prevents the interaction of Rad23 and Dsk2 with the proteasome *in vivo*. As shown in Figure 5B, overexpression of Pth2 in yeast decreased the amount of Dsk2 associated with the proteasome (lanes 3 and 4). Under the same conditions, we could not detect a decreased amount of Rad23 associated with the proteasome, consistent with the results in Figure 5A (lanes 6–8). Therefore, we examined the effect of greater amounts of Pth2 on the proteasome–Rad23 interaction. Figure 5C shows that a higher dose of Pth2 inhibited the interaction of Rad23 with the proteasome in the cell extracts (lanes 3 and 4). This result was confirmed by a GST pull-down assay of the proteasome from extracts with Rad23-GST, followed by

Figure 2 Pth2 associates with polyubiquitinated proteins via Dsk2 and Rad23. (A) Gel filtration of yeast extracts on a Superdex 200 column. Each fraction was immunoblotted with the indicated antibodies. The bar at the bottom indicates the position of monomeric Pth2 (~25 kDa) (Rosas-Sandoval *et al*, 2002). (B) Binding of Pth2, Dsk2, Rad23 and polyubiquitin conjugates. GST-Pth2 was expressed in wild-type (YPH499), *dsk2 Δ* , *rad23 Δ* or *dsk2 Δ rad23 Δ* strains. The GSH beads precipitates were immunoblotted as indicated. (C) *In vitro* GST pull-down assays with Pth2 and polyubiquitin. In total, 1 μ g of tetraubiquitin (lanes 1–5) or polyubiquitin (lanes 6–10) was incubated with GST-Pth2, GST-Dsk2, GST-Rad23, GST-Ddi1 or GST alone (1 μ g each). The samples were precipitated with GSH beads and immunoblotted as indicated. (D) *In vivo* GST pull-down assays with Pth2 and polyubiquitin. GST-Pth2, GST-Dsk2, GST-Rad23, GST-Ddi1 or GST alone was expressed in wild-type cells. Cell extracts were precipitated with GSH beads and immunoblotted as indicated. (E) Growth of yeast overexpressing Pth2. A single copy of *PTH2* was expressed from a galactose-inducible pGAL1-YEplac181 ($\times$ 1), or two copies of *PTH2* were expressed from both pGAL1-YEplac181 and pGAL1-YEplac195 ($\times$ 2) in wild-type, *dsk2 Δ* , *rad23 Δ* or *dsk2 Δ rad23 Δ* strains. Cells were incubated for 2–5 days at 30°C, the permissive temperature for growth of *dsk2 Δ rad23 Δ* (Biggins *et al*, 1996). Expression levels were determined by Pth2 immunoblotting (on the right).

immunoblotting for the proteasomal subunits, FLAG-tagged Rpt1 and Rpt3 (lanes 7 and 8). Pth2 itself did not associate with the proteasome; no Pth2 signal was detected in the 26S proteasome-precipitated fraction, even in cells overexpressing Pth2 (Figure 5D). Taking these results together, it appears that Pth2 competitively inhibits the interaction between UBL-UBA proteins and polyubiquitin receptors on the proteasome.

Pth2 binds directly to the UBL domains of Rad23 and Dsk2

We tested which domain of UBL-UBA proteins is required for binding to full-length Pth2. A series of deletion mutants of Rad23 and Dsk2 were constructed (Figure 6A), and their binding to Pth2 was tested *in vitro* and *in vivo*. Figure 6B shows that the N-terminal UBL domain of Rad23 (lane 5) bound to Pth2 *in vitro*, but the N-terminal Rad23 truncation

Figure 3 Pth2 inhibits the ubiquitin–proteasome pathway. (A) Degradation of the N-end rule substrate was accelerated in *pth2Δ*. Leu-β-gal and Ala-β-gal (indicated by arrowheads) were induced with galactose in wild-type (YPH499) or *pth2Δ* cells. Cell samples were collected at the indicated times after the induction was terminated, and degradation was assessed by immunoblotting with anti-β-gal. The quantification of the experiments is shown on the right. Note that, despite our expectations, Pth2 overexpression barely inhibited degradation of Leu-β-gal (data not shown), which might be indicative that Pth2 is involved in substrate specificity for degradation (cf Figure 3B). (B) Degradation of Sic1 in *pth2Δ* and *PTH2*-overexpressing cells. Wild-type (YPH499) and *pth2Δ* cells were arrested by α -factor treatment and then Sic1-HA was expressed for 1 h by galactose induction. For overexpression, Sic1-HA and GST-Pth2 were coexpressed in synchronized wild-type cells for 2 h by galactose induction. After release into the cell cycle, cell samples were collected at the indicated times and Sic1-HA protein was followed by immunoblotting with anti-HA. (C) Rescue of the proteasome defect of *pre9Δ* by *PTH2* deletion. Growth was tested for 2–3 days at 30°C, at which temperature the *pre9Δ* mutant shows impaired growth. (D) Effects of *PTH2* deletion on sensitivity to amino-acid analogs and UV irradiation. Wild-type (YPH499) and *pth2Δ* were spotted onto SD plates containing 0.5 mM AZC or 3 μg/ml canavanine in serial dilutions (upper panels). Wild-type and the mutants (*dsk2Δrad23Δ* and *dsk2Δrad23Δpth2Δ*) were spotted onto YPD plates containing 5 mM AZC in serial dilutions (bottom left panel). Cells were also spotted on YPD plates and exposed to 50 J/m² UV irradiation (bottom right panel). The plates were incubated at 30°C for 2–4 days.

(lane 6) did not. The requirement of the UBL domain of Dsk2 for Pth2 binding was also shown by deletion analysis of Dsk2 *in vitro* (Figure 6B, lanes 12–16). Thus, Pth2 binds directly to the UBL domains of Rad23 and Dsk2.

Pth2 bound *in vivo* to the UBL domain of Rad23 (Figure 6C, lane 5) and Dsk2 (lane 11); Pth2 also bound weakly to the

N-terminal truncation mutants of Rad23 and Dsk2 (lanes 6, 13, 15). However, when a double deletion *dsk2Δrad23Δ* mutant was tested in the *in vivo* binding assay (Figure 6D), the Rad23 mutant fragment (78–398) did not interact with Pth2 (lane 4), indicating that the weak signal is due to an indirect interaction that polyubiquitin chains bridge between the GST-UBA

Figure 4 Pth2 inhibits the interaction between UBL-UBA proteins and ubiquitin receptors. (A) Rpn1–Rad23 and Rpn10–Dsk2 interactions *in vitro*. GST-Dsk2, GST-Rad23, GST-Ddi1 or GST alone (1 µg each) was incubated with 1 µg of either His₆-T7-Rpn1 (lanes 1–7) or His₆-T7-Rpn10 (lanes 8–14) for 1 h, followed by precipitation with GSH beads and immunoblotting as indicated. Inputs represent 5% of the amounts used for the assay. (B) Effect of Pth2 on the Rad23–Rpn1 interaction *in vitro*. GST-Rpn1 (1 µg) was incubated with His₆-T7-Rad23 (1 µg) in the presence of increasing amounts of His₆-T7-Pth2 (× 1 = 1 µg) for 1 h, followed by precipitation with GSH beads and immunoblotting as indicated. (C) Effect of Pth2 on the Dsk2–Rpn10 interaction *in vitro*. GST-Rpn10 (1 µg) was incubated with Dsk2-His₆ (1 µg) in the presence of increasing amounts of His₆-T7-Pth2 (× 1 = 1 µg) for 1 h, followed by precipitation with GSH beads and immunoblotting as indicated.

domain fusion proteins and endogenous Rad23 or Dsk2, which then binds to Pth2.

Notably, Pth2 also interacted with the Dsk2 UBA domain *in vivo* but not with Rad23 UBA domain (Figure 6C and D). Pth2 appeared to bind both the Dsk2 UBL and the UBA domains at equivalent levels (Figure 6C, lanes 11 and 13). Even in *dsk2Δrad23Δ* (Figure 6D), Pth2 co-precipitated with the UBA domain alone (lane 9) *in vivo*. By contrast, a Dsk2 mutant fragment (78–335) that lacks both the UBL and UBA domains did not bind to Pth2 (lane 8). Therefore, in addition to the UBL domain, Pth2 interacts with the Dsk2 UBA domain *in vivo*. However, this Pth2–UBA interaction seems to be mediated by an additional cellular factor(s) in yeast cells because Pth2 did not bind the UBA domain *in vitro* (Figure 6B) (see Discussion).

Pth2 function in the ubiquitin–proteasome pathway is independent of its C-terminal peptidyl-tRNA hydrolase activity

The Pth2 C-terminal domain, which contains the peptidyl-tRNA hydrolase activity, is conserved across species; however, the N-terminal domain is not conserved, and its function is unknown (Rosas-Sandoval *et al*, 2002). We investigated whether the peptidyl-tRNA hydrolase activity of Pth2 is required for its binding to UBL-UBA proteins.

Guided by a structural analysis of the catalytic domain (de Pereda *et al*, 2004), we produced a series of Pth2 peptidyl-tRNA hydrolase mutants by site-directed mutagenesis of the putative active center (Figure 7A). The peptidyl-tRNA hydrolase activity of these mutants was tested by complementation of the *Escherichia coli* mutant, *pth^{ts}* (Rosas-Sandoval *et al*, 2002). The yeast Pth2 mutants D171A, D171N, DRTQ171AAAA, DRTQ171NLVL and RTQ174LVL failed to rescue the temperature-sensitive growth of *pth^{ts}* (summarized in Figure 7A), indicating a lack of peptidyl-tRNA hydrolase activity in the Pth2 mutants. When we examined the ability of these Pth2 mutants to bind to Rad23 (Figure 7B, lanes 1–9) and Dsk2 (lanes 10–18), we found that these Pth2 mutants could bind to Rad23 (lanes 5–9) and to Dsk2 (lanes 14–18). Overexpression of mutant Pth2 (D171A) inhibited growth to a similar degree as wild-type Pth2 (Figure 7C). We also confirmed this result by complementation test for cell sensitivity to amino-acid analog: *pth2Δ* expressing Pth2 mutant (D171A) became sensitive to azetidine compared with *pth2Δ* alone (Figure 7D, see also Figure 3D). These data, therefore, demonstrated that Pth2 function in the ubiquitin-mediated pathway is independent of its peptidyl-tRNA hydrolase activity. In addition, this complementation assay also shows that FLAG-tagged Pth2 expressing from *PTH2*'s own promoter in *pth2Δ* made more sensitive to azetidine than *pth2Δ*, supporting that the tagged Pth2 is functional in yeast cells (Figure 7D). Like FLAG-Pth2, GST-Pth2 expressing in *pth2Δ* at endogenous level rescued the azetidine-resistant phenotype of *pth2Δ* (data not shown). As shown in Figure 7E, the C-terminal half (83–208) of Pth2 bound to Rad23 (lane 12) and Dsk2 (lane 24) *in vivo*; however, the N-terminal domain (1–82) did not bind these proteins (lanes 11 and 23). Furthermore, overexpression of the N-terminal domain (1–82), which lacks the Rad23/Dsk2-binding site, did not inhibit the growth of yeast, unlike wild-type Pth2 (Supplementary Figure 3). Therefore, the C-terminal domain of Pth2 is necessary for binding to Rad23 and Dsk2.

Discussion

We have found a novel role for yeast Pth2 in the ubiquitin-mediated protein degradation pathway through its interaction with UBL domains of Rad23 and Dsk2. This Pth2 function is independent of its peptidyl-tRNA hydrolase activity.

Yeast Pth2 negatively regulates substrate delivery via its interaction with shuttle proteins

Polyubiquitinated proteins are targeted to the 19S proteasome regulatory particle via either the proteasome subunit Rpn10 or the shuttle proteins Rad23 and Dsk2. The Rad23/Dsk2-mediated shuttling pathway thus is redundant with Rpn10, and it remains controversial as to which is the primary pathway to the proteasome (Elsasser *et al*, 2004; Verma *et al*, 2004). We present evidence that Pth2 inhibits the binding of proteasome receptors to UBL-UBA proteins Rad23 and Dsk2 (Figures 4 and 5) and that Pth2 inhibits degradation of the authentic *in vivo* substrate Sic1 and the N-end rule substrate Leu-β-gal (Figure 3), suggesting that Pth2 suppresses ubiquitin-mediated degradation. The interaction of Pth2 with UBL-UBA protein exists in yeast cells (Figures 1 and 2). Interactions between Pth2 and the UBL domain of UBL-UBA proteins (Figure 6) may ultimately prevent delivery

Figure 5 Pth2 inhibits the interaction between UBL-UBA proteins and the proteasome. (A) Effect of Pth2 on the interaction between the proteasome and Dsk2/Rad23 *in vitro*. 19S regulatory particle was affinity-purified with FLAG-Rpt1 from yeast cells in which endogenous *RPT1* had been replaced with FLAG-RPT1. Rad23-GST UBL domain (1–77) protein or Dsk2-GST UBL domain (1–77) protein (1 µg) was mixed with the 19S proteasome (19S RP) and incubated with GSH beads for 1 h in the presence of increasing amounts of His₆-T7-Pth2 ($\times 1 = 1$ µg) followed by immunoblotting of the precipitates with anti-Rpt3, anti-FLAG, anti-T7 and anti-GST. (B) Effect of Pth2 on the interaction between the proteasome and Dsk2/Rad23 in yeast. GST or GST-Pth2 was overexpressed (O/E) by the galactose-inducible pGAL1-YEplac112-GST or pGAL1-YEplac112-GST-Pth2 for 4 h in yeast in which endogenous *RPT1* was replaced with FLAG-His₆-tagged *RPT1*. Extracts were precipitated with anti-FLAG M2 beads, followed by immunoblotting with anti-Rpt3, anti-GST, anti-FLAG and anti-Rad23. Extracts were immunoblotted as a control (lanes 1–2). (C) Effect of Pth2 on the interaction between the proteasome and Rad23 in yeast extracts. Lanes 1–4: GST or GST-Pth2 was overexpressed in yeast cells as in (B). Cell extracts were precipitated in the presence of purified GST-Pth2 (lane 4, 15-fold in molar excess; asterisk) with anti-FLAG M2 beads, followed by immunoblotting with anti-Rpt3, anti-GST, anti-FLAG and anti-Rad23 (lanes 1–4). Extracts were immunoblotted as a control (lanes 1–2). Lanes 5–8: experimental conditions were the same as (A), except that cell extracts were used instead of a purified 19S proteasome. Purified His₆-T7-Pth2 (30-fold excess) was added to the cell extract (lane 8). (D) The proteasome does not contain Pth2. Endogenous *PRE1* was replaced with FLAG-His₆-tagged *PRE1*. Pth2 was overexpressed either in wild-type (YPH499) or the FLAG-His₆-tagged *PRE1*-integrated strain (lanes 2 and 4, respectively). Extracts were precipitated with anti-FLAG M2 beads, followed by immunoblotting with anti-Rpt1, anti-20S, anti-FLAG and anti-Pth2 (lanes 5–8). Extracts were immunoblotted as a control (lanes 1–4).

of polyubiquitinated substrates to the proteasome receptor. Such a negative cascade might be important in ensuring a proper rate of substrate degradation by preventing a high flux of substrates from reaching the proteasome.

A proper rate of delivery of polyubiquitinated substrates to the proteasome presumably requires an optimal concentration of UBL-UBA proteins. Although UBL-UBA proteins are positive factors in proteasomal degradation, at higher concentrations, they inhibit protein degradation (Raasi and Pickart, 2003). Such inhibitory effects could be explained by an overflow of polyubiquitinated substrates onto the proteasome due to an excess of UBL-UBA proteins. In this regard, we suspect that Pth2 binding may decrease the affinity of the UBL domain for the proteasomal receptor. This interpretation is supported by our data that Pth2 itself

did not bind to the proteasome (Figure 5D) and that the majority of Pth2 was associated with Rad23 and Dsk2 that were bound to polyubiquitinated substrates in cells rather than to free Rad23 or Dsk2 (Figure 2A). Moreover, overexpression of Pth2 accumulated polyubiquitinated substrates in cells (Figures 2B and D), although the accumulation is less than cells overexpressing Rad23, Dsk2 or Ddi1 (cf lanes 2–5), implying that Pth2 functions indirectly in the ubiquitin–proteasome pathway through the UBL-UBA proteins. Therefore, we propose that Pth2 plays a role in regulating the flow of polyubiquitinated substrates to the proteasome by interfering with the interaction between UBL-UBA proteins and proteasome receptors. An efficient substrate flux to the proteasome also requires a transient interaction of UBL-UBA proteins with proteasomal receptors, so as not to occupy the

Figure 6 Pth2 binds to the UBL domains of Rad23 and Dsk2. (A) A diagram of Rad23 and Dsk2 constructs used for *in vitro* and *in vivo* binding assays. Summary of Pth2 binding to the mutants are shown on the right. Asterisks indicate an indirect interaction detected in cells (see text). (B) Binding of Pth2 to Rad23 and Dsk2 mutants *in vitro*. His₆-T7-Pth2 (1 µg) was incubated with various GST-Rad23 mutants (lanes 1–6) or Dsk2 mutants (lanes 7–16) for 1 h, precipitated with GSH beads, and immunoblotted with the indicated antibody. Because Dsk2 contains only a single C-terminal UBA domain, we constructed and used a series of Dsk2 mutants for domain analysis. (C) Binding of Pth2 to Rad23 and Dsk2 mutants *in vivo*. T7-Pth2 was coexpressed with GST-Rad23 (lanes 1–6) or GST-Dsk2 (lanes 7–15) mutants in wild-type (YPH499) cells, and the cell extracts were precipitated with GSH beads followed by immunoblotting with the indicated antibodies. (D) Binding of Pth2 to Rad23 and Dsk2 mutants *in vivo* in *dsk2Δrad23Δ*. The *in vivo* assay was carried out as in (C), using cell extracts of *dsk2Δrad23Δ*.

receptor site permanently. Therefore, Pth2 may be involved in the release of UBL-UBA proteins on the proteasome because overexpression of Pth2 decreases the amounts of Rad23 and Dsk2 associated with the proteasome (see Figure 5B). However, it is unlikely that Pth2 itself occupies the receptor site, because Pth2 associated with the proteasome was not detected even when *PTH2* was overexpressed (Figure 5D).

Interaction of Pth2 with the UBL domain in the shuttling pathway

To date, several UBL domain-associated proteins have been reported to modulate substrate delivery: ataxin-3 (Wang *et al*, 2000; Doss-Pepe *et al*, 2003), Ufd2 (E3/E4) (Koegl *et al*, 1999; Kim *et al*, 2004) and 19S subunit Rpn1 (Elsasser *et al*, 2002; Saeki *et al*, 2002). Similarly, Pth2 binding may induce a structural change in the UBL domain and hinder its subsequent docking to proteasome receptors. Indeed, Pth2 overexpression prevented the association of the proteasome with Rad23 and Dsk2 *in vitro* and Dsk2 *in vivo* (Figures 4 and 5). Although we could not observe a competitive inhibition by Pth2 in cells in the case of Rad23 *in vivo* (Figure 5B), it is conceivable that Rad23 is insensitive to Pth2 overexpression because Rad23 is much more abundant than Dsk2 (Figure 5, Elsasser *et al*, 2002).

In addition to the direct interaction of Pth2 with the UBL domain, we observed that Pth2 interacts with polyubiquitinated proteins. This Pth2–polyubiquitin interaction seen

in vivo is indirectly mediated via the UBA domains of Rad23 and Dsk2 (Figure 6). We suspect that polyubiquitin chains bridge an interaction between the GST-UBA domain fusion proteins and endogenous Rad23 or Dsk2, which then binds to Pth2. Interestingly, we observed that Pth2 is also capable of interacting with the Dsk2 UBA domain *in vivo* (Figure 6C and D). By contrast, Pth2 appears not to interact with the Rad23 UBA domain (compare lanes 4 and 9 in Figure 6D). Because Pth2 itself does not bind the UBA domain *in vitro* (Figure 6B), it thus appears that the interaction of Pth2 with the Dsk2 UBA domain in cells is mediated by some additional factors. Such putative Pth2-associated factors could bridge the UBA domain and the UBL domain, which may give Dsk2 a distinct function in the shuttling pathway.

The structure of the UBL domain has been determined (Mueller and Feigon, 2003; Lowe *et al*, 2006), and the N-terminal UBL domain can interact intramolecularly with the C-terminal UBA domain (Ryu *et al*, 2003; Walters *et al*, 2003; Lowe *et al*, 2006). The UBL-UBA protein hHR23a undergoes a conformational change between ‘closed’ and ‘open’ states defined by an intramolecular interaction of the UBL and UBA domains (Walters *et al*, 2003). On the contrary to the ‘open’ conformation predicted for the S5a receptor, it is plausible that Pth2 binding alters the conformation of Rad23 so as to form the ‘closed’ states, causing reduced substrate flow to the proteasome. In the case of Dsk2, the interaction of the UBA domain with Pth2 might cause Dsk2 to adopt more ‘closed’ conformation.

Figure 7 The function of Pth2 in the ubiquitin–proteasome pathway is independent of its peptidyl-tRNA hydrolase activity. (A) Pth2 mutants and the sequences of the peptidyl-tRNA hydrolase active site. Conserved residues of the active site (D171, R174, T175 and Q176) were changed by site-directed mutagenesis as indicated in italics. A summary of the hydrolase activity and Rad23/Dsk2 binding of these mutants is shown on the right. (B) Binding of Pth2 hydrolase mutants to Rad23 and Dsk2. Each T7-tagged Pth2 mutant was coexpressed with GST-Rad23 (lanes 1–9) or with GST-Dsk2 (lanes 10–18) in yeast, and cell extracts were precipitated with GSH beads and immunoblotted with anti-GST or anti-T7. Note that the expression of the mutant protein DRTQ171N (lane 17) is rather low when it was coexpressed with GST-Dsk2 but not with GST-Rad23. (C) Overexpression of Pth2 hydrolase mutants in yeast. Pth2 hydrolase mutant (D171A) or wild-type Pth2 was overexpressed in YPH499 cells harboring a single ($\times 1$) pGAL1-YEplac112 or both ($\times 2$) pGAL1-YEplac112 and pGAL1-YEplac195 at 30°C for 2–4 days. (D) Complementation test for drug sensitivity of Pth2 hydrolase mutant. *pth2Δ* expressing D171A from *PTH2* promoter was spotted onto SD plate (minus uracil) containing 0.5 mM AZC in serial dilutions (lower panel). *pth2Δ* expressing FLAG-Pth2 from *PTH2* promoter were spotted in parallel. The plates were incubated at 30°C for 2 days. (E) Requirement of the Pth2 C-terminal domain for binding to Rad23 and Dsk2 *in vivo*. GST-fused Rad23 (lanes 1–6) or GST-Dsk2 (lanes 7–12) was coexpressed in yeast with T7-tagged full-length, N-terminal (1–82) or C-terminal (83–208) domains of Pth2. The extracts were precipitated with GSH beads and immunoblotted with anti-GST and anti-T7.

Pth2 function and redundancy of proteasomal ubiquitin receptors

Three different proteasomal ubiquitin receptors, the Rad23 and Dsk2 shuttle proteins and the Rpn10 regulatory particle

subunit, have been analyzed extensively, and it has been shown that they have overlapping functions in the ubiquitin–proteasome pathway. According to the alternative receptor model (Elsasser *et al*, 2004; Verma *et al*, 2004), Rad23 or Dsk2

acts as a shuttle receptor pathway via the Rpn1 scaffolding protein, whereas Rpn10 seems to prefer the intrinsic proteasomal gateway via its direct binding to polyubiquitin chains. Moreover, another ubiquitin receptor(s) seems to exist in cells, because double deletion mutants and the triple deletion mutant of these receptors are still viable under certain growth conditions (Lambertson *et al*, 1999; Saeki *et al*, 2002; Elsasser *et al*, 2004). The proteasomal ATPase subunit Rpt5 (Lam *et al*, 2002) and elongation factor 1A (eEF1A) (Chuang *et al*, 2005) are candidates for additional ubiquitin receptors. However, it seems that Pth2 is not involved in an Rpt5 or eEF1A pathway, but rather interacts with Rad23 and Dsk2. Pth2-induced growth inhibition is mediated mainly by Rad23 and Dsk2. In the shuttling pathway, Rpn1 seems to be the major receptor pathway for Rad23 and Dsk2. We cannot exclude the direct involvement of Dsk2 in the Rpn10-mediated pathway in cells; however, it appears that Dsk2 interacts with the large scaffolding protein Rpn1 *in vivo*, whose association may be facilitated by its interaction with Rpn10. In light of these aspects, it is conceivable that Pth2 mainly affects Rad23 and Dsk2 through an Rpn1-mediated pathway.

Recent studies suggest that the Cdc48 receptor is involved in ER-associated degradation (ERAD) pathway for misfolded proteins (reviewed by Elsasser and Finley, 2005), where unfolded substrates are recruited to Cdc48 and polyubiquitinated by the E4 enzyme Ufd2, and this complex is subsequently delivered to the proteasome (Koegl *et al*, 1999; Kim *et al*, 2004; Medicherla *et al*, 2004; Richly *et al*, 2005). Dsk2 and Rad23 also mediate delivery in the ERAD pathway (Medicherla *et al*, 2004); therefore, our expectation is that Pth2 may also participate in the degradation of ERAD substrates. More recently, the shuttling pathway involving elongation factor 1A was shown to participate in the ubiquitin-mediated degradation of nascent damaged proteins (Chuang *et al*, 2005). A link between ubiquitin-mediated degradation and protein synthesis has been reported (Sato *et al*, 1998; Schubert *et al*, 2000; Turner and Varshavsky, 2000). During translational elongation, a significant proportion of nascent peptidyl-tRNA dissociates from the ribosome prematurely, and the prematurely dissociated polypeptides are degraded by proteasome-mediated proteolysis. Considering these points, we hypothesize that yeast Pth2 may play bifunctional roles by switching between hydrolysis of peptidyl-tRNA and an interaction with UBL-UBA proteins in protein degradation.

Possible role of the C-terminal and N-terminal domains of yeast Pth2 in the ubiquitin–proteasome pathway

Pth2 homologs have been found in archaea and eukaryotes but not in bacteria (Rosas-Sandoval *et al*, 2002). Although the C-terminal peptidyl-tRNA hydrolase catalytic site is not necessary for Rad23 or Dsk2 binding, another region in the C-terminal domain is required for binding (Figure 7). In addition to the C-terminal domain, the Pth2 N-terminal half may also contribute to full binding of Rad23 and Dsk2 (see Figure 7E). The N-terminal domain has phylogenetically diverged in length and sequence: a protein database search indicated that the N-terminal domains of Pth2 from different eukaryotes, such as yeast, nematode, fly and mammal, are distinct, implying that this domain could define species-specific functions of Pth2. Indeed, mammalian Pth2 has an N-terminal signal sequence for mitochondrial localization mediating apoptosis in humans (Jan *et al*, 2004), while

archaeal Pth2 lacks the N-terminal domain entirely (Rosas-Sandoval *et al*, 2002). *Caenorhabditis elegans* Pth2 (C24G6.8) contains the UBA domain in its N-terminal half and its sequence shows high homology to the deubiquitinating enzyme Ubp14. In yeast, it is possible that the N-terminal domain also plays a part in ubiquitin-mediated degradation, together with the C-terminal Rad23/Dsk2-binding domain.

Materials and methods

Strains and media

The yeast strains used in this study were in the *S. cerevisiae* YPH499 (*MATa ade2-101 leu2-Δ1 trp1-Δ63 ura3-52 lys2-801 his3-Δ200*) background (Sikorsky and Hieter, 1989). Yeast strains were grown in YPD or selective medium. The yeast strain deleted for the *PTH2* gene was generated by PCR-mediated gene disruption (Adams *et al*, 1997) and verified by PCR and immunoblot analysis. For over-expression experiments, yeast cells transformed with a galactose-inducible plasmid were cultured in minimal medium containing 2% raffinose at 30°C to $A_{600} = 0.8$. Galactose (2%) then was added to the medium and the cells were incubated at 30°C for 4 h. The *E. coli* DH5 α strain was used for DNA manipulation, and *E. coli* BL21 (DE3) was used for the expression of recombinant protein. The yeast strain Y190 (*MATa ade2-101 his3-Δ200 leu2-3,112 trp1-901 ura3-52 gal4Δ gal80Δ cyh2 URA3::Gal-lacZ LYS2::GAL-HIS3*) and PJ69-4A (*MATa trp1-901 leu2-3,112 ura3-52 his3Δ200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*) were used for the two-hybrid screening. *DSK2* lacking the N terminus (72–373) was subcloned into pAS404 and used as bait in the two-hybrid screen using a yeast Gal4AD-cDNA library (Funakoshi *et al*, 2002). Transformants that grew on the SD-His-Trp-Leu + 25 mM 3-aminotriazole plate were selected and the library clones were obtained and sequenced.

Plasmids and mutants

For the binding assays, the PCR-amplified *PTH2* ORF was subcloned into a *Bam*HI–*Hind*III site in the galactose-inducible vector pGAL1-YEplac112-T7. For overexpression, *PTH2* DNA was subcloned into a *Bam*HI–*Hind*III site in the galactose-inducible vectors, pGAL1-YEplac112, pGAL1-YEplac181 and pGAL1-YEplac195. For the expression of GST-fused Pth2, the PCR-amplified *PTH2* ORF was subcloned into a *Xba*I–*Hind*III site in the galactose-inducible vector pEG(KG) and expression vector pGEX(KG). For the expression of His₆-T7 tagged Pth2, *PTH2* DNA was subcloned into a *Bam*HI–*Hind*III site in the expression vector pET-28a(+). *PTH2* deletion mutants were constructed by the PCR method and subcloned into pGAL1-YEplac112-T7. For the complementation test, the PCR-amplified *PTH2* ORF was subcloned into an *Eco*RI–*Hind*III site in the expression vector pKQV4. *PTH2* gene (1.1 kb) that has 5'UTR containing its promoter, ORF, 3'UTR, and terminator was generated by PCR amplification from yeast genome DNA and subcloned into a *Bam*HI–*Hind*III site in pRS316 vector. FLAG-*PTH2* in pRS316 was made by the whole-plasmid PCR amplification method (Parikh and Guengerich, 1998). *PTH2* hydrolase mutants were generated by the whole-plasmid PCR amplification method similarly, and subcloned into either pKQV4 and pGAL1-YEplac112-T7 or pRS316.

The PCR-amplified *RAD23* and *DDI1* ORFs were subcloned into a *Bam*HI–*Sal*I site in pEG(KG) and pGEX(KG) to construct GST-tagged Rad23 and GST-Ddi1, and *RAD23* deletion mutants were constructed by the PCR method and subcloned into a *Xba*I–*Xho*I site in pEG(KG) and pGEX(KG). The *RPN1* ORF was generated by the PCR method and subcloned into a *Bam*HI–*Xho*I site in pGEX(KG) and pET-28a(+). The PCR-amplified *RPN10* ORF was subcloned into an *Xba*I–*Hind*III site in pGEX(KG), and into an *Sac*I–*Hind*III site in pET-28a(+). For the expression of 3'-His₆-tagged Dsk2, the PCR-amplified *DSK2* ORF was subcloned into a *Nde*I–*Hind*III site in the expression vector pET-21a, and the other plasmids carrying *DSK2* were described previously (Funakoshi *et al*, 2002). Plasmids carrying Ub-Leu- β -galactosidase (β -gal) and Ub-Ala- β -gal were originally provided by Dr A Varshavsky.

Pull-down assays and immunoblotting

Cell extracts were prepared using glass beads in lysis buffer: 20 mM HEPES pH 7.5, 100 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA,

1 mM *p*-aminophenylmethanesulfonyl fluoride and 1 µg/ml each of leupeptin, pepstatin and chymostatin. For the GST pull-down assay, cell extracts (5.0 A₆₀₀ cells equivalent) or recombinant proteins (1 µg) were incubated with 20 µl glutathione-Sepharose 4B (Pharmacia) in 500 µl lysis buffer for 1 h at 4°C. The beads were washed three times with lysis buffer, followed by SDS–PAGE of the retained proteins and immunoblotting with anti-GST. For FLAG pull-down, cell extracts (5.0 A₆₀₀) were immunoprecipitated with anti-FLAG M2 beads (Sigma). Cell extracts equivalent to 0.2 A₆₀₀ were used for immunoblotting of cell extracts, except for anti-Dsk2 antibody (0.02 A₆₀₀). Recombinant GST-fused proteins and His₆-tagged proteins were prepared from *E. coli* BL21 (DE3) that was transformed with expression vectors. Proteasomes were affinity-purified from the cell lysates prepared by manually ground powder methods with pestle and mortar (Verma *et al*, 2000).

Gel filtration

Gel filtration chromatography was carried out using an AKTA FPLC system equipped with a Superdex 200 HR 10/30 column (Pharmacia). Yeast cell extract was applied to the column and eluted with a buffer (20 mM HEPES pH 7.5, 100 mM NaCl, 1 mM EDTA) at a flow rate of 0.4 ml/min. Then, each fraction was analyzed by immunoblotting. The Gel Filtration Standard (Bio-Rad) containing thyroglobulin (670 kDa), gamma globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and vitamin B-12 (1.35 kDa), and bovine serum albumin (67 kDa) was used for molecular size markers.

Degradation assay of N-end rule substrate and Sic1

The plasmid carrying the Ub-LeuZ fusion gene (Bachmair *et al*, 1986) was transformed into strain YPH499 and *pth2Δ*. Ub-Leu-β-gal and Ub-Ala-β-gal (Varshavsky, 1996) were expressed in yeast under the control of the *GAL1* promoter. Cells were collected by centrifugation and transferred to galactose medium lacking uracil. The incubation was continued for 2 h to induce Ub-Leu-β-gal or Ub-Ala-β-gal, and then dextrose and cycloheximide were added to a final concentration of 2% and 0.5 mg/ml, respectively. Cell extracts were prepared (Runder *et al*, 2000), followed by immunoblotting with anti-β-gal. The plasmid carrying HA-tagged Sic1, pMF920, was transformed into YPH499 and *pth2Δ*. The transformants were pregrown at 30°C overnight in raffinose medium lacking tryptophan. Cells were transferred to fresh raffinose medium and incubated 2 h at 26°C; succinate and α-factor were then added to the culture at a concentration of 0.5% and 10 µg/ml, respectively. After incubating another 4 h at 26°C, galactose (2%) was added to the medium and the incubation was continued for 1 h to induce Sic1-HA protein. Cells were washed twice with sterile water, transferred to glucose medium, and incubated for cell sampling at the time intervals. For overexpression of GST-Pth2 in Sic1-expressing cells, both pMF920 and pEG(KG)-*PTH2* were trans-

formed into YPH499 cells. After synchronization with α-factor, the cells were incubated in galactose medium for 2 h to induce Sic1-HA and GST-Pth2.

Growth sensitivity to amino-acid analogs and UV irradiation

Yeast strains were cultured in synthetic complete medium (for the canavanine assay, lacking arginine) at 30°C for 14–16 h and their cell densities were adjusted to OD₆₀₀ = 1.0. Ten- and three-fold serial dilutions were prepared in sterile water, and 10 µl was spotted on duplicate synthetic complete medium plates (SD) and synthetic complete medium plates supplemented with either 0.5 mM azetidine-2-carboxylic acid (AZC) or 3 µg/ml canavanine. In case of YPD medium, cells were spotted on YPD plates supplemented with 5 mM AZC. Yeast cells on YPD plates were exposed to UV irradiation (50 J/m²). The sensitivity to amino-acid analogs and UV irradiation was determined after 2–4 days of growth at 30°C.

Complementation of *E. coli* strain C600 *pth^{ts}*

Competent *E. coli* C600 *pth^{ts}* cells (Rosas-Sandoval *et al*, 2002) were prepared and transformed with the pKQV4 vector (Strauch *et al*, 1989) containing wild-type *PTH2* and its mutants. Expression in each construct was controlled by the IPTG-inducible *tac* promoter. The transformants were plated on LB agar containing 0.1 mg/ml ampicillin and 1 µM IPTG, and duplicate plates were incubated at 32 and 42°C.

Antibodies and ubiquitins

Anti-Pth2 polyclonal antiserum was raised in rabbits using purified Pth2 prepared from thrombin-digested recombinant GST-Pth2. Other reagents included anti-Dsk2 (Funakoshi *et al*, 2002), anti-Ub, anti-GST, anti-Rad23 and anti-Cdc28 from Santa Cruz Biotechnology (Santa Cruz, CA), anti-T7 and anti-β-gal (Promega), anti-polyubiquitin (FK1, Nippon Bio-Test Laboratories, Tokyo), anti-20S proteasome 'core', anti-Rpt1, anti-Rpt3, tetra-Ub and poly-Ub chains from Affiniti (Exeter, UK), anti-FLAG (M2, Sigma) and anti-HA (Babco).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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