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Endoplasmic Reticulum-associated Degradation of Pca1p, a Polytopic Protein, via Interaction with the Proteasome at the Membrane*

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Endoplasmic reticulum-associated degradation (ERAD) plays a critical role in the destruction of terminally misfolded proteins at the secretory pathway. The system also regulates expression levels of several proteins such as Pca1p, a cadmium exporter in yeast. To gain better insight into the mechanisms underlying ERAD of Pca1p and other polytopic proteins by the proteasome in the cytosol, our study determined the roles for the molecular factors of ERAD in dislodging Pca1p from the endoplasmic reticulum (ER). Inactivation of the 20S proteasome leads to accumulation of ubiquitinated Pca1p in the ER membrane, suggesting a role for the proteasome in extraction of Pca1p from the ER. Pca1p formed a complex with the proteasome at the membrane in a Doa10p E3 ligase-dependent manner. Cdc48p is required for recruiting the proteasome to Pca1p. Although the Ufd2p E4 ubiquitin chain extension enzyme is involved in efficient degradation of Pca1p, Ufd2p-deficient cells did not affect the formation of a complex between Pca1p and the proteasome. Two other polytopic membrane proteins undergoing ERAD, Ste6*p and Hmg2p, also displayed the same outcomes observed for Pca1p. However, poly-ubiquitinated Cpy1*p, a luminal ERAD substrate, was detected in the cytosol independent of proteolytic activities of the proteasome. These results indicate that extraction and degradation of polytopic membrane proteins at the ER is a coupled event. This mechanism would relieve the cost of exposed hydrophobic domains in the cytosol during ERAD.

Buildup of misfolded proteins in the endoplasmic reticulum (ER)[2] induces the unfolded protein response to enhance folding capacity and reduce new protein synthesis (1–4). Terminally misfolded proteins at the secretory pathway are targeted to the ubiquitin-proteasome-dependent removal system, which is known as ER-associated degradation (ERAD) (3, 5–9). Accumulation of misfolded proteins or excess turnover is attributed to multiple diseases, such as cystic fibrosis, diabetes, and amyotrophic lateral sclerosis as well as Alzheimer and Parkinson diseases (3, 6, 7).

Several molecular factors involved in ERAD have been characterized (10–13). In the yeast *Saccharomyces cerevisiae*, misfolded ER luminal proteins and proteins carrying misfolding(s) at the transmembrane region(s) are ubiquitinated by the E3 ubiquitin ligase Hrd1p, whereas proteins carrying misfolding(s) at the cytosolic region(s) are ubiquitinated by Doa10p. Although these E3 enzymes display substrate specificity, some overlap has also been observed (14–17). In congress with the E3 ubiquitin ligases, several other components, such as E2 ubiquitin-conjugating enzymes, E4 ubiquitin extension enzymes, and molecular chaperones, work for recognition and direction of substrates to ubiquitin ligases and the proteasome (3, 13, 16). Because the proteasome is in the cytosol, ERAD substrates must be mobilized from the ER lumen or dislodged from the membrane to be destroyed (18). The Cdc48p AAA-ATPase (p97 in mammals) is thought to provide a primary driving force in the process (10, 14, 19). It has been proposed that translocation of luminal ERAD substrates to the cytosol may occur through a translocon (translocation channel) formed with several proteins, such as Sec61p (20–28), and E3 ubiquitin ligases possessing multitransmembrane domains (*e.g.* Hrd1p) (29–31). E4 ubiquitin chain extension enzymes (*e.g.* Ufd2p and Hul5p) facilitate ERAD through poly-ubiquitination (10, 32, 33).

Despite significant progress in the identification and characterization of molecular factors involved in ERAD, the mechanisms of how proteins in the ER are targeted to the cytoplasmic proteasome remained to be elucidated (3, 10, 32). Integral membrane proteins might be dislodged from the membrane and escorted to the proteasome for destruction (10), which requires the cells to extract proteins containing hydrophobic regions and maintain their solubility in the cytosol. Thus, direct loading of full-length substrates or fragmented pieces into the proteasome during extraction from the membrane could be a mechanism resolving the problem (32).

Our previous study showed that expression of the Pca1p cadmium efflux transporter in the yeast *S. cerevisiae* is dependent upon the ERAD pathway (34). In the absence of cadmium, Pca1p is rapidly turned over through a Doa10p- and proteasome-dependent mechanism. However, when cadmium is present, Pca1p escapes from ERAD and is secreted to the plasma membrane to export cadmium (34, 35). A degron at the N-terminal

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[2] The abbreviations used are: ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated degradation; IP, immunoprecipitation; DTBP, dimethyl 3,3′-dithiobispropionimidate; 3HA, triple HA; 2Myc, two c-myc; Ub, ubiquitin; S, supernatant; N, nonsoluble fraction; P, pellet.

cytosolic domain is responsible for the ERAD of Pca1p and also senses cadmium to rescue Pca1p from ERAD. Because of its rapid turnover ($t_{½} < 5$ min) (34), a degron- rather than misfolding-dependent ERAD, and control of this process by cadmium, Pca1p is a unique example among ERAD substrates. Moreover, the well established experimental systems in yeast and conserved mechanisms for ERAD between yeast and mammals allow Pca1p to be a useful model substrate for gaining a better understanding of the mechanisms underlying ERAD of polytopic membrane proteins.

Here we characterized the roles for the molecular factors involved in dislodging Pca1p from the ER for ERAD. Subcellular location, physical interaction with the proteasome, and turnover rates of Pca1p were determined in yeast cells using genetic and biochemical approaches. Our data indicate that extraction and degradation of Pca1p and two other polytopic membrane proteins, Ste6*p and Hmg2p, are coordinated by complex formation with the proteasome while they reside at the ER membrane. The mechanism is likely significant for avoiding the release of membrane proteins to the cytosol.

Experimental Procedures

Yeast Strains and Growth Conditions—A BY4741 haploid *S. cerevisiae* strain (*MATa his3_1*, *leu2_0*, *met15_0*, and *ura3_0*) and null mutants of particular gene(s), including *hul5*::KanMX6 (*hul5*Δ), *ufd2*::KanMX6 (*ufd2*Δ), *rpn5*::KanMX6 (*rpn5*Δ), and *doa10*::KanMX6 (*doa10*Δ), were obtained from OpenBiosystems. A *pdr5*::KanMX6 (*pdr5*Δ) strain background was used for experiments in which cells were co-cultured with cycloheximide, MG132, or bortezomib. Strains of *doa10*::*His3* or *ufd2*::*His3* in the *pdr5*Δ background were generated by homologous recombination of a deletion cassette as described previously (36).

Yeast cells were cultured in synthetic complete medium (2% (v/v) dextrose, 0.2% (v/v) amino acid mixture, and 0.67% (v/v) yeast nitrogen base) lacking specific amino acid(s) when plasmid selection was necessary. Cells were cultured at 30 °C unless specified otherwise. To inactivate Cdc48p and Cim3p, strains expressing a temperature-sensitive *CDC48* (*cdc48–3*) or *CIM3* (*cim3–1*) allele were cultured at a permissive temperature (23 °C) and then shifted to a restrictive temperature (37 °C) for 30 min (37, 38).

To inhibit translation, cells at mid-log phase were co-cultured with cycloheximide (Sigma, 100 μg/ml) for a period as indicated in each experiment. To inhibit the proteasome, cells were co-cultured with MG132 (Cayman Chemical, 20 μM) (39) or bortezomib (Selleck Chemicals, 20 μM) (40) for 2 h.

Plasmid Construction—A single-copy yeast vector, p416-GPD (41), was used for glyceradehyde-3-phosphate dehydrogenase gene promoter-mediated constitutive expression of *PCA1*, N-terminal truncated *PCA1* (35), *CPY1**, *STE6**, and *HMG2*. Plasmids for HA epitope tagging and GFP fusion at the N terminus of Pca1p were conducted as described previously (35). C-terminal c-myc epitope tagging of Pca1p was achieved by PCR cloning using a primer containing a c-myc sequence prior to the stop codon. Cpy1*p possesses the G255R substitution to be targeted to ERAD (13). Site-directed mutagenesis was accomplished by a primer overlap extension method (42). Two c-myc epitopes were inserted in the C terminus of *CPY1** for Western blotting analysis using anti-myc antibodies. *HMG2* containing one c-myc epitope (43) was subcloned in the p416-GPD vector. Common molecular biology techniques, including plasmid amplification using *Escherichia coli* and purification, followed methods established previously (46). Plasmid transformation into yeast was performed using the lithium acetate method (44).

Fluorescence Microscopy—Cells cultured in synthetic complete medium were mixed with PBS containing NaN_3 (15 mM) and NaF (15 mM) to inactivate cells. Cells were collected by centrifugation and subjected to confocal microscopy (Olympus FV500) as described previously (35).

Fractionation of Cell Lysates—Yeast cells were broken by vortexing (1 min, five times) with glass beads in PBS lysis buffer containing 1 mM PMSF, 5 mM EDTA, and 50 mM protease inhibitor mixture (Halt™, Thermo Scientific). Centrifugation (4 °C, 1000 × *g*, 10 min) removed large cell debris and unbroken cells. Soluble and membrane-associated proteins were separated by centrifugation (4 °C, 100,000 × *g*, 1 h).

To differentiate membrane association *versus* membrane integration of Pca1p, membrane fractions of *prd5*Δ, *cdc48–3* (cultured at 37 °C), and *doa10*Δ cells expressing 3HA epitope-tagged Pca1p were resolubilized in PBS with and without Na_2CO_3 or Triton X-100. After incubation at 4 °C for 30 min, samples were fractionated by centrifugation (4 °C, 100,000 × *g*, 30 min). Supernatant (S) was precipitated using TCA and denatured in SDS sample buffer. The unsoluble pellet (N) was denatured in SDS sample buffer. Both S and N samples were subjected to Western blotting analysis.

Flotation sucrose gradient fractionation was performed as described previously (45). Yeast cells were broken using glass beads in lysis buffer (50 mM HEPES, 150 mM NaCl, 5 mM EDTA, 1 mM DTT, 1 mM PMSF, 50 mM protease inhibitor mixture). Sucrose gradient (0.25 M, 1.5 M, ~1.7 M (mixture of 200 μl of cell lysate and 600 μl of 2.3 M sucrose), and 2.3 M in descending order) was spun at 4 °C at 100,000 × *g* for 5 h. Twelve fractions (300 μl each) were then taken from the top.

In Vitro Deubiquitination—The flotation sucrose gradient fractions (10–12) were subjected to buffer exchange using a Slide-A-Lyzer dialysis cassette (Thermo Scientific) in 500 ml of deubiquitination buffer (50 mM HEPES, 150 mM NaCl, 5 mM EDTA, 1 mM DTT, 1 mM PMSF, and protease inhibitor mixture without sucrose), followed by incubation with 5 ng of USP2 catalytic domain (BML-UW9850-0100, Enzo Life Sciences) while rocking at 37 °C for 1 h. Samples were then subjected to a second round of buffer exchange in the same buffer without DTT. Immunoprecipitation was conducted using a kit (Profound™ c-Myc tag IP/co-IP application set, Thermo Scientific) according to the specifications of the manufacturer.

In Vivo Cross-linking and Immunoprecipitation—Cells expressing Pca1p tagged with the HA epitope were co-cultured with a 20S proteasome inhibitor, MG132 (20 μM, 2 h). Cells were collected by washing twice in ice-cold PBS and resuspended in PBS containing MG132 and a membrane-permeable thiol-reversible cross-linker, dimethyl 3,3′-dithiobispropionimidate (DTBP) (100 μg/ml final concentration), for 30 min at room temperature with gentle rocking. Cells were then

washed in lysis buffer (50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 50 mM protease inhibitor mixture, and 1 mM PMSF) to quench the cross-linking reaction. Cells were then broken by glass bead disruption (10 × 1 min on ice). Lysates were collected and fractionated by centrifugation (100,000 × *g*, 30 min). The resulting pellet was washed twice in ice-cold PBS and resuspended in PBS containing Triton X-100 (1%, v/v) and protease inhibitors. Immunoprecipitation was performed using a kit (Profound™ c-Myc tag IP/co-IP application set, Thermo Scientific) according to the specifications of the manufacturer. DTT in the SDS sample buffer cleaved the cross-links.

Immunoblotting—Yeast cells were broken by vortexing (5 × 1 min) with glass beads in lysis buffer (PBS containing 1 mM PMSF, 50 mM protease inhibitor mixture, 5 mM EDTA, and 1% Triton X-100). The soluble fraction was obtained by centrifugation (21,000 × *g*, 15 min). Protein concentrations were measured using a kit (BCA, Pierce) according to the specifications of the manufacturer. Samples were denatured in a reducing SDS sample buffer containing DTT (25 mM) for 15 min at 37 °C and then subjected to PAGE. This mild denaturation condition avoided aggregation of Pca1p, which contains eight predicted transmembrane helices. The gels were transferred to a nitrocellulose membrane and hybridized with primary antibodies against the HA epitope (Rockland, 600-401-384), the Rpn5p subunit of the 19S proteasome (Abnova, PAB 15594), the α and β subunits of the 20S proteasome (Abcom, ab22673), the c-myc epitope (ABM, G019), or ubiquitin (Covance, MMS-257P). 3-Phosphoglycerate kinase (Pgk1p) was detected using anti-Pgk1p antibodies (Invitrogen, 459250) to determine equal loading. Horseradish peroxidase-conjugated goat anti-rabbit IgG and sheep anti-mouse IgG (Santa Cruz Biotechnology Inc.) were used as secondary antibodies. Western Pico chemiluminescence (Pierce) was used to detect antibody-bound proteins.

Statistical Analysis—Experiments were conducted using a minimum of two independent clones, and representative data are shown. Results are presented as mean ± S.D. Student's *t* test determined the statistical differences. $p < 0.05$ was considered to be significant.

Results

Pca1p Remains in the Membrane When the Proteasome Is Inactivated—Pca1p, a cadmium-exporting P_{1B}-type ATPase in yeast, is a polytopic protein (Fig. 1*A*) that undergoes ERAD in the absence of cadmium (34). A degron identified within amino acids 250 and 350 at the N-terminal cytosolic domain (1–557 amino acids, ~61 kDa) (Fig. 1*A*), Doa10p, an E3 ligase, and the proteasome are required for Pca1p turnover (35). Given the cadmium-induced rescue of Pca1p from ERAD (34, 35) and its eight predicted transmembrane helices, we sought to determine whether ERAD of Pca1p relies on known molecular factors for ERAD of misfolded proteins and how this polytopic protein is degraded by the proteasome. To determine the subcellular localization and turnover rates of Pca1p, we fused GFP or an epitope, such as triple hemagglutinin (3HA) and two c-myc (2Myc) at the N or C terminus (Fig. 1*A*). These *PCA1* alleles are fully functional and display cadmium-dependent ERAD (data not shown).

We first determined the subcellular distribution of GFP-Pca1p when proteolytic activities of the proteasome are inhibited by MG132 co-culture (39) or when Cdc48p AAA-ATPase is inactivated (Fig. 1*B*). Cdc48p is a known critical player in dislodging ERAD substrates from the ER (47). A strain possessing a temperature-sensitive *CDC48* allele (*cdc48–3*) was used to inactive this essential gene (37) at a restrictive temperature, 37 °C. Cells were precultured with cycloheximide to inhibit new Pca1p synthesis. Pca1p fused with GFP at the N terminus (GFP-Pca1p) is not detectable in WT control cells because of rapid turnover, as demonstrated previously (35). However, Pca1p is highly expressed when the proteasome or Cdc48p is inactivated (Fig. 1*B*). Co-localization of Pca1p-GFP with Sec63p, an ER-resident protein, indicates that Pca1p remains in the ER membrane under the experimental conditions. These results suggest that ubiquitinated Pca1p cannot be extracted from the ER membrane without proteolytic activities of the proteasome or upon inactivation of Cdc48p AAA-type ATPase.

We next ascertained the cellular levels of Pca1p and its fragments when the proteasome or Cdc48p is inactivated. Pca1p fused with 3HA and 2Myc (Fig. 1*A*) was expressed in WT, *cdc48–3*, and *doa10*Δ cells. Western blotting analyses of cell lysates using anti-HA or c-myc antibodies displayed signals at the locations corresponding to full-length Pca1p (Fig. 1*C*, *arrowheads*). Given that the half-life of Pca1p is less than 5 min (35), the majority of remaining Pca1p in WT cells co-cultured with cycloheximide should be ubiquitinated species. Pca1p fragments below full-length Pca1p, including ~60-, 70-, 75-, and 90-kDa bands, were detected in WT, *cdc48–3*, and *doa10*Δ cells (Fig. 1*C*, *top panel*). We previously reported that Pca1p ubiquitination is absent in *doa10*Δ cells (34, 35). Therefore, fragmentation of Pca1p, observed in *doa10*Δ cells, is not likely to be a ubiquitination-dependent process. When probing with anti-myc antibodies, only full length was detectable, and all lower molecular weight fragments were nonspecific, as seen by the empty vector control (Fig. 1*C*, *center panel*, *first lane*). To determine the distribution of Pca1p, total cell lysates were fractionated by ultracentrifugation (100,000 × *g* for 30 min) to supernatant (S) and pellet (P) containing soluble proteins and membranes, respectively (Fig. 1*D*). Western blotting analysis detected full-length and higher molecular weight Pca1p species at the P fraction but not the S fraction (Fig. 1*D*). Immunoprecipitation of Pca1p from the S and P fractions with anti-HA antibodies followed by Western blotting using anti-HA and anti-ubiquitin (Ub) antibodies (Fig. 1*E*) further suggested that most ubiquitinated Pca1p species, except a portion of the ~75-kDa fragment, reside in the membrane.

To rule out any off-target effect of MG132 on proteasome inhibition, we also conducted experiments using another proteasome inhibitor, bortezomib (40), and a strain expressing a temperature-sensitive allele of *CIM3* (*cim3–1*) encoding a proteasome subunit (38). Fluorescent confocal microscopy of cells co-cultured with bortezomib displayed virtually identical results to those obtained using MG132. However, cells expressing *CIM3–1* manifested Pca1p stabilization at the ER at both permissive (23 °C) and restrictive (37 °C) temperature (data not shown), which was confirmed by Western blotting analysis (Fig. 1*F*, *fourth versus fifth lane*). This indicates that Cim3–1p is not

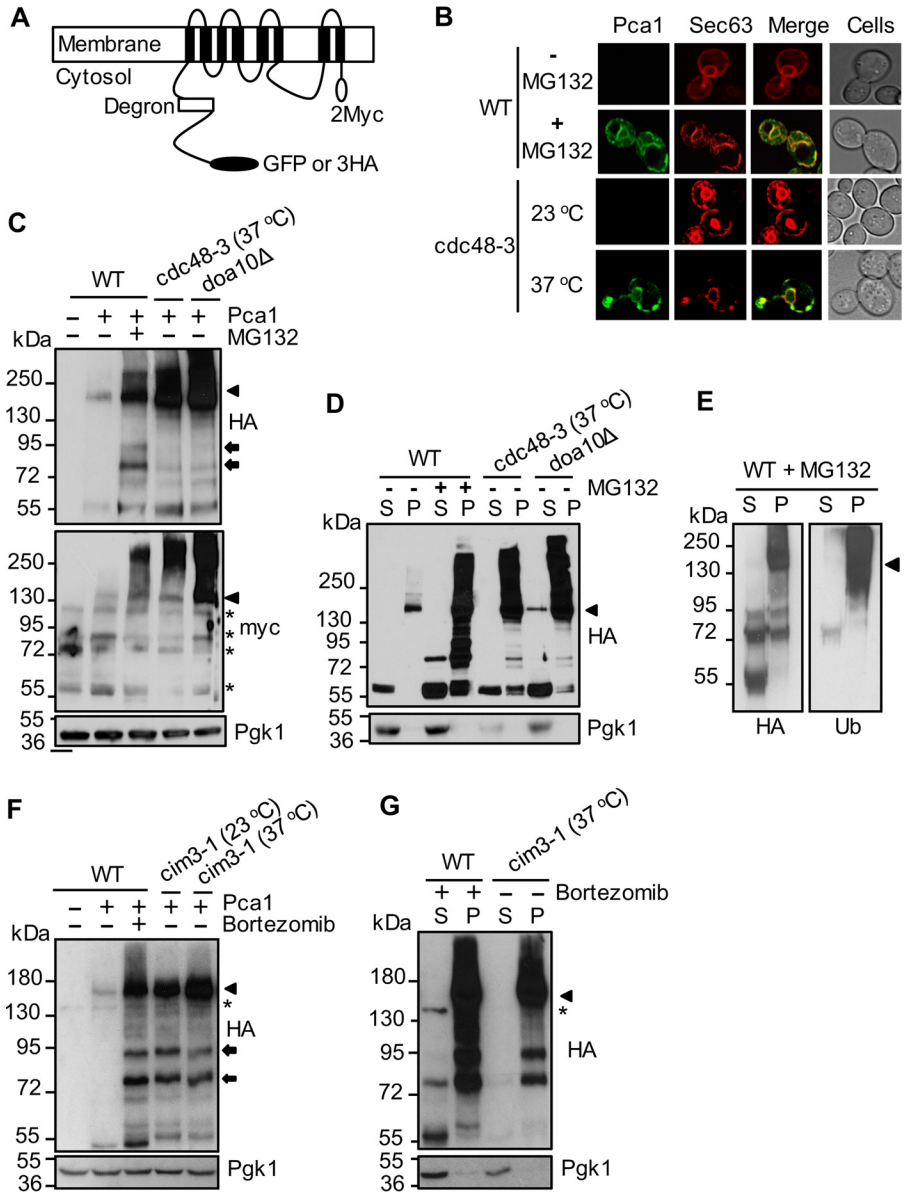

FIGURE 1. **Localization of Pca1p at the membrane of the cells in which the proteasome or Cdc48p is inactivated.** *A*, schematic of Pca1p. The *black squares* indicate eight transmembrane helices. GFP or 3HA is fused at the N terminus (*filled oval*). 2Myc is fused at the C terminus (*empty oval*). The *empty square* indicates the amino acid 250–350 region containing a degron. *B*, subcellular distribution of Pca1p determined by fluorescent microscopy. Expression plasmids of GFP-fused Pca1p and red fluorescent protein-fused Sec63p, an ER-resident protein, were co-expressed in a control strain lacking *PDR5* (*WT*) and a strain containing a temperature-sensitive allele of *CDC48* (*cdc48–3*). Mid-log phase cells were cultured with and without MG132 for 2 h. Cycloheximide was added to the medium 1 h before collecting cells. The *cdc48–3* cells cultured at 23 °C were shifted to 37 °C for 30 min. Subcellular distribution of Pca1p-GFP was visualized by confocal fluorescent microscopy. *C*, detection of Pca1p and its fragments by immunoblotting. Pca1p fused with 3HA and 2Myc was expressed in a WT, a strain expressing the *cdc48–3* allele, and a *doa10*Δ strain. WT and *cdc48–3* cells were precultured with MG132 for 2 h and at 37 °C for 30 min, respectively. Total cell extracts prepared by glass bead disruption and Triton X-100 (1%) solubilization were subjected to Western blotting using anti-HA and anti-myc antibodies. The blots were also probed with anti-Pgk1p antibodies to determine equal loading. *D*, determination of the subcellular distribution of Pca1p. Lysates of the cells that were cultured as described in *C* were prepared by glass bead disruption, followed by removing unbroken cells (centrifugation at 300 × *g* for 10 min). The samples were separated to soluble and pellet fractions by centrifugation (100,000 × *g* for 30 min). The S and P fractions were solubilized (1% Triton X-100) and subjected to Western blotting analysis using anti-HA antibodies. *E*, detection of the majority of ubiquitinated Pca1p at the P fraction. Pca1p in the S and P fractions obtained from WT cells as described above was immunoprecipitated using anti-HA antibodies. The expression levels and ubiquitination status of Pca1p were determined by Western blotting using anti-HA and anti-Ub antibodies, respectively. *F* and *G*, the experiments presented in *C* and *D* were conducted using bortezomib, a proteasome inhibitor, and a strain carrying a temperature-sensitive *cim3–1* allele. The *arrowheads* (*C–G*), *asterisks* (*C*, *F*, and *G*), and *arrows* (*C* and *F*) indicate the location of full-length non-ubiquitinated Pca1p, nonspecific bands detected by anti-myc (*C*) or anti-HA (*F*) antibodies, and a Pca1p fragment dominant in proteasome-inactive WT cells relative to other cells, respectively. Each experiment was conducted three or more times using different clones, and representative data are presented.

fully functional at both 23 °C and 30 °C. Fractionation of soluble and membrane proteins followed by Western blotting of Pca1p also displayed similar results in bortezomib and MG132 co-cultures (Fig. 1*G*). Collectively, the data presented in Fig. 1 suggests that most of Pca1p and its fragments, except for an ~60-kDa fragment, are not dislodged from the membrane without the activities of the proteasome and Cdc48p.

Pca1p in the Pellet Fraction Exists as Membrane-integrated Species—We next considered the possibilities that Pca1p could be extracted out of the lipid bilayer but attached to the mem-

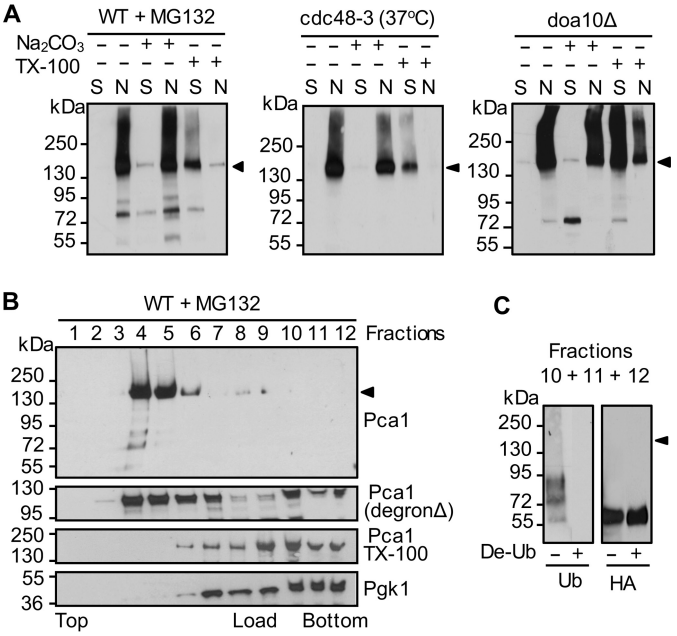

FIGURE 2. **Detection of fragmented or ubiquitinated Pca1p by subcellular fractionation.** *A*, the majority of Pca1p and its fragments are embedded in the membrane. Pca1p fused with triple HA at the N terminus was expressed in a control strain lacking *PDR5* (*WT*), a strain expressing a *cdc48–3* temperature-sensitive allele, and a *doa10*Δ strain. WT and *cdc48–3* cells were cultured with MG132 (20 μM, 2 h) and at 37 °C (30 min), respectively. Membrane fractions obtained by centrifugation (100,000 × *g*, 30 min) of the total cell lysate were resuspended in PBS with and without Na_2CO_3 (0.2 M, pH 11) or Triton X-100 (1%). The samples were incubated for 30 min on ice and then centrifuged at 100,000 × *g* for 30 min. The supernatant was precipitated in trichloroacetic acid (10%) and washed twice with acetone. The nonsoluble pellet was resuspended in PBS containing detergent (1% Triton X-100). Samples were denatured in SDS sample buffer (37 °C, 15 min) and analyzed by Western blotting using anti-HA antibodies. *B*, fractionation of Pca1p by flotation sucrose gradient. WT cells expressing 3HA-tagged Pca1p or Pca1p(degronΔ) lacking the first 392 amino acids containing a degron were co-cultured with MG132 for 2 h. Cycloheximide was added to the medium 1 h before collecting cells. Total cell lysates were prepared by glass bead disruption followed by centrifugation (300 × *g*, 10 min) to remove unbroken cells. Samples were loaded at the layer of 1.7 M sucrose density at a 0.25–2.3 M gradient from *top* to *bottom*. After centrifugation (100,000 × *g*, 5 h), the collected fractions were subjected to Western blotting. An aliquot was incubated with Triton X-100 (1%) on ice for 30 min before the fractionation to extract Pca1p out of the membrane. Obtained fractions were subjected to Western blotting analyses of Pca1p and Pca1p(degronΔ) using anti-HA antibodies. Pgk1p was detected as a marker of fractions containing soluble proteins. *C*, distribution of full-length, ubiquitinated, and fragmented Pca1p species. Fractions obtained as described in *B* were subjected to Western blotting analysis of Pca1p with and without treatment of deubiquitination enzyme. Fractions 10, 11, and 12 containing cytosolic proteins were pooled, deubiquitinated (*De-Ub*), and then solubilized by detergent (1% Triton X-100). Pca1p was immunoprecipitated using anti-HA antibodies. The samples were subjected to Western blotting using anti-Ub and anti-HA antibodies. The *arrowhead* indicates the location where full-length non-ubiquitinated Pca1p migrates. Results were confirmed by three or more repeats, and representative data are presented.

brane or form large cytosolic complexes that could be pulled down to the pellet fraction. To address these concerns, the membrane fractions were incubated with Na_2CO_3 (0.2 M, pH 11) (48) to release peripheral membrane proteins to the supernatant (*S*, Fig. 2*A*). However, all Pca1p species, including Pca1p fragments that migrate faster than full-length Pca1p, remained in the non-soluble pellet (N) factions of WT cells co-cultured with MG132 (Fig. 2*A*, *left panel*). Na_2CO_3 dissociates only a small potion (<5%) of full-length and fragmented Pca1p from the membrane (Fig. 2*A*, *left panel*, *third lane*). Only a small portion of Pca1p detected in the membrane fraction was dissociated by Na_2CO_3 in Cdc48p-inactive cells and in *doa10*Δ cells (Fig. 2*A*, *center* and *right panels*), indicating membrane integration of Pca1p species. Triton X-100 (1%, v/v) released Pca1p to the S fraction nearly completely (Fig. 2*A*), suggesting that most Pca1p and its fragments are integrated in the membrane.

To confirm the localization of Pca1p in the membrane rather than aggregated complexes, cell lysates were subjected to sucrose density gradient fractionation (45) (Fig. 2*B*). If Pca1p is in membrane vesicles, it will "float" to the low-density fractions. WT cells expressing 3HA-tagged Pca1p or Pca1p(degronΔ) lacking the first 392 amino acids containing the degron were co-cultured with MG132 for 2 h. Glass bead-disrupted cells were loaded at the layer of 1.7 M sucrose. Centrifugation followed by Western blotting analysis of collected fractions showed that the majority of full-length Pca1p and Pca1p(Δ392) were within the membrane-containing low-density fractions, unlike Pgk1p, a soluble protein (Fig. 2*B*, *fourth panel*). Membrane solubilization of cell lysate with Triton X-100 (1%, v/v) resulted in co-fractionation of Pca1p with Pgk1p (Fig. 2*B*, *third panel*).

Pca1p species with different degrees of ubiquitination may exist in the cytosol below the detectable limit by Western blotting. To address this concern, we assessed the ubiquitination status of Pca1p in fractions 10, 11, and 12 containing soluble proteins. Pca1p was immunoprecipitated using anti-HA antibodies and then probed with antibodies against ubiquitin and HA epitopes (Fig. 2*C*). The soluble protein fractions contained three major ubiquitin-conjugated Pca1p fragments (~60, ~75, and ~90 kDa) (Fig. 2*C*, *first panel*). It was further determined that poly-ubiquitinated full-length Pca1p species were not present in the soluble fractions because deubiquitination by the purified catalytic subunit of the deubiquitinating enzyme, USP2, did not lead to an enrichment of full-length Pca1p (Fig. 2*C*, *second panel*). Anti-HA antibodies detected primarily a fragment of ~60 kDa. Given the predicted N-terminal cytosolic domain (61 + ~3 kDa corresponding to tagged 3HA epitopes), the ubiquitinated Pca1p fragment detected in the soluble fraction could be a cleaved N-terminal cytosolic domain. However, a portion of the fragment was also detected in the membrane fraction as a peptide integrated into the membrane (Figs. 1, *D* and *G*, and 2, *A* and *B*). Therefore, it is likely that the fragment contains one or more transmembrane helices (Fig. 1*A*) and/or domain(s) incorporated into the lipid bilayer. Collectively, these results indicate that, in the absence of proteasomal activities, Pca1p and most of its fragments, except for the ~60-kDa fragment, are not dislodged from the membrane.

Pca1p Interacts with the Proteasome at the ER Membrane in an E3 Ligase- and Degron-dependent Manner—Given the potential role for the proteasome in extraction of Pca1p from the ER, we next determined whether the proteasome forms a complex with Pca1p at the ER membrane. WT and *doa10*Δ cells expressing C-terminal two c-myc epitope-tagged wild-type control Pca1p and Pca1p lacking the N-terminal 392 amino acids that contain a degron (Pca1(degronΔ)) were co-cultured with MG132 to inactivate the 20S proteasome. Membrane fractions were isolated as described in Fig. 1*D*, washed, and solubilized (Triton X-100, 1% (v/v)). Samples were then subjected to immunoprecipitation of Pca1p. The formation of a complex

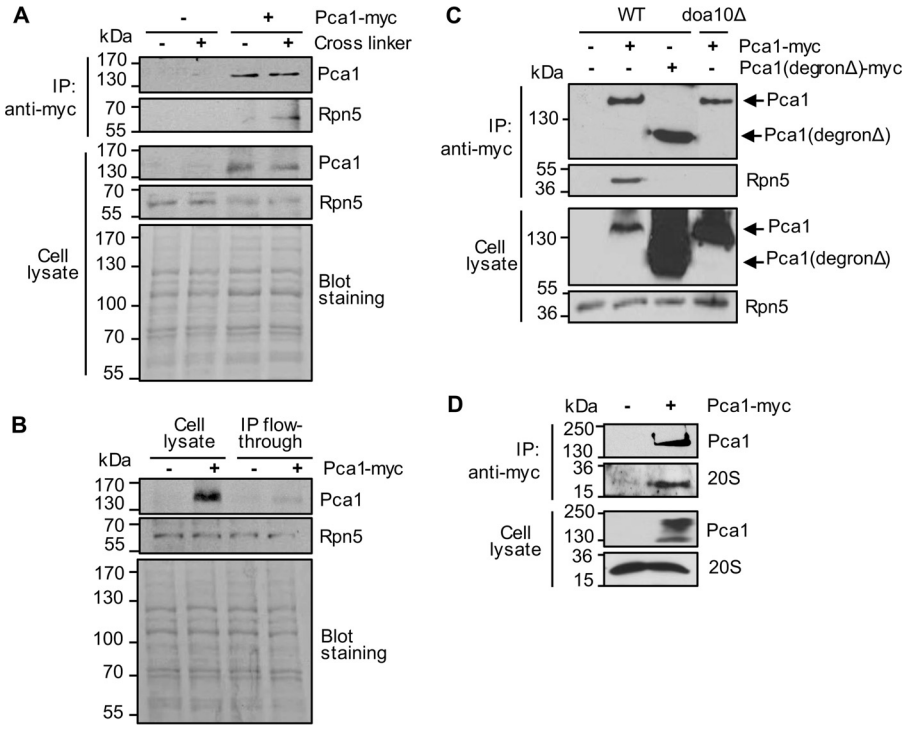

FIGURE 3. **Pca1p forms a complex with the proteasome at the membrane in a Doa10p-dependent manner.** Pca1p, Pca1p(degronΔ) deleting the first 392 amino acids, and control empty vector were expressed in WT and *doa10*Δ strains. Two c-myc epitopes were tagged at the C terminus of Pca1p. Cells were cultured with and without a cell-permeable and reversible cross-linker (DTBP, 100 μg/ml) and MG132 (20 μM) for 2 h. Cell lysates were obtained by Triton X-100 (1%) solubilization of the membrane fraction. *A*, *C*, and *D*, the samples were subjected to immunoprecipitation using anti-myc antibodies. Co-immunoprecipitation of Pca1p with Rpn5p, a subunit of the 19S regulatory particle (*A* and *C*), and the α and β subunits of the 20S catalytic core (*D*) was determined by Western blotting. *B*, Pcalp and Rpn5 levels in the input (*cell lysate*) and flow-through of Pca1p immunoprecipitation (*IP flow-through*) were compared by Western blotting. Each experiment was conducted twice using two independent clones, and representative data are presented.

between Pca1p and Rpn5, a proteasome subunit (49), was visualized by Western blotting. However, no detectable association was observed without cross-linker co-culture (Fig. 3*A*, *lane 3*). This could reflect a transient binding between Pca1p and the proteasome in the process of degradation. To address this concern, we conducted the experiment using a membrane-permeable thiol-reversible cross-linker, DTBP. After breaking the cross-links by denaturing samples in dithiothreitol-containing SDS sample buffer, the physical interactions of the proteasome subunits (39) with Pca1p were visualized by Western blotting. Indeed, Pca1p was found to form a complex with Rpn5p, a 19S proteasomal subunit (Fig. 3, *A*, *fourth lane*, and *C*, *second lane*), and the α and β subunits of 20S the proteasome (Fig. 3*D*) at the membrane. Although the immunoprecipitation efficiency of Pca1p was over 90% (Fig. 3*B*, *top panel*, *second versus fourth lane*), most Rpn5p (>81%) still remained in the flow-through (Fig. 3*B*, *center panel*, *second versus fourth lane*). This suggests that Rpn5p is associated not only with Pca1p but also with other membrane proteins.

The complex formation between Pca1p and Rpn5p was dependent on Doa10p and the degron in Pca1p (Fig. 3*C*). Although the expression levels of Pca1p in *doa10*Δ cells and Pca1p lacking the degron (Pca1p(degronΔ)) were strikingly higher than Pca1p levels in wild-type control cells (Fig. 3*C*, *third lane*) (34, 35), immunoprecipitation of Pca1p and Pca1p(degronΔ) in *doa10*Δ and WT cells, respectively, did not pull down Rpn5p at detectable levels (Fig. 3*C*, *second panel*). These results confirm the specificity in complex formation between Pca1p and Rpn5p and indicate that, when Pca1p is ubiquitinated by Doa10p, the proteasome is recruited while Pca1p resides at the membrane.

Cdc48p Is Required for Complex Formation between Pca1p and the Proteasome—Cdc48p is a critical molecular factor for ERAD of Pca1p (Figs. 1, 2*A*, and 4*A*) and other proteins (10, 34, 37). The ATPase activities of Cdc48p are believed to contribute to the retrotranslocation of ubiquitinated luminal proteins for ERAD (37), and Cdc48p may escort them to the proteasome, as demonstrated by a physical interaction between Cdc48p and the proteasome (37). To determine the role of Cdc48p in the ERAD of Pca1p in conjunction with the interaction between Pca1p and the proteasome, we ascertained whether the proteasome forms a complex with Pca1p in cells expressing inactive Cdc48p. The levels of ubiquitinated proteins in cells expressing non-functional Cdc48p (37 °C culture) are comparable (1.26- $\pm$ 0.62-fold, $n = 3$, $p = 0.53$) with those of control (23 °C culture) cells (Fig. 4*A*, *first panel*). Despite higher Pca1p expression in Cdc48p-defective cells (Fig. 4*A*, *second panel*), immunoprecipitation of Pca1p followed by Rpn5p detection revealed that formation of a complex between Pca1p and the proteasome is dramatically reduced in cells expressing non-functional Cdc48p (Fig. 4*B*). This result suggests that Cdc48p recognizes ubiquitinated Pca1p for recruitment to the proteasome.

It was known that Cdc48p also interacts with other molecular factors involved in ERAD, such as the E4 ubiquitin chain extension enzyme (50). The observed defect of Pca1p ERAD (Fig. 1*B*) and the lack of Pca1p interaction with the proteasome

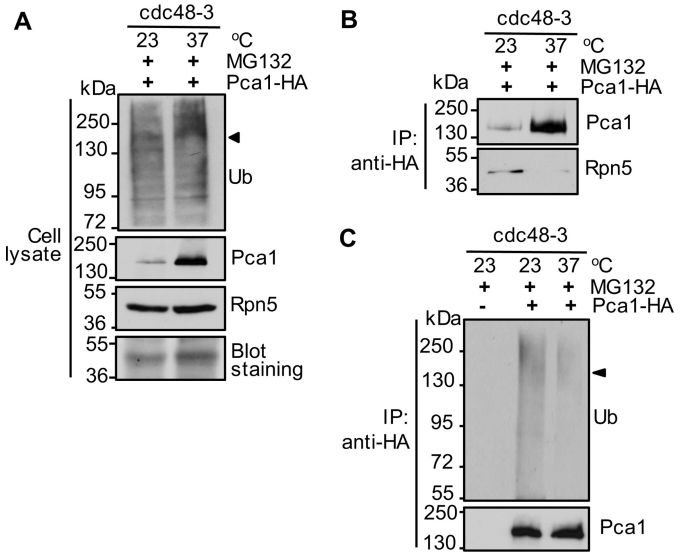

FIGURE 4. **Cdc48p plays a role in Pca1p complex formation with the proteasome but not its poly-ubiquitination.** *A*, no significant change of protein poly-ubiquitination in cells expressing non-functional Ccd48p. Pca1p tagged with the triple HA epitope was expressed in a strain carrying temperature-sensitive *cdc48–3*. Cells at mid-log phase were cultured at a permissive temperature (23 °C) or shifted to a restrictive temperature (37 °C) for 30 min with a cell-permeable and reversible cross-linker (DTBP, 100 μg/ml) and MG132 (20 μM), a proteasome inhibitor, for 2 h. Membrane fractions were solubilized with Triton X-100 (1%). The expression levels of Pca1p and Rpn5p and the overall ubiquitination status were determined by Western blotting. *B*, Cdc48p-dependent physical association between Pca1p and Rpn5p, a subunit of the 19S regulatory particle, was determined by co-immunoprecipitation. The Triton X-100 (1%)-solubilized membrane fraction was used for immunoprecipitation of Pca1p using anti-HA antibodies. Co-immunoprecipitation of Rpn5p, a subunit of the 19S regulatory particle, was detected by Western blotting. *C*, poly-ubiquitination of Pca1p as a function of Cdc48p activities. To determine the ubiquitination status of Pca1p, the samples obtained as described in *B* were subjected to Western blotting using anti-ubiquitin antibodies. To compare the relative ubiquitination of Pca1p, a 5-fold amount of immunoprecipitated Pca1p from cells cultured at 23 °C relative to those at 37 °C was loaded. Data were confirmed by triplicate experiments.

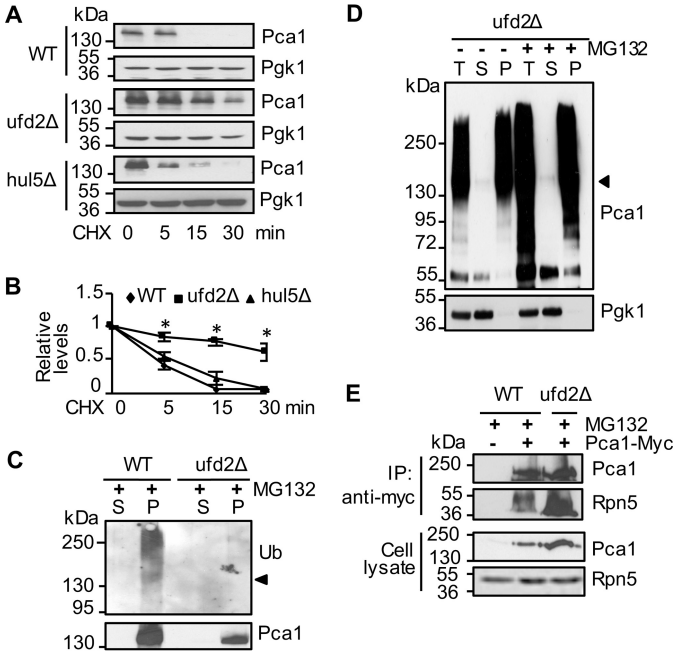

FIGURE 5. **Formation of a complex between Pca1p and the proteasome is independent of poly-ubiquitination of Pca1p.** *A*, significant reduction of turnover rate of Pca1p in *UFD2* gene knockout (*ufd2*Δ) cells. Pca1p tagged with the two c-myc epitope at the C terminus was expressed in WT, *ufd2*Δ, and *hul5*Δ strains. Cycloheximide (*CHX*) chase and Western blotting determined Pca1p levels. Protein extracts were prepared by glass bead disruption and detergent (1% Triton X-100) solubilization. Pgk1p was probed to determine equal loading. *B*, the Pca1p levels presented in *A* were quantitated ($n = 4$). Pca1p levels relative to those at time 0 are presented. *, $p < 0.01$. *C*, defect in poly-ubiquitination of Pca1p in an *ufd2*Δ strain. The Pca1p-tagged c-myc epitope was expressed in an *ufd2*Δ strain. Total cell lysates were obtained with and without co-culture with MG132 (20 μM, 2 h). Samples were subjected to separation to soluble and pellet fractions by centrifugation (100,000 × *g* for 30 min). After solubilization with Triton X-100 (1%), the S and P fractions were subjected to immunoprecipitation using anti-myc antibodies, followed by Western blotting using anti-Ub and anti-myc antibodies to detect Pca1p. *D*, distribution of Pca1p species in the S and P fractions in *ufd2*Δ cells. Total cell lysates (*T*) and the soluble and pellet fractions described in *C* were subjected to Western blotting using anti-myc and -Pgk1p antibodies. *E*, formation of a complex between Pca1p and Rpn5p, a subunit of the 19S proteasome. After co-culturing the cells with a permeable and reversible cross-linker (DTBP, 100 μg/ml) and MG132, an inhibitor of proteasomal proteolytic activities, cell lysates were obtained by glass bead disruption and detergent solubilization. The samples were subjected to immunoprecipitation using anti-myc antibodies. Rpn5p in the samples was detected by Western blotting using anti-Rpn5p antibodies. The *arrowheads* (*C* and *D*) indicate the location where full-length Pca1p migrates. The experiment (*B–D*) was conducted twice using two independent clones, and representative data are presented.

(Fig. 4*B*) in cells expressing non-functional Cdc48p may be attributed to a deficiency in recruiting E4 ligase(s) and/or presenting monoubiquitinated Pca1p for poly-ubiquitination. To address this, we determined the ubiquitination status of Pca1p in Cdc48p-inactive cells. Immunoprecipitation of Pca1p using anti-HA antibodies followed by Western blotting analysis of Pca1p using anti-ubiquitin antibodies determined poly-ubiquitination of Pca1p in cells expressing non-functional Cdc48p (Fig. 4*C*). Given that Pca1p levels in cells expressing nonfunctional Cdc48p are 5-fold higher relative to those in control cells (Fig. 4, *A* and *B*), for the experiment presented in Fig. 4*C*, we loaded a 5-fold higher immunoprecipitated sample obtained from cells cultured at 23 °C. Our data showed that cells expressing non-functional Cdc48p accumulate poly-ubiquitinated Pca1p comparable with control cells (Fig. 4*C*). Therefore, the higher Pca1p expression (Fig. 4*A*, *second panel*) and the reduced association of Pca1p with the proteasome (Fig. 4*B*) in Cdc48p-inactive cells are not likely attributable to a defect in poly-ubiquitination of Pca1p.

Ufd2p, an E4 Ubiquitin Extension Enzyme for Pca1p, and Poly-ubiquitination of Pca1p Did Not Affect Complex Formation between Pca1p and the Proteasome—Following ubiquitination by E3 ligase(s), E4 ubiquitin chain extension enzymes such as Ufd2p and Hul5p in yeast have been implicated in the degradation of several ERAD substrates (10, 32, 50, 51). Ufd2p interacts with Cdc48p and Rad23p in the process of substrate delivery to the proteasome (28). It is intriguing that Ufd2p regulates turnover of only a subset of Doa10p substrates (33), and Hul5p has been found to be associated with the 19S proteasome (32, 52, 53). We determined the $t_{1/2}$, poly-ubiquitination of Pca1p, and formation of a complex between Pca1p and the proteasome in *ufd2*Δ and *hul5*Δ strains. Cycloheximide chase of Pca1p revealed no significant difference in Pca1p degradation in WT and *hul5*Δ cells; however, *UFD2* deletion dramatically extended the $t_{1/2}$ of Pca1p (Fig. 5, *A* and *B*). This result suggests that Ufd2p is required for efficient turnover of Pca1p. Immunoprecipitation of Pca1p followed by immunoblotting using anti-ubiquitin antibodies supported that Ufd2p is the major E4

enzyme of Pca1p (Fig. 5*C*). In *ufd2*Δ cells, most Pca1p species were detected at the membrane fraction but not at the soluble fraction (Fig. 5*D*), indicating that, in *ufd2*Δ cells, the majority of Pca1p remains in the membrane.

Because Ufd2p is required for efficient degradation of Pca1p (Fig. 5*B*), we next sought to determine whether poly-ubiquitination by Ufd2p is necessary for the interaction of Pca1p with the proteasome. *In vivo* cross-linking and co-immunoprecipitation followed by Western blotting analysis showed that Pca1p is still able to interact with the proteasome in the absence of Ufd2p (Fig. 5*E*). This indicates that Ufd2p-dependent ubiquitin chain extension (Fig. 5*C*) is not critical for the formation of a complex between Pca1p and the proteasome. This result also further confirmed that the reduced interaction of Pca1p with the proteasome in cells expressing nonfunctional Cdc48p (Fig. 4, *B* and *C*) is not relevant to the poly-ubiquitination status of Pca1p.

Proteasome-dependent Extraction of ERAD Substrates Is Specific for Polytopic Proteins—We next determined whether other polytopic membrane proteins undergoing ERAD manifest an interaction with the proteasome at the membrane as observed for Pca1p. Ste6*p is a mutated form of the α factor transporter Ste6p in yeast (54), which causes a premature stop codon and a change in *N*-glycosylation. Ste6*p is also targeted for degradation via Doa10p, Cdc48p, and a proteasome-dependent pathway (10, 54, 55). Subcellular fractionation and flotation sucrose gradient fractionation showed that Ste6*p remained in the membrane fraction under proteasome inhibition conditions (data not shown), as observed for Pca1p. The same experiment described in Fig. 3*A* showed that Ste6*p pulled down a proteasome subunit, Rpn5p, in a Doa10p-dependendent manner (Fig. 6*A*). Therefore, Ste6*p also interacts with the proteasome at the ER membrane when it is ubiquitinated by Doa10p.

The expression of Hmg2p, an ER-resident membrane enzyme required for cholesterol synthesis, is controlled through the ERAD-M pathway. When the proteasome is inactivated by MG132 co-culture, Hmg2p also forms a complex with the proteasome at the membrane and not in the cytosol (Fig. 6*B*). Therefore, as observed for Pca1p and Ste6*p, inhibition of the proteolytic function of the proteasome prevents Hmg2p release from the ER membrane.

Vacuolar carboxypeptidase Y (Cpy1p) containing a G255R mutation (Cpy1*p) is degraded through the ERAD-C pathway (13, 56–59). We have employed Cpy1*p as an example of an ER luminal protein to elaborate the hypothesis that soluble ERAD substrates may be retrotranslocated prior to being targeted to the proteasome. When WT yeast cells expressing c-myc epitope-tagged Cpy1*p were co-cultured with MG132, a proteasome inhibitor, Cpy1*p was detected as full-length and poly-ubiquitinated forms in the soluble fraction (Fig. 6*C*). Rpn5p, a subunit of the 19S proteasome, was co-immunoprecipitated with Cpy1*p in the soluble fraction (Fig. 6*C*, *second panel*), indicating that Cpy1*p forms a complex with the proteasome in the cytosol. However, Cpy1*p in cells lacking Hrd1p E3 ligase targeting Cpy1*p for ERAD remains at the pellet fraction along with Kar2p, an ER-luminal protein (Fig. 6*D*). Therefore, detection of Cpy1*p in the soluble fraction (Fig. 6*C*) is a consequence of ERAD progress of Cpy1*p rather than its leaking from the ER

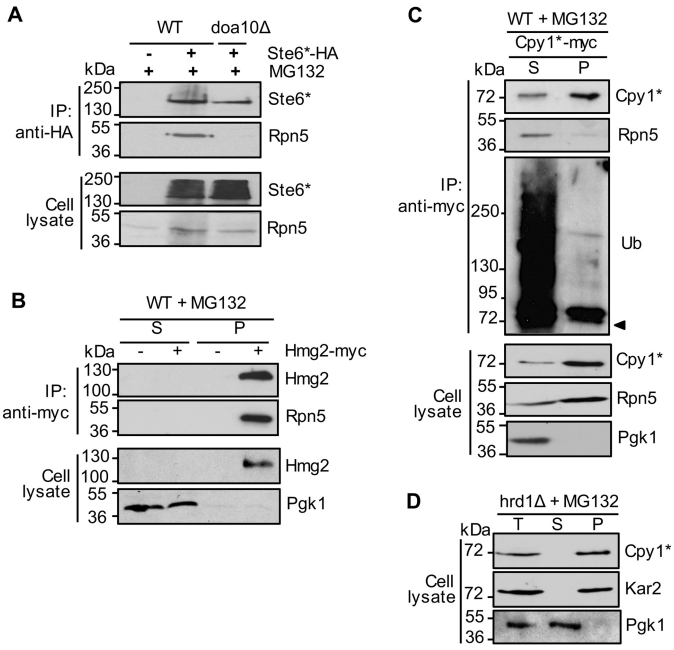

FIGURE 6. **Ste6*p, Hmg2p, and Cpy1*p form a complex with the proteasome at the membrane.** Ste6*p, Hmg2p, or Cpy1*p tagged with either HA or the c-myc epitope was expressed in WT control (*A–C*) *doa10*Δ (*A*) and *hrd1*Δ (*D*) cells. Cells were co-cultured with MG132 (20 μM, 2 h), an inhibitor of proteasomal proteolytic activities, and a cell-permeable reversible cross-linker (DTBP, 100 μg/ml, 30 min). Total cell lysates (*T*) were prepared by glass bead disruption. Samples were subjected to separation of soluble and membrane (*P*) fractions by centrifugation (100,000 × *g*, 30 min). Protein extracts were obtained by solubilizing samples with detergent (1% Triton X-100). *A*, the membrane fractions were used to determine an interaction of Ste6*p with the proteasome. HA-Ste6*p was subjected to anti-HA immunoprecipitation followed by Western blotting using anti-HA and anti-Rpn5 antibodies. *B* and *C*, lysates of WT cells expressing Hmg2p-myc or Cpy1*p-myc were subjected to fractionation (S and P) followed by detergent solubilization. Anti-myc immunoprecipitation and Western blotting using anti-Rpn5p and anti-myc antibodies determined their physical interaction. The ubiquitination status was determined by probing the blots with anti-Ub antibodies (*C*, *third panel*). *D*, Cpy1*p expressed in total cell lysate (*T*) and S and P fractions of *hrd1*Δ cells was detected by Western blotting using anti-myc antibodies. The blots were also probed with antibodies against Kar2p and Pgk1p, an ER-resident protein, and cytosolic protein, respectively. Representative data of two or more independent experiments are presented.

during sample preparation followed by interaction with the proteasome. These results suggest that Cpy1*p ubiquitinated by Hrd1p is retrotranslocated to the cytosol without proteolytic activities of the proteasome. This is distinct from other examined polytopic proteins (Pca1p, Ste6*p, and Hmg2p).

Discussion

ERAD involves retrotranslocation of ER luminal proteins and dislodging of membrane proteins. Our data suggest that polytopic ERAD substrates undergo proteasomal degradation at the ER membrane via recruiting the proteasome rather than being extracted to the cytosol prior to delivery to the proteasome. This is distinct from ERAD of Cpy1*p, a soluble protein in the lumen of the ER. The coupling of dislodging and destruction of membrane proteins would be advantageous for cells by minimizing the energy cost associated with preventing detrimental effects of hydrophobic proteins in the cytosol. These results shed new light on this vital but undercharacterized process.

Cdc48p (p97/VCP in higher eukaryotes), a hexameric AAA-type ATPase, plays diverse roles in ERAD (60, 61), although the mechanistic details remain to be defined. Despite experimental evidence arguing for a non-essential role of Cdc48p in the ERAD process (62), our study confirmed that Cdc48p is a significant player for ERAD of Pca1p by promoting its binding to the proteasome. In cells expressing inactive Cdc48p, the complex formation between the proteasome and Pca1p and Pca1p degradation is impaired. This result is consistent with a previous report displaying cooperation between Cdc48p and the proteasome in turnover of Insig-1p, an ERAD-M substrate (63). Several lines of evidence support that Cdc48p and its cofactors Ufd1p and Npl4p are recruited to the ER via Ubx2p to disassemble ubiquitin-conjugated substrates from the membrane and for the delivery of those to the proteasome (60, 61). Therefore, it is likely that Cdc48p facilitates the interaction between ubiquitinated Pca1p and the proteasome.

Distinct from the ERAD-L pathway dealing with soluble substrates, a concern in ERAD of integral membrane proteins (ERAD-M and -C pathways) would be the extraction of proteins from the lipid bilayer and solubilization at the cytosol (10, 64). Although several potential mechanisms have been proposed (3, 10, 32), the results reported previously were not consistent with each other. An *in vitro* reconstitution assay found that, prior to degradation, Ste6*p, a well characterized ERAD-C substrate, is dislodged from the membrane (10). However, it is worth pointing out that only a small fraction of Ste6*p was released from the membrane under the experimental conditions. Secondly, an *in vitro* ERAD assay of cystic fibrosis transmembrane conductance regulator (CFTR) displayed relatively minor effects of proteasome inactivation on extraction and release of its degradation intermediates from the ER membrane (65). Our study was conducted in cells, and most ubiquitinated Pca1p and its fragments remained in the membrane and formed a complex with the proteasome. This finding was confirmed with two other polytopic proteins. Therefore, our data support the hypothesis that dislodging of membrane proteins does not occur prior to their delivery to the proteasome.

Detection of the complex containing Pca1p and subunits of the 19S and 20S proteasomes from the membrane fractions is dependent on Doa10p, the major E3 ubiquitin ligase of Pca1p. This suggests that ubiquitinated Pca1p attracts the proteasome while residing in the ER membrane. The proteasomes are localized mainly in the nucleus and cytosol as free or nuclear envelope- and ER network-attached forms (66–69). The clustering of the proteasomes on the ER membrane is particularly high in the yeast *S. cerevisiae* (>80% total proteasome) relative to mammals (<20%) (67). The proteasome might be associated with the ER via physical interaction with ER membrane protein(s), which is reminiscent of Cue1 and Ubx2p recruiting Ubc6p/7p and Cdc48p to the ER membrane, respectively (71–73). This may allow for rapid recognition of ERAD substrates by the proteasome. Sts1p is critical for enriching the proteasome at the nuclear envelope (70). However, the counterpart at the ER membrane remains to be identified. Alternatively, the enrichment of the proteasome at the ER membrane might reflect the complexes between the proteasome and proteins undergoing ERAD. High demand of ERAD might control the distribution of the proteasomes for an efficient completion of the task. The 26S, 20S, and 19S proteasomal particles are known to exist in a dynamic equilibrium (68). The assembly, subunit composition, posttranslational modifications of amino acid residues, and interaction of the proteasome with regulators(s), if any, could be changed in response to cellular cues and stresses (66). It would be interesting to examine whether ER stresses would lead to an enrichment of the proteasomes to the ER membrane. This could be better accessed in mammals displaying relatively minor steady-state ER localization of the proteasome (74, 75).

The significance of poly-ubiquitination of membrane proteins in ERAD remains an intriguing question. The regulatory particles of the proteasome contain several functional components, including AAA-type ATPase, E4 ubiquitin ligase(s), and deubiquitination enzyme(s). Hul5p, an E4 enzyme associated with the regulatory particle of yeast, is involved in ERAD of reporter substrates (*e.g.* CTL* and Sec61–2L) (32) and proteasome-dependent degradation of cytosolic proteins that are damaged under heat stress (52). However, knockout of the *UFD2* gene encoding another E4 ligase did not affect the heat-induced ubiquitination (52). Our study on the turnover rate of Pca1p in the *hul5*Δ and *ufd2*Δ strains showed that Ufd2p, but not Hul5p, is involved in ERAD of Pca1p, confirming the target specificity of E4 ligases. Nevertheless, it is worth noting that the relative stability of Pca1p in the *ufd2*Δ strain was still significantly lower than that of the *doa10*Δ strain ($t_{1/2}$ = 30 min *versus* >2 h), which suggests that poly-ubiquitination is not essential for ERAD but enhances efficiency. One might argue that, given three other E4 ligases in addition to Hul5p and Ufd2p in the genome of the yeast *S. cerevisiae* (76), there may be some redundancy. However, we disproved this by showing a near absence of poly-ubiquitinated Pca1p in *ufd2*Δ cells. It is also interesting to note that, although Pca1p turnover is slow in *ufd2*Δ cells and a drastic reduction of poly-ubiquitination of Pca1p in the cells, Ufd2p deficiency does not affect the formation of a complex between Pca1p and the proteasome. These results indicate that poly-ubiquitination of Pca1p is not a critical determinant of the initial interaction with the proteasome but, rather, promotes efficient processing of substrates by the proteasome.

This study reflects a comprehensive *in vivo* study determining how polytopic proteins are targeted to the ERAD pathway in yeast. The recruitment of the proteasome to ubiquitinated proteins while they reside in the membrane could be a conserved mechanism in ERAD of higher eukaryotes. It would be also interesting to define whether the proteasome-dependent destruction of membrane proteins in other organelles occurs in a similar manner.

Author Contributions—N. S., D. J. A., and J. L. designed the research. N. S., D. J. A., M. Z., X. Q., and H. K. performed the experiments. N. S., D. J. A., and J. L. analyzed the data. N. S., D. J. A., and J. L. wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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