Characterization of the proteasome interaction with the Sec61 channel in the endoplasmic reticulum

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Summary

Biogenesis of secretory proteins requires their translocation into the endoplasmic reticulum (ER) through the Sec61 channel. Proteins that fail to fold are transported back into the cytosol and are degraded by proteasomes. For many substrates this retrograde transport is affected by mutations in the Sec61 channel, and can be promoted by ATP and the 19S regulatory particle of the proteasome, which binds directly to the Sec61 channel via its base. Here, we identify mutations in *SEC61* which reduce proteasome binding to the channel, and demonstrate that proteasomes and ribosomes bind differently to cytosolic domains of the channel. We found that Sec63p and BiP coprecipitate with ER-associated proteasomes, but Sec63p does not contribute to proteasome binding to the ER. The 19S base contains six AAA-ATPase subunits (Rpt proteins) that have non-equivalent functions in proteasome-mediated protein turnover and form a hetero-hexamer. Mutations in the ATP-binding sites of individual Rpt proteins all reduced the affinity of 19S complexes for the ER, suggesting that the 19S base in the ATP-bound conformation docks at the Sec61 channel.

Key words: ER translocation, ER quality control, ERAD, 19S regulatory particle, Rpt proteins, Yeast, Ribosome binding

Introduction

Proteasomes are responsible for misfolded secretory protein turnover after export of these proteins through a channel in the ER membrane to the cytosol (ER-associated degradation, ERAD) (Romisch, 2005). The composition of this channel is controversial, but retrograde transport of many soluble substrates is dependent on the Sec61 channel which also mediates protein import into the ER during secretory and transmembrane protein biosynthesis (Romisch, 2005). The Sec61 channel consists of three proteins, Sec61p, Sbh1p, and Sss1p in yeast, equivalent to Sec61α, β, γ in mammals (Johnson and van Waes, 1999). This channel on its own mediates cotranslational protein translocation into the ER, during which the ribosome binds to Sec61p and Sbh1p (Kalies et al., 2005; Levy et al., 2001). A mutation in the cytosolic loop 8 of Sec61p reduces its affinity for ribosomes (Cheng et al., 2005). The crystal structure of an archaeal homologous channel in the closed conformation consists of a single Sec61α/β/γ heterotrimer, but the presence of ribosome-nascent chain complexes with signal peptides provokes the assembly of three to four heterotrimers in the ER membrane which can also be co-isolated with ribosomes from ER membranes and fluorescence quenching experiments suggest that in the open state the channel is formed by several Sec61 complexes (van den Berg et al., 2004; Hanein et al., 1996; Hamman et al., 1997). For posttranslational protein import into the ER, the Sec61 channel associates with the heterotetrameric Sec63 complex (Sec63p, Sec62p, Sec71p, Sec72p) (Johnson and van Waes, 1999). Sec63p also functions independently of the other subunits of the Sec63 complex in nuclear fusion and cotranslational import (Ng and Walter, 1996; Young et al., 2001). The ER lumenal loop of Sec63p interacts with the chaperone Kar2p (BiP in mammals) which is involved in both protein import into the ER and in export from the ER for degradation (Kabani et al., 2003). Kar2p and a second ER lumenal chaperone required for ERAD, protein disulfide isomerase, Pdi1p (PDI in mammals), keep ERAD substrates export-competent by preventing their aggregation (Gillece et al., 1999; Kabani et al., 2003). The chaperones may also target substrates to the export channel or trigger channel opening from the lumenal side (Romisch, 2005; McCracken and Brodsky, 2005).

A number of reports have been published on the isolation of ER membrane protein complexes containing components of the ERAD machinery. These suggest that there are two major complexes in the ER membrane, the Doa10p complex and the Hrd1p complex, named after their central E3 ubiquitin ligase subunits (Carvalho et al., 2006; Denic et al., 2006; Gauss et al., 2006). The Doa10p complex consists of Ubc7p and Cue1p (an E2 enzyme and its membrane anchor), the cytosolic AAA-ATPase Cdc48p and its cofactors, and its ER membrane anchor Ubx2p (Carvalho et al., 2006). Cdc48p is required for release of defective, ubiquitinated proteins from the ER membrane and escorting them to the proteasome (Romisch, 2005; McCracken and Brodsky, 2005). The Hrd1p complex contains the transmembrane Hrd3 protein which stabilizes Hrd1p and recognizes ERAD substrates via its lumenal domain, in concert with the ER lumenal lectin Yos9p, which itself is bound to Hrd3p and Kar2p (Carvalho et al., 2006; Denic et al., 2006; Gauss et al., 2006). This complex also contains Usa1p, a recently identified ER membrane protein required for ERAD, and Ubx2p/Cdc48p (Carvalho et al., 2006; Denic et al., 2006).

In one purification regimen, Emp47, which is required for vesicle budding from the ER, copurified with the complex (Denic et al., 2006). Using a different protocol the Hrd1p complex identified by Carvalho et al. was found to be lacking Kar2p and Emp47, but also contained Cue1p and Ubc7p, and Der1p (Derlin-1 in mammals), a transmembrane protein of unknown function (Carvalho et al., 2006). Because it consists mostly of transmembrane domains, and interacts with the MHC class I heavy chain during its virally induced retrotranslocation from the ER, Derlin-1 has been proposed to form the protein export channel in the ER membrane (Lilley and Ploegh, 2004; Ye et al., 2004). *DER1* can be deleted in yeast, however, with limited consequences for turnover of most ERAD substrates. The protein contains probably only three transmembrane domains, and is expressed at low levels, therefore it is more likely to be an accessory protein required for export of only a subset of substrates (Kim et al., 2006; Romisch, 2005; Carvalho et al., 2006).

Two subparticles, the 20S proteolytic core particle (CP) and the 19S regulatory particle (RP), which contains a de-ubiquitating enzyme, 6 AAA-ATPases, and a number of proteins of unknown function, form the 26S proteasome (Voges et al., 1999). Recent data suggest that at the end of proteasomal substrate turnover in a cell, the 19S and 20S particles dissociate from each other in a process dependent on ATP hydrolysis, and that the 19S particle dissociates further into its base and lid subparticles (Babbitt et al., 2005). This assembly/disassembly cycle of the proteasome may allow for exchange of the 19S lid with the structurally homologous COP9 signalosome (Sharon et al., 2006). Individual proteasome 19S subparticles can also function on their own in transcription (Gonzalez et al., 2002; Lee et al., 2005).

In a cell-free system, proteasomes in the presence of ATP promote export and degradation of a soluble degradation substrate from ER to the cytosol (Lee et al., 2004). Export and degradation of this substrate can be uncoupled, and the 19S RP on its own is sufficient for export in this system (Lee et al., 2004). Proteasomes bind to the Sec61 channel in yeast and mammalian ER, and compete with ribosomes for channel binding (Kalies et al., 2005). The interaction is ATP dependent, and mediated by the 19S RP and protease-sensitive cytosolic loops of the Sec61 complex (Kalies et al., 2005). Here we further examined the interaction of the 19S RP with the Sec61 channel. We asked whether we could isolate mutations in *SEC61* defective in proteasome binding, and whether a *sec61* mutant defective in ribosome binding had any effect on proteasome binding. We investigated which other ER proteins were present in the proteasome/Sec61 channel complex, and whether one of the identified proteins, Sec63p, contributed to proteasome binding to the ER. Finally, we determined the contribution of individual 19S RP subunits to interaction of the complex with the Sec61 channel in the ER membrane.

Results

Proteasomes and ribosomes bind to different sites in the Sec61 channel

Proteasomes can bind directly to the Sec61 channel via the 19S RP and compete with ribosomes for binding to ER membranes (Kalies et al., 2005). Ribosome binding to the Sec61 channel is required during cotranslational import. We therefore asked if mutations in *SEC61*, which reduce cotranslational protein import into the ER, have any effect on proteasome 19S RP binding to ER membranes. After mutagenizing the *SEC61* gene by error prone PCR we first selected mutants that were viable by expressing them in a strain in which the wild-type *SEC61* gene was expressed from the *GAL1* promoter and screening for growth on glucose (not shown). We then transformed these *sec61* mutants with reporter plasmids for cotranslational translocation, pPHO8-URA3 (this study), and for posttranslational translocation, pCPY-URA3 (Ng et al., 1996). If translocation of the reporter fusion protein is reduced in a *sec61* mutant, the Ura3p part of the chimaeric protein allows growth on plates without uracil. As shown in Fig. 1A, we identified three *sec61* mutants that were defective in translocation of the cotranslational import substrate alkaline phosphatase (Pho8p), but not in posttranslational import of carboxypeptidase Y (CPY). The mutant *sec61* genes were sequenced. We found that *sec61-301* carried two point mutations which led to conversion of R67C and D227G (filled circles, Fig. 1B). D227G is located in a loop close to the lumenal end of transmembrane helix 5 (Fig. 1B). Although both substitutions are non-conservative, R67C is more likely to be responsible for the functional defect in Sec61p since it is located in a domain of Sec61p that is homologous to the so-called 'plug' in archaeal *SecY*, which blocks the central pore of the channel in the closed conformation, but it is surprising that it exclusively affects co-translational import (Fig. 1B) (van den Berg et al., 2004). Junne et al. (Junne et al., 2006) found a pronounced defect in post-translational import in a 'plug' deletion mutant of yeast Sec61p and only minor defects in import of co-translationally imported substrates. The differences in our observations may be a difference between a point mutation and complete deletion of the plug, or due to a contribution of the D227G mutation, or to the strain backgrounds used. Nevertheless our finding confirms the observation by Junne and colleagues that the 'plug' is not essential for general Sec61p function (Junne et al., 2006).

The *sec61-302* allele contains four point mutations: D168G, S179P, F263L, and S353C (asterisks, Fig. 1B). Three of these mutations are at or close to the cytoplasmic end of transmembrane helices 4, 5 and 6, whereas S353C is located in the large ER lumenal loop between transmembrane domains 7 and 8 (Fig. 1B). Another mutation in this loop, *sec61-3*, strongly affects all transport through the *sec61* channel, but several other mutations in this domain have no effects (Stirling et al., 1992) (not shown). The *sec61-303* mutant contains two of the same point mutations, D168G and F263L (asterisks in transmembrane domains 4 and 6, Fig. 1B).

For proteasome binding experiments, we isolated microsomes from wild type and the three isogenic *sec61* mutant yeast strains with defects in co-translational import, and a strain with a point mutation at the lumenal end of transmembrane domain 4, *sec61-32* (C150Y), which has been shown previously to have general defects in import into the ER and export to the cytosol for ERAD (Pilon et al., 1997). After removal of ribosomes with puromycin and high salt, stripped membranes (PK-RM) were incubated with purified 19S RP; binding was analyzed by flotation of the membranes in sucrose gradients and immunoblotting of gradient fractions for the FLAG-tagged 19S RP subunit. Total proteasome binding to yeast PK-RM was relatively low, which made it difficult to interpret differences between wild-type and mutant membranes

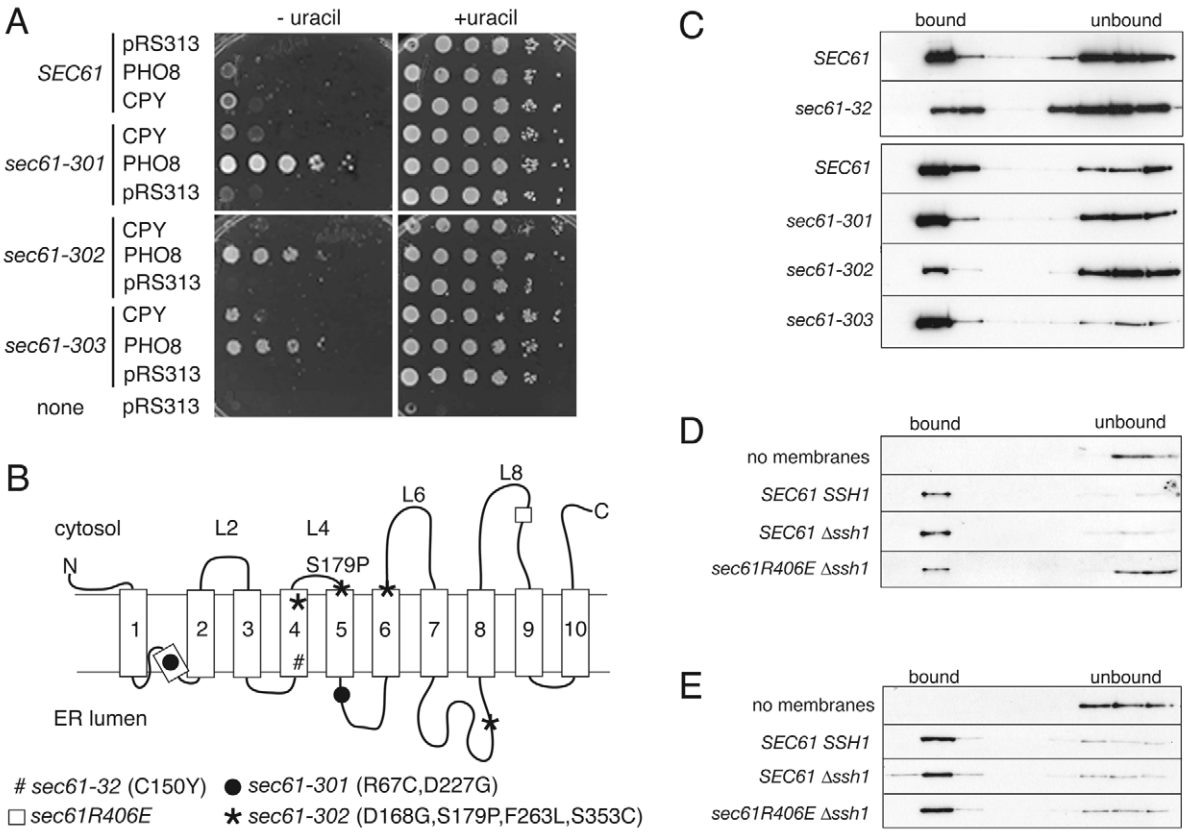

Fig. 1. Proteasomes and ribosomes bind to different sites in the Sec61 channel. (A) JDY683 (pGAL-*SEC61*) derivatives with pCEN-*LEU2-SEC61*, or the same plasmid containing the indicated *sec61* mutants, or the empty vector, were transformed with a reporter plasmid for co-translational import, pRS313-*PHO8-URA3*, or the reporter plasmid for post-translational import, pRS313-*CPY-URA3*, or the empty vector. Growth was monitored on SD containing glucose and uracil, but lacking leucine and histidine (right panels; this controls for Sec61p function) and the same plates without uracil (left panels). Growth in the absence of uracil indicates a translocation defect for the *URA3* reporter. (B) Topology model of Sec61p. Position of mutations in mutant alleles used in the proteasome and ribosome binding experiments in C-E is indicated. (C) Yeast 19S RP (2 pmol) FLAG-tagged on Rpn11p were incubated with 20 eq of proteoliposomes from puromycin/high salt-treated yeast microsomes as described in Materials and Methods. 1 eq equals 1 μl of microsomes of A_{280}=50. The membranes were floated through 1.8 M sucrose cushions and gradients fractionated from the top. Proteasomes in individual fractions were detected by SDS-PAGE and immunoblotting with anti-FLAG antibody. Sec61p was also detected, and was equal in wild-type and corresponding *sec61* mutant membranes (not shown). The positions of proteasomes bound to membranes and of unbound proteasomes are indicated. (D) Dog pancreas ribosomes (1.25 pmol) were bound to 20 eq wild-type or *sec61* mutant proteoliposomes under the same conditions as proteasomes above, and analyzed as above using an antibody against the small ribosomal subunit S6. (E) Yeast 19S RP (2 pmol) were bound to 20 eq of the same wild-type and mutant proteoliposomes used for ribosome binding in D, and binding analyzed as in C.

(not shown) (see also Kalies et al., 2005). We had observed previously that solubilization of yeast membranes and reconstitution of total protein into proteoliposomes improved the binding of proteasomes to the membranes (Kalies et al., 2005). We therefore prepared proteoliposomes from wild-type and *sec61* PK-RM and incubated these with excess 19S RP. Sec61p content of proteoliposomes was monitored by immunoblotting and found to be equal in wild-type and corresponding mutant membranes (not shown). Overall 19S RP binding was increased compared to PK-RM, thus we were able to detect a significant reduction in 19S RP binding to *sec61-302* membranes compared to wild type (Fig. 1C, lower panel). The mutations in *sec61-303* did not affect 19S RP interaction with the ER membrane, therefore D168G and F263L are not responsible for reduced interaction of 19S RP with mutant Sec61p in *sec61-302* (Fig. 1C, lower), but they had a strong negative effect on co-translational import, suggesting that proteasomes and ribosomes bind differently to the protein translocation channel. S353C, which is located in a lumenal loop of Sec61p, and *sec61-301*, which is mutated in two other lumenal loops that are inaccessible from the cytosolic side of the membrane, had no effect on proteasome binding (Fig. 1C, lower). We therefore conclude that the S179P mutation at the beginning of transmembrane domain 5 is the probable cause for reduced proteasome binding to *sec61-302* membranes.

We also observed reduced proteasome association with *sec61-32* proteoliposomes, although the effect here was not as strong as in *sec61-302* membranes (Fig. 1C, top panel). This mutation is located towards the lumenal end of transmembrane domain 4 and this region of Sec61p cannot make direct contact with the proteasome 19S RP in the cytosol. The mutation may

instead change the conformation of transmembrane helix 4 which in turn may affect proteasome binding.

Proteasomes and ribosomes compete for binding to the Sec61 channel, but may bind to different domains (Kalies et al., 2005). The ribosome binding site in Sec61p has been identified in cytosolic loop 8 (Kalies et al., 2005; Cheng et al., 2005). We therefore asked, whether a mutation in this loop, *sec61*R406E, which has been shown to drastically lower the affinity of ribosomes for the Sec61 channel, had any effect on proteasome binding to ER membranes (Fig. 1B) (Cheng et al., 2005). The gene encoding the *SEC61* homologue, Ssh1p, which can also bind to ribosomes, was deleted in this strain (Cheng et al., 2005). We prepared PK-RM from *sec61*R406E and the corresponding wild-type strain, and first assayed ribosome binding under conditions identical to those used for proteasome binding. Samples were analyzed by flotation in sucrose gradients and immunoblotting with an antibody against the small ribosomal subunit S6. As shown in Fig. 1D ribosome binding to the *sec61*R406E membranes was reduced compared to binding to wild-type membranes (Fig. 1D, compare bound to unbound *SEC61* versus *sec61*R406E). Ssh1p is only present at about 10% of the level of Sec61p in ER membranes, and the resolution of our assays was not high enough to address its contribution to ribosome or proteasome binding to the ER (Fig. 1D and E, *SSH1* vs *ssh1*). In contrast to ribosomes, proteasome 19S RP bound equally well to wild-type and *sec61*R406E proteoliposomes (Fig. 1E). We conclude that proteasome binding to the cytosolic face of the protein translocation channel differs from ribosome binding, and that R406 in cytosolic loop 8, which is essential for ribosome binding, is not required for proteasome binding to the Sec61 channel.

Sec63p and Kar2p co-precipitate with ER-associated proteasomes

Proteasomes can bind to proteoliposomes containing only the heterotrimeric Sec61 channel (Sec61p, Sbh1p, Sss1p), and we were previously unable to detect coprecipitation of a subunit of the heptameric Sec complex, Sec72p, with ER-associated proteasomes containing a protein-A-tagged lid subunit (Rpn11-Protein A) (Kalies et al., 2005). Here we asked whether other ER proteins were associated with the proteasome-Sec61 channel complex in the ER. Proteasome binding to the ER membrane is ATP dependent, which is why our buffers for solubilization and precipitation of proteasome-associated proteins contained 5 mM ATP (Kalies et al., 2005). During purification from yeast cell lysate, proteasomes hydrolyze ATP rapidly, which leads to dissociation of 26S proteasomes into 19S and 20S particles, and 19S particles into base and lid (Babbitt et al., 2005). Replacement of ATP with the non-hydrolyzable analogue ATP-γ-S, however, had no effect on the amount of Sec61p co-precipitating with Rpn11-Protein-A-tagged proteasomes, even if we added ATP-γ-S during the disruption of spheroplasts and during the isolation of microsomes (not shown). Since the base of the 19S particle binds to the ER, and some dissociation of lid and base may occur during the precipitation we decided to repeat the co-precipitation experiments using membranes from a strain in which the 19S base subunit Rpt1p was tagged with protein A (Rpt1p-Protein A, Fig. 2A). Membranes were solubilized in 3% DeoxyBigCHAP, and proteins precipitated with IgG-Sepharose beads washed with buffer. Proteasomes

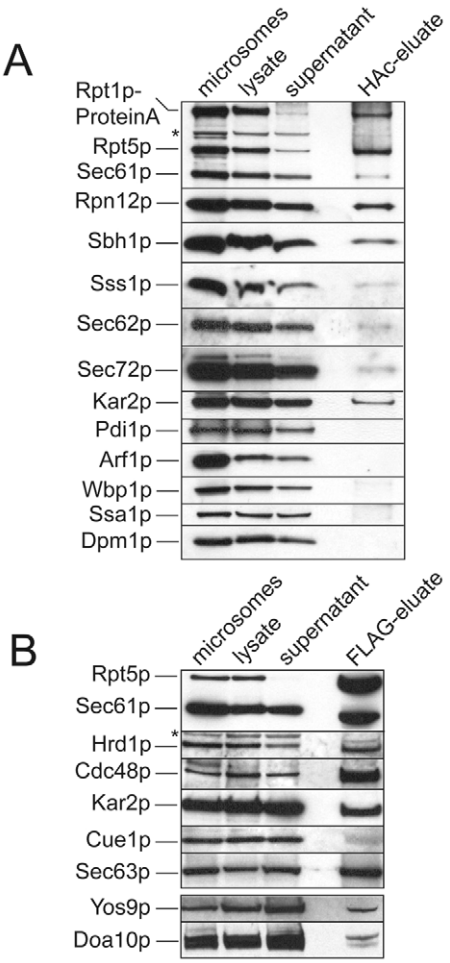

Fig. 2. Sec63p and Kar2p co-precipitate with ER-associated proteasomes. (A) Yeast microsomes were isolated from KRY665 in which the Rpt1p subunit of the 19S RP base was tagged with protein A. Membranes were solubilized in DeoxyBigCHAP, and protein-A-tagged Rpt1p and associated proteins isolated by batch absorption to IgG-Sepharose. Protein A-Rpt1p and associated proteins were eluted from washed beads with 0.5 M acetic acid, pH 3.4 (HAc-eluate). Equivalent amounts of microsomes, lysate, material not bound to IgG-Sepharose (supernatant) and 50× HAc-eluate were separated by SDS-PAGE and individual proteins detected by immunoblotting with polyclonal antisera. The asterisk marks a band non-specifically labelled by the Rpt5 antibody. (B) Yeast microsomes were isolated from RJD1171 in which Rpt1p is FLAG tagged. Microsomes were solubilized as above and Rpt1p and associated proteins isolated by adsorption to anti-FLAG agarose. Proteins were eluted with 200 μg/ml FLAG peptide for 1 hour at 4°C. Equivalent amounts of microsomes, lysate, material not bound to anti-FLAG-agarose (supernatant) and 100× of the FLAG eluate were analyzed by SDS-PAGE and immunoblotting as above. The asterisk marks a band non-specifically labelled by the Hrd1 antibody. Note that data in the top and bottom panels are from two separate experiments.

and associated proteins were eluted with acetic acid and samples analyzed by immunoblotting (Fig. 2A). Rpt1p-Protein A is quantitatively incorporated into proteasomes, and two 19S RP subunits, Rpt5p and Rpn12p, were detected in the eluate (Fig. 2A) (Kalies et al., 2005). A fraction of the Sec61 channel (Sec61p, Sbh1p, Sss1p) was associated with

proteasomes (Fig. 2A). We consistently co-precipitated a higher proportion of Sbh1p than of Sss1p, suggesting that Sbh1p itself, which in contrast to Sss1p has a cytoplasmic domain protruding into the cytosol that also binds to ribosomes, may interact with proteasomes directly (Fig. 2A) (Levy et al., 2001). Both Kar2p (BiP in mammals) and Pdi1p (PDI in mammals) are required for ER-associated degradation (Kabani et al., 2003; Gillece et al., 1999). We detected a strong association of the ER lumenal Hsc70 Kar2p with cytosolic ER-bound proteasomes, but not of the equally abundant ER lumenal Pdi1p nor the cytosolic Hsp70 Ssa1p, nor an abundant cytosolic ER-membrane-associated protein, Arf1p (Fig. 2A). Kar2p does not have a transmembrane domain but binds to the lumenal domain of the Sec63p subunit of the Sec63 complex (Sec63p, Sec62p, Sec71p, Sec72p). We detected weak signals for Sec72p and Sec62p in the proteasome-associated complex, but not for a subunit of the translocon-associated oligosaccharyl transferase, Wbp1p, or a non-translocon associated-ER membrane protein, dolichol phosphate mannosyl transferase (Dpm1p, Fig. 2A). By contrast, Sec63p itself was strongly associated with proteasomes (not shown, but see Fig. 2B).

The relatively large protein A tag on Rpt1p may reduce the affinity of the 19S RP base for Sec61 channels in the ER membrane, which is why we repeated our co-precipitation experiments with membranes from a strain in which Rpt1p was FLAG tagged (Verma et al., 2000). The FLAG tag at Rpt1p does not interfere with proteasome function and also allows a more specific elution of co-precipitated proteins from the matrix with FLAG-peptide (Verma et al., 2000). Experiments were scaled up twofold and a higher proportion of the FLAG eluate loaded onto the gel in order to improve detection of co-precipitating proteins. Sec61p and the proteasome 19S RP subunit Rpt5p co-precipitated with FLAG-Rpt1p proteasomes from solubilized microsomes (Fig. 2B, top). Two proteins involved in ER-associated degradation, the E3 ubiquitin ligase Hrd1p and the AAA-ATPase Cdc48p, were also present in the complex (Fig. 2B). By contrast, the E3 ubiquitin ligase required for turnover of transmembrane proteins, Doa10p, was only weakly associated with the complex, and Cue1p, which serves to anchor the ubiquitin-conjugating enzyme Ubc7p at the ER membrane, was barely detectable (Fig. 2B) (Biederer et al., 1997). We were unable to detect Der1p or the mannose-binding lectin Htm1p in our complexes (not shown) (Jakob et al., 2001). A second lectin involved in targeting ERAD substrates for degradation, Yos 9p, however, was clearly present in the complex (Fig. 2B). As for Rpt1p-Protein A proteasomes (Fig. 2A), Sec63p and Kar2p were prominently present in co-precipitates with FLAG-Rpt1p proteasomes from solubilized ER membranes (Fig. 2B). Our data suggest that Sec63p and Kar2p may constitute part of the protein export complex in the ER membrane.

Sec63p does not contribute to proteasome binding to the ER

We have shown previously that the Sec61 complex is sufficient for proteasome binding to proteoliposomes, but we did not investigate if binding was enhanced if the entire Sec complex was present (Kalies et al., 2005). We therefore next asked whether the Sec63 complex or Sec63p on its own contribute to proteasome binding to the ER. A mutation in *SEC63*, *sec63-1*, which is strongly defective in protein import into the ER, has only modest effects on protein export for degradation (Stirling et al., 1992; Pilon et al., 1997; Plemper et al., 1997). Mutations in *sec62*, and the absence of Sec71p and Sec72p have no effect on export at all (Pilon et al., 1997; Plemper et al., 1997). We therefore first investigated whether a mutation in *SEC63*, T352A, which reduces phosphorylation of Sec63p and abolishes interaction of Sec63p with Sec62p, had any influence on proteasome binding to the ER (Wang and Johnsson, 2005). We prepared microsomes and proteoliposomes containing total ER protein from the *sec63* mutant strain and performed binding experiments with 19S RP as described for Fig. 1. As shown in Fig. 3A, we detected only a slight reduction in proteasome interaction with *sec63*T652A membranes. Deletion of the C-terminal 27 amino acids of Sec63p including the phosphorylation site had a similar effect (not shown).

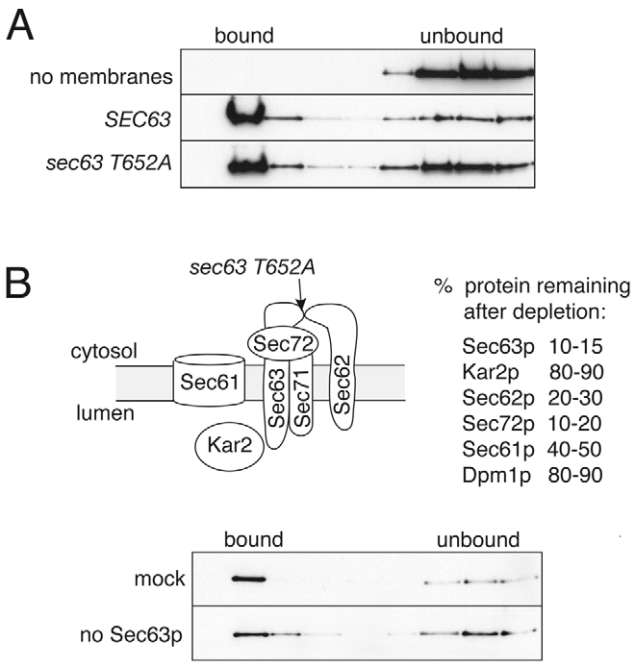

Fig. 3. Sec63p does not contribute to proteasome binding to the ER. (A) Yeast 19S RP (2 pmol) were incubated in the absence or presence of 20 eq of SEC63 wild-type or *sec63* T652A proteoliposomes as indicated, and binding assessed by flotation in sucrose gradients and SDS-PAGE followed by immunoblotting for the FLAG-tagged Rpn11p subunit as described for Fig. 1. The positions of proteasomes bound to membranes and of unbound proteasomes are indicated. (B) The upper panel shows a schematic drawing of the Sec61 channel and the Sec63 complex; the position of the T652A mutation in the cytosolic domain of Sec63p that disrupts the interaction with Sec62p, is indicated. Membranes from a *SEC63* wild-type strain (KRY333) were solubilized in DeoxyBigCHAP and Sec63p depleted by immunoprecipitation, or lysates mock-incubated without antibodies. Depleted and mock-depleted lysates were reconstituted into proteoliposomes, and the degree of depletion of Sec63 and associated proteins assessed by quantitative immunoblotting. (Lower panel) Yeast 19S RP (2 pmol) were incubated with 15 eq of mock-depleted or Sec63p-depleted proteoliposomes as indicated, and binding assessed by flotation in sucrose gradients and SDS-PAGE followed by immunoblotting for the FLAG-tagged Rpt1p subunit.

Therefore, the intact Sec63 complex does not contribute significantly to proteasome binding to the ER.

In addition, we depleted Sec63p from solubilized wild-type microsomes using a polyclonal Sec63p antibody. After overnight incubation with the antibody, or mock incubation, the depleted and mock-depleted lysates were reconstituted into proteoliposomes. The relative amounts of subunits of the Sec complex, Kar2p, and a non-translocon-associated transmembrane protein, Dpm1p, were determined by quantitative immunoblotting and are shown in Fig. 3B. Although close to 90% of Sec63p was depleted, proteasomes were still able to bind to the Sec63p-depleted membranes (Fig. 3C). There was a reproducible reduction in proteasome binding compared to mock-depleted membranes, but the reduction was more proportional to the partial depletion of Sec61p (40-50%) from the membranes than to the almost complete depletion of Sec63p. We conclude that neither the Sec63 complex nor Sec63p itself contribute significantly to proteasome binding to the ER.

The 19S RP base in the ATP-bound conformation mediates proteasome binding to the ER

Proteasomes bind to the ER membrane via the 19S RP, whereas the 20S core particle has no affinity for the ER (Fig. 4A,B) (Kalies et al., 2005). The 19S RP consists of at least 17 proteins, including ubiquitin-binding proteins, deubiquitylation enzymes, AAA-ATPases, two proteins with leucine-rich repeats involved in binding proteasome accessory proteins, and a number of proteins whose function remains unknown (Sharon et al., 2006). Here, we investigated which 19S RP subunits are responsible for its interaction with the ER membrane. The 19S RP can be dissociated into two subcomplexes, the base and the lid, using high salt (Fig. 4A,B). Using yeast and dog pancreas PK-RM we had shown previously that it is primarily the base that binds to the ER, whereas the lid has substantially lower affinity for the ER (Fig. 4C) (Kalies et al., 2005). Most of our lid preparations were slightly contaminated with intact 19S particles, so the residual binding observed may be due to this contamination (note the presence of faint Rpn1 and Rpn2 bands in the Lid lane, Fig. 4A) (see also Kalies et al., 2005). Several of the lid Rpn proteins also have affinity for nuclear membranes of which a small proportion is present in dog pancreas microsomes (not shown). In order to rule out a genuine contribution of the lid to 19S binding to the ER, we prepared GST-fusion proteins for all lid subunits, and investigated their binding to the ER (Elsasser et al., 2002). Although GST-Rpn5p, GST-Rpn6p and GST-Rpn8p bound to dog pancreas PK-RM, this interaction could not be competed with intact 19 RP (not shown). By contrast, association of the 19S RP base with PK-RM could be competed with excess intact 19S RP (Fig. 4D). We conclude that solely the 19S base is responsible for proteasome binding to the ER.

The 19S RP base consists of nine subunits: six AAA-ATPases, two proteins with long leucine-rich repeats, Rpn1p and Rpn2p, which have been shown to be interaction partners of proteasome accessory proteins, and Rpn10p, which forms the hinge between lid and base (Elsasser et al., 2002). Individual GST-Rpn1p and GST-Rpn2p fusion proteins can also interact with these same binding partners (Elsasser et al., 2002). We purified GST-fusion proteins with Rpn1p, Rpn2p and Rpn10p, and found that GST-Rpn2p had affinity for dog-pancreas PK-RM, but this interaction could not be competed with excess 19S RP, and does therefore not contribute to binding of the intact 19S RP to the ER (not shown).

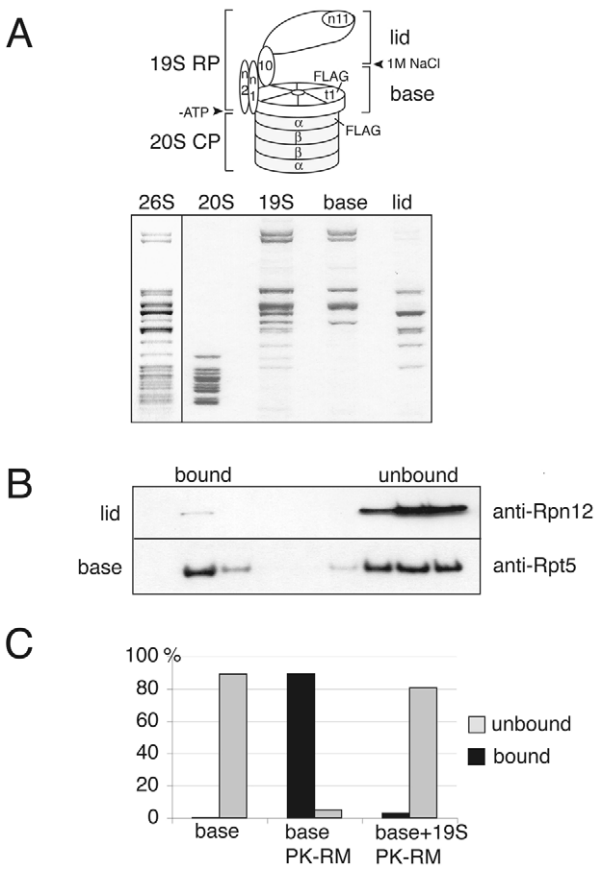

Fig. 4. 19S RP and base compete with each other for binding to the ER. (A) Upper panel: schematic representation of 26S proteasome subparticles. Positions of FLAG tags for purification are indicated; the Pre1p-FLAG strain was used for purification of 20S particles, all other particles were purified from the Rpt1p-FLAG strain RJD1171. Lower panel: Coomassie Blue-stained gel of purified 26S proteasomes and subparticles. Note the slight contamination of the lid fraction with intact 19S RP. (B) Dog PK-RM (10 eq) were incubated with 5 pmol lid or 1 pmol base and analyzed by flotation in a sucrose gradient, followed by SDS-PAGE and immunoblotting for Rpn12 (lid) or Rpt5p (base). (C) Dog PK-RM (10 eq) were incubated with 2.5 pmol base in the absence or presence of 10× excess 19S RP, and analyzed as in B. Base binding was detected using the anti-FLAG antibody, 19S RP binding with anti-Rpn12 antibody, and quantified using chemiluminescence and a CCD camera system (Raytest, Germany).

The six AAA-ATPases in the base, Rpt1-6p, are homologous, but functionally non-equivalent, and form a hetero-hexameric ring (Rubin et al., 1998). In order to investigate the role of individual Rpt proteins in binding to the ER we purified 19S particles in which the ATP-binding sites of one or two individual ATPases had been mutated. To this end we introduced a FLAG tag into the Rpn11p lid subunit of strains with equivalent mutations in the ATP-binding sites of Rpt1p, Rpt5p and Rpt3p/Rpt6p, which had been characterized

previously (Fig. 5) (Rubin et al., 1998). We found that all *rpt* mutants investigated had reduced affinity for ER membranes (Fig. 5 and not shown). We therefore conclude that the ATP-bound conformation of the hetero-hexameric AAA-ATPase ring of the proteasome 19S RP base is required for proteasome binding to the ER.

Discussion

Proteasomes are responsible for protein turnover in ER-associated degradation (Romisch, 2005). The proteasome 19S RP is sufficient to promote export of a soluble degradation substrate from the ER and can bind directly to the Sec61 channel (Kalies et al., 2005; Lee et al., 2004). Here we have characterized the interaction between the 19S RP of the proteasome and the Sec61 channel in the ER membrane. We have shown that ribosomes and the 19S RP bind to different sites in the Sec61 channel, and have identified mutations in *SEC61* that reduce proteasome binding to ER membranes (Fig. 1). We found that Sec63p and Kar2p are associated with the proteasome-bound Sec61 channel and are therefore likely to be part of the protein export complex in the ER membrane (Fig. 2), but Sec63p was not required for the proteasome-channel interaction (Fig. 3). Finally, we have shown that the heterohexameric AAA-ATPase complex of the 19S RP base binds to the Sec61 channel in the ATP-bound conformation (Fig. 5).

We have demonstrated previously that a protease-sensitive cytoplasmic domain of the protein translocation channel is required for proteasome binding to the ER (Kalies et al., 2005). Judging from a crystal structure of the closed conformation of a related channel from *Methanococcus jannaschii* and limited protease digests of the mammalian Sec61 channel, the only large exposed domains of Sec61p on the cytoplasmic face of the ER are loop 6, loop 8, the C terminus and possibly the N terminus (Raden et al., 2000; van den Berg et al., 2004). The *sec61*R406E mutation is located on the tip of an exposed loop in cytosolic loop 8 of Sec61p and reduces ribosome binding to the Sec61 channel, but did not affect proteasome binding to the membranes (Fig. 1D,E). Interestingly, this mutation and two other *sec61* mutants in loops L6 and L8, which have a negative effect on protein import into the ER, led to an enhanced turnover of the ER-degradation substrate CPY*, which suggests that transport through the Sec61 channel is limiting, and if import into the ER is reduced, more CPY* can be exported and degraded (Cheng et al., 2005). Our *sec61-303* mutant, which contains a mutation at the beginning of L6, was competent for proteasome binding (Fig. 1B,C). The data presented here suggest that neither L6 nor L8 of Sec61p are important docking sites for the proteasome 19S RP, or if they are involved, interaction of the 19S RP with these loops is fundamentally different from that of the ribosome.

Proteoliposomes from *sec61-32* yeast which are cold-sensitive for protein import into the ER, and strongly defective in protein export to the cytosol for degradation, are competent for proteasome binding, but have a reduced affinity for the 19S RP (Fig. 1C) (Pilon et al., 1998; Pilon et al., 1997). The mutation in *sec61-32*, C150Y, is located close to the lumenal end of transmembrane domain 4 (Fig. 1B), and is therefore likely to affect proteasome binding to the cytoplasmic domains of the channel indirectly, perhaps by changing the orientation of helix 4 in the membrane. Mutations in lumenal loops of

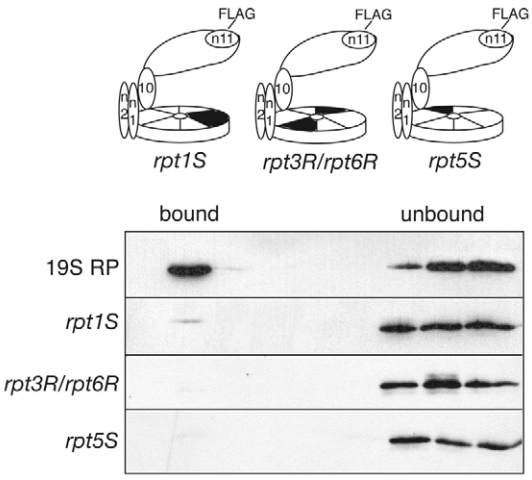

Fig. 5. The 19S RP base in the ATP-bound conformation mediates proteasome binding to the ER. Wild-type 19S RP (1 pmol) or 19S RP with the indicated mutations was incubated with 10 eq dog PK-RM and analyzed by sucrose gradient centrifugation, SDS-PAGE and immunoblotting for the FLAG-tagged Rpn11p subunit. Positions of membrane-bound and unbound 19S RP are indicated.

Sec61p had no effect on proteasome interaction with the channel (*sec61-301*, Fig. 1B,C), but a *sec61* mutant with four point mutations strongly reduced 19S RP binding to proteoliposomes (*sec61-302*, Fig. 1B,C). Since membranes from *sec61-303*, which contains two of the same point mutations, were competent for 19S RP binding, and the third point mutation in *sec61-302* is located in a flexible ER lumenal loop, the reduced binding in *sec61-302* is probably due to the S179P substitution in the short cytoplasmic loop L4 at the beginning of transmembrane helix 5 (Fig. 1B,C). Since this loop is protease-insensitive, it is unlikely to constitute the entire cytoplasmic proteasome binding site of Sec61p (Kalies et al., 2005; Raden et al., 2000). L4 has been proposed to act as hinge region, allowing a rearrangement of transmembrane helices 4 and 5 during channel opening (van den Berg et al., 2004). A conformational change in Sec61p may also be required for exposure of a proteasome binding site at the cytoplasmic face of the Sec61 channel, as postulated previously (Kalies et al., 2005). Movement of transmembrane domain 4 of Sec61p would affect the positions of the Sec61p N terminus and of the cytosolic domain of Sbh1p, which is also protease-sensitive and co-precipitates strongly with ER-bound 19S RP (Fig. 2A) (van den Berg et al., 2004). Movement of transmembrane domain 5 in Sec61p would reposition its C terminus (van den Berg et al., 2004). Yeast proteasomes bind well to mammalian ER, but of the three cytosolic termini which may serve as binding sites for the proteasome only the Sec61p N terminus is well conserved between yeast and mammals (Kalies et al., 2005; van den Berg et al., 2004). After a conformational change in Sec61p that involves movement around the hinge between transmembrane domains 4 and 5, the N terminus of Sec61p may become more exposed and serve as a binding site for the proteasome 19S RP.

Using yeast strains in which 19S RP subunits were tagged with protein A or the FLAG epitope we have shown that

Table 1. Yeast strains

Strain	Relevant genotype	Source/reference
RJD1144	*MATa his3Δ200 leu2-3,112 lys2-801 trp1Δ63 ura3-52 PRE1^{FH}::Ylplac211(URA3)*	Verma et al., 2000
RJD1171	*MATa his3Δ200 leu2-3,112 lys2-801 trp1Δ63 ura3-52 RPT1^{FH}::Ylplac211(URA3)*	Verma et al., 2000
KRY665	*MATa his3Δ200 leu2-3,112 lys2-801 trp1-1 ura3-52 rpt1::HIS3 pep4::URA3* [*pCEN-TRP1-RPT1TEVProA*]	This study
JDY683	BMA38a, *MATa his3-Δ200 leu2-3,112 ura3-1 trp1-Δ1 ade2-1 can1-100* [*kanr-pGAL-SEC61*]	This study
KRY706	JDY683, except [pCEN-*LEU2-SEC61*]	This study
KRY709	as JDY683, except [pCEN-*LEU2-sec61-301*]	This study
KRY712	as JDY683, except [pCEN-*LEU2-sec61-302*]	This study
KRY715	as JDY683, except [pCEN-*LEU2-sec61-303*]	This study
BWY12	*trp-1 ade2 leu2-3, 112 ura3 his3-11 can1 sec61::HIS3* [pCEN-*SEC61-URA3*] (=KRY674)	Cheng et al., 2005
RGY400	*trp-1 ade2 leu2-3, 112 ura3 his3-11 can1 sec61::HIS3* [p*SEC61-LEU2*] *ssh1::KanMX4* (=KRY675)	Cheng et al., 2005
RGY400R406E	*trp-1 ade2 leu2-3, 112 ura3 his3-11 can1 sec61::HIS3* [p*sec61*R406E-*LEU2*] *ssh1::KanMX4* (=KRY676)	Cheng et al., 2005
JD53	MATα *his3-Δ200 lys2-801 leu2-3,112 trp1-Δ63 ura3-52*	Wang and Johnson, 2005
NJY148	MATα *his3-Δ200 lys2-801 leu2-3,112 trp1-Δ63 ura3-52 sec63*T652A*::pRS304*	Wang and Johnson, 2005
KRY694	*MATa his3-Δ200 lys2-801 leu2-3,112 trp1-1 ura3-52 RPN11^{FLAG}::pRS306-URA3*	This study
KRY695	as KRY694, except *rpt1::HIS3* [*pCEN-LEU2-rpt1S*]	This study
KRY697	as KRY694, except *rpt3:: HIS3 rpt6* [*pCEN-LEU2-rpt3R*] [*pCEN-TRP1-rpt6R*]	This study
KRY698	as KRY694, except *rpt5:: HIS3* [*pCEN-LEU2-rpt5S*]	This study

Sec61p, Sbh1p and Sss1p co-precipitate with ER-associated proteasomes (Kalies et al., 2005) (see also Fig. 2A). Only small amounts of two subunits of the Sec63 complex, Sec62p and Sec72p, were bound to proteasomes, but we detected consistently strong signals for Sec63p itself and Kar2p (Fig. 2) (see also Kalies et al., 2005). The heterotetrameric Sec63 complex is responsible for post-translational protein import into the ER, and mutations in *SEC62* and *SEC71*, which are defective in post-translational import, have no effect on export from the ER (Pilon et al., 1997; Plemper et al., 1997). Sec63p and Kar2p can function independently of the Sec63 complex, both in nuclear membrane fusion and in cotranslational protein import into the ER (Young et al., 2001; Ng and Walter, 1996). The *sec63-1* mutation, which disrupts the interaction between Kar2p and Sec63p, reduces ERAD of two substrates, CPY* and mutant alpha-factor precursor (Pilon et al., 1997; Plemper et al., 1997). Kar2p is required for keeping ERAD substrates export competent, and mutations in the peptide binding domain of Kar2p specifically affect export from the ER (Kabani et al., 2003). Together with our observation that Sec63p and Kar2p co-precipitate with ER-associated proteasomes, these data suggest that Sec63p and Kar2p are part of the protein export complex in the ER membrane.

Proteoliposomes from which up to 90% of Sec63p had been depleted, were competent for proteasome binding (Fig. 3B). During depletion, about 50% of Sec61p was also removed from the membranes because of its association with the Sec63 complex. The reduction in proteasome binding to the depleted membranes was approximately proportional to the amount of Sec61p remaining (Fig. 3B, compare unbound to bound for mock-depleted and Sec63p-depleted membranes). Our data suggest that although Sec63p is part of the export complex, the cytosolic domain of Sec63p does not contribute to proteasome binding to the ER.

Three other proteins involved in ERAD, the ubiquitin-ligase Hrd1p, the mannose-lectin and chaperone Yos9p, and the AAA-ATPase Cdc48p also co-precipitated with ER-associated proteasomes (Fig. 2B). Kar2p has been shown to be anchored to the Hrd1 complex via Yos9p, so Kar2p interaction with proteasome-associated export channels may be mediated by either Sec63p, or Yos9p, or both. Cdc48p can associate directly with proteasomes and also co-precipitates with ERAD substrates, and with the Doa10p and Hrd1p complexes in the ER membrane (Carvalho et al., 2006; Lilley and Ploegh, 2004; Verma et al., 2000; Denic et al., 2006). It remains unclear which of these interactions is responsible for the presence of Cdc48p in the proteasome-associated fraction of ER proteins in our experiments, but the fact that Hrd1p and Kar2p also co-precipitate with 19 RP from solubilized ER membranes suggest that 19S RP is associated with the Hrd1p complex. This is consistent with the observations of Carvalho et al. (Carvalho et al., 2006) who detected 19S RP subunits co-migrating with the solubilized Hrd1p complex in sucrose gradients. The relatively weaker association of proteasome 19S particles with Doa10p that we observed (Fig. 2B) may reflect the lower concentration of the Doa10p complex in the ER membrane since, in contrast to the Hrd1 complex, the Doa10p complex can be transported to and is present in the inner nuclear membrane (Deng and Hochstrasser, 2006). Alternatively, the Doa10p complex may not interact strongly with the Sec61 channel or the 19S RP under our experimental conditions. We raised a polyclonal antibody against the C terminus of Der1p, but were unable to detect Der1p in the complex associated with 19S RP, so either the protein is not present in the complex under our solubilization conditions or the untagged protein cannot be detected due to its low endogenous expression levels (not shown). Cue1p was barely detectable in our complexes, and Der1p, Cue1p and Ubc7p were also absent from the Hrd1p complexes isolated by Denic et al., whereas all three proteins were present in the Hrd1p complex purified by Carvalho (Fig. 2B) (Carvalho et al., 2006; Denic et al., 2006). The Sec61 channel, however, was absent from Hrd1p complexes purified by Gauss, Denic and Carvalho (Denic et al., 2006; Gauss et al., 2006; Carvalho et al., 2006). Gauss et al. performed their co-precipitation experiments in the presence of NP40, in which the Sec61 complex is unstable, whereas Denic and colleagues solubilized their membranes in Triton X-100, in which the Sec61 complex is not associated with the 19S RP in our hands (Denic et al., 2006; Gauss et al., 2006) (data not shown). None of the other investigators included ATP in their co-immunoprecipitations which is essential for the association 19S RP complex with the Sec61 channel and may also be important for the interaction of the channel with the Hrd1p complex (Kalies et al., 2005). Taken

together these data suggest that the ER export complex is an unstable and probably dynamic assembly of a number of subcomplexes which, depending on the membrane source, experimental and solubilization conditions, can be co-precipitated or not.

We show that the ATP-bound conformation of the hetero-hexameric AAA-ATPase ring of the 19S RP base is required for the interaction with the Sec61 channel (Fig. 5B) (Rubin et al., 1998). This is reminiscent of the ATP-dependent interaction of the 19S RP with the 20S core particle (CP) of the proteasome (Voges et al., 1999). The points of contact between 19S RP and the 20 CP are not known, but a crystal structure of an archaeal proteasome with the bound 11S activator complex suggests that the three C-terminal residues of the activator AAA-ATPase subunits dock in conserved pockets in the proteasome CP surface (Forster et al., 2005). This interaction is primarily stabilized by main chain interactions, the only side chain involved is that of Lys66 of the alpha subunits of the proteasome CP (Forster et al., 2005). There is a mismatch between the hexameric AAA-ATPase ring of the 11S activator complex and the heptameric alpha subunit ring, but only six of the seven alpha subunits contain the critical lysine, and as few as four pockets can be occupied by 11S C termini (Forster et al., 2005). The interaction of the 19S RP with the 20S CP is proposed to be similarly mediated by the Rpt protein C termini docking into the lysine-containing pockets in the 20S CP (Forster et al., 2005). The surface of the protein translocation complex in the ER membrane is formed by the assembly of several (three or four) heterotrimeric Sec complexes in the ER membrane, and is structurally very different from that of the 20S CP (van den Berg et al., 2004; Forster et al., 2005; Hanein et al., 1996). Docking to the Sec61 channel mediated by only a few C-terminal amino acids of the Rpt proteins into lysine-containing pockets in the Sec61 complexes would explain how the 19S RP can interact with such dissimilar protein complexes, but it is hard to reconcile with the binding of intact 26S proteasomes to the ER membrane that we observed previously (Kalies et al., 2005). An alternative possibility is that the coiled-coil domains in the N termini of the Rpt proteins (except Rpt2p) interact with the Sec61 channel, similar to the ATP-dependent interaction of the N-terminal coiled-coil domains of the AAA-ATPase complex formed by Vps4p with the ESCRT-III complex at endosomal membranes (Scott et al., 2005). The orientation of binding of the 19S RP to the Sec61 channel, and the contribution of individual cytosolic domains of the channel to this interaction remain to be determined.

Materials and Methods

Yeast strains

Yeast strains used in this paper are listed in Table 1.

Generation of *SEC61* mutants

A PCR product was amplified from pBW11 (LEU, CEN SEC61) (Wilkinson et al., 1996) comprising the whole *SEC61* open reading frame plus flanking sequences (244 5′ and 215 3′ nucleotides), using oligos 61c (gtt acg gtg gta acg tag) and 61d (aga tcg cgt atg ata ttg), in a series of PCR reactions in which each nucleotide in turn was provided at increased (1.0 mM) concentration, the remaining three being at 0.2 mM. Products were pooled and transformed into yeast strain JDY638 containing plasmid pJEY203 [pRS313 containing a *PHO5* promoter-driven fusion of sequences encoding the first 70 amino acids – including the signal-anchor – of *PHO8* to the whole *URA3* ORF derived from pMPY1 (kind gift from Martin Pool, University of Manchester, UK)], along with *Bgl*II-linearized plasmid pJNY258 (pBW11 modified to contain a *Bgl*II restriction site in place of the *SEC61* ORF). In vivo gap repair generated a library of *sec61* mutants. These were screened for growth on –Leu–His dextrose medium to test for function of the mutant Sec61 protein, and –Leu–His-URA dextrose medium to identify those transformants in which translocation of PHO8-URA3 into the ER was inefficient. *sec61* plasmids were recovered from ura+ colonies into *E. coli* and re-transformed into fresh JDY638 cells alongside either pJNY203 or pDN106 (containing CPY-URA3) (Ng et al., 1996), and re-tested on the same set of media to identify those *sec61* alleles that conferred SRP-dependent (i.e. PHO8-URA3-specific) translocation defects. Western blotting confirmed that the level of Sec61p expressed in the mutants (on glucose, when transcription of the genomic *pGAL-SEC61* was repressed) similar to that in wild-type cells. The mutants were sequenced and the following mutations identified: *sec61-301*: plasmid 12: C-T at 199 and A-G at 680 yielding Arg-Cys at 67, Asp-Gly at 227; *sec61-302*: plasmid 14: A-G at 503, t-C at 535, T-C at 787, C-G at 1058 yielding D-G at 168, S-P at 179, F-L at 263, S-C at 353; *sec61-303*: plasmid 18: A-G at 503, T-C at 787 yielding D-G at 168, F-L at 263.

Antibodies

Polyclonal rabbit antibodies against Sec61p, Rpn12p, Sbh1p, Kar2p and Pdi1p have been described previously (Kalies et al., 2005). Antiserum against overexpressed Cdc48p purified from *E. coli* was raised using an expressing construct from M. Ghislain (Decottignies et al., 2004). Antiserum against Der1p was raised against the C-terminal 14 amino acids, and affinity-purified. Rpt5 antibody was from D. Finley (Harvard, MA) or from Affiniti (Exeter, UK), Sss1p, Sec62p, Sec63p, Sec72p antibodies were from R. Schekman (UC Berkeley, CA), Sec63p antibody used in Fig. 2 was from Colin Stirling (University of Manchester, UK), antibodies against Arf1p from R. Duden (Royal Holloway, UK), Wbp1p from M. Aebi (ETH, Zurich, Switzerland), Ssa1p from J. Brodsky (University of Pittsburgh, PA), Cue1p from T. Sommer (MDC Berlin, Germany), Hrd1p from R. Hampton (UC San Diego, CA), Anti-Yos9p, Anti-Htm1p and anti-Doa10p from D. Ng (TLL, Singapore). Anti-Dpm1p was from Molecular Probes, anti-S6 from Cell Signalling/NEB.

Yeast and dog pancreas microsomes, and proteoliposome preparation

Yeast microsomes were prepared as described by Pilon et al. (Pilon et al., 1997). Dog pancreas microsomes and microsomes stripped of ribosomes by puromycin/high salt treatment (PK-RM) were prepared as described by Gorlich et al. (Gorlich et al., 1992). Proteoliposomes were prepared as described previously (Kalies et al., 1994) from either mock-depleted ER-lysates or lysates that had been incubated overnight with a polyclonal antiserum against Sec63p from Randy Schekman (20 μl serum per 75 eq lysate; after depletion this resulted in about 40 eq reconstituted membranes quantified by amount of Dpm1p).

Purification of proteasomes and proteasome subparticles

26S proteasomes, 19S particles, base and lid were purified from RJD1171 as described previously (Kalies et al., 2005). For investigation of the effect of *rpt* mutants, 19S particles were purified from KRY694-698.

Ribosome purification

Ribosomes stripped from dog pancreas microsomes by puromycin high salt treatment (above) were collected by sedimentation through a 1.6 M sucrose cushion by centrifugation at 100,000 ***g*** for 2 hours. Ribosomes were washed three times in buffer A (50 mM Hepes-KOH, pH 7.6, 250 mM sucrose, 150 mM potassium acetate, 5 mM magnesium acetate, 1.5 mM dithiotreitol, 1× protease inhibitors (Roche), 1.5 mM puromycin, 0.1 mM GTP); the final pellet was resuspended in B88 (50 mM Hepes-KOH, pH 6.8, 150 mM potassium acetate, 5 mM magnesium acetate, 250 mM sorbitol), and aliquots snap frozen and stored at –80°C.

Native co-precipitations

Co-precipitations from lysed KRY665 microsomes (ProteinA-RPN11; 500 μg protein) or lysed RJD1171 (FLAG-RPT1; 1 mg protein) were done as in (Kalies et al., 2005), but with 200 mM potassium acetate in the solubilization buffer [50 mM Hepes-KOH, pH 7.4, 200 mM potassium acetate, 5 mM magnesium acetate, 10% glycerol, 5 mM ATP, 3% DeoxyBigCHAP (Calbiochem) plus protease inhibitors], and precipitation buffer (same as for solubilization, but 0.7% DeoxyBigCHAP); precipitated proteins were eluted from IgG-Sepharose with 0.5 M acetic acid, pH 3.4, or from anti-FLAG M2 agarose (Sigma) with 200 μg/ml FLAG peptide in precipitation buffer without glycerol for 1 hour at 4°C. Eluates from IgG-Sepharose were dried under vacuum, from anti-FLAG agarose concentrated in Centricon YM-10. Samples were heated in 2× SDS sample buffer and analyzed by SDS-PAGE and immunoblotting.

Proteasome and ribosome binding assays and analysis

Proteasome and subparticle binding experiments were done as described previously (Kalies et al., 2005). Briefly, ribosome-stripped membranes (PK-RM) or proteoliposomes were mixed with the indicated concentrations of 26S proteasomes, 19S particles or base or lid in the presence of 5 mM ATP, incubated for 20 minutes on ice followed by 10 minutes at room temperature. Samples were analyzed by

flotation in 1.8 M sucrose for 1 hour at 4°C at 200,000 ***g*** in a TLS-55 rotor. Fractions were collected from the top and analyzed by SDS-PAGE and immunoblotting. Ribosome binding was done as above with 1.25 pmoles of ribosomes per 20 eq of PK-RM.

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