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Uncoupling retro-translocation and degradation in the ER-associated degradation of a soluble protein

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Aberrant polypeptides in the endoplasmic reticulum (ER) are retro-translocated to the cytoplasm and degraded by the 26S proteasome via ER-associated degradation (ERAD). To begin to resolve the requirements for the retro-translocation and degradation steps during ERAD, a cell-free assay was used to investigate the contributions of specific factors in the yeast cytosol and in ER-derived microsomes during the ERAD of a model, soluble polypeptide. As ERAD was unaffected when cytoplasmic chaperone activity was compromised, we asked whether proteasomes on their own supported both export and degradation in this system. Proficient ERAD was observed if wild-type cytosol was substituted with either purified yeast or mammalian proteasomes. Moreover, addition of only the 19S cap of the proteasome catalyzed ATP-dependent export of the polypeptide substrate, which was degraded upon subsequent addition of the 20S particle.

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Introduction

Protein quality control in the eucaryotic secretory pathway ensures that only properly folded proteins transit through cellular organelles (Ellgaard and Helenius, 2001). Misfolded proteins may be degraded or may accumulate in the endoplasmic reticulum (ER) and induce the unfolded protein response (UPR). Whereas the UPR leads to an increased cellular capacity to handle misfolded proteins (Kaufman, 1999; Patil and Walter, 2001), the degradation of aberrant polypeptides in the ER, termed ER-associated degradation (ERAD; McCracken and Brodsky, 1996), rids the secretory pathway of misfolded proteins. Many UPR-induced genes encode factors that catalyze ERAD, and cells defective for ERAD or the UPR exhibit compromised growth in the absence of the complementary system (Casagrande *et al*, 2000; Friedlander *et al*, 2000; Ng *et al*, 2000; Travers *et al*, 2000).

Although it was anticipated that an ER lumenal protease would destroy aberrant secreted proteins, the first glimpses into the molecular mechanism of ERAD emerged when it was found that mis-folded membrane proteins and mis-folded soluble polypeptides in the ER were degraded by cytoplasmic proteasomes (Fewell *et al*, 2001; Tsai *et al*, 2002; Kostova and Wolf, 2003). The 26S proteasome is an $\sim$2 MDa complex composed of one or two 19S ‘caps’ (also known as the ‘regulatory particle’ or proteasome activator (PA) 700 in mammals) and a 20S core in which three distinct proteolytic activities reside (Voges *et al*, 1999). The 19S cap contains 18 unique subunits in yeast, retains aggregation-prone polypeptides in solution, and can re-model polypeptide conformation, suggesting chaperone-like activity (Glickman *et al*, 1998; Braun *et al*, 1999; Strickland *et al*, 2000; Verma *et al*, 2000; Liu *et al*, 2002). Six proteins in the 19S particle are AAA proteins that mediate protein unfolding and catalyze ATP-dependent degradation in other systems (Zwickl and Baumeister, 1999). Most proteasome substrates are ubiquitinated, and components in or associated with the 19S cap bind to ubiquitin-conjugated proteins (Hershko and Ciechanover, 1998; Dai and Li, 2001; Lam *et al*, 2002). After de-ubiquitination, the AAA proteins in the 19S cap might unfold and/or ‘drive’ polypeptides into the proteolytic chamber of the 20S component (Horwich *et al*, 1999; Lee *et al*, 2001). For a number of ERAD substrates, export and degradation are tightly coupled, which may explain why proteasomes are associated primarily with the ER/nuclear membrane (Enenkel *et al*, 1998).

ERAD should exhibit exquisite substrate specificity, as ERAD substrates must be selected from properly folded proteins and folding intermediates of wild-type proteins en route to their native conformations. Mediators of substrate selectivity include the Hsp70 molecular chaperones, which recognize mis-folded polypeptides. Specifically, the ER lumenal Hsp70 BiP and two Hsp40 co-chaperones facilitate ERAD in yeast (Plemper *et al*, 1997; Brodsky *et al*, 1999; Nishikawa *et al*, 2001). Calnexin, a lectin that retains improperly folded proteins in the ER by binding to mono-glucosylated oligosaccharyl side chains, also plays a role during ERAD, and it has been proposed that the trimming of mannose residues from the core oligosaccharide functions as a ‘timer’ to remove ERAD substrates from calnexin and target them to another putative lectin, known as EDEM, prior to retro-translocation (Ellgaard and Helenius, 2001; Sifers, 2003). Whether this mechanism operates for all glycosylated ERAD substrates, and how non-glycosylated ERAD substrates are targeted for degradation, is unclear.

Three models have been presented to describe how polypeptides may be driven from the ER. First, cytoplasmic molecular chaperones might interact with polypeptides emer-

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ging from the ER. This model derives from the fact that BiP and mitochondrial Hsp70 drive the post-translational translocation of polypeptides into the ER and the mitochondria, respectively (Fewell *et al*, 2001; Pfanner and Geissler, 2001). Second, as many ERAD substrates are polyubiquitinated, and polyubiquitination and export are coupled, ubiquitin conjugation might serve as a 'ratchet' (Biederer *et al*, 1997; de Virgilio *et al*, 1998; Shamu *et al*, 2001). Third, the 26S proteasome may drive retro-translocation (Mayer *et al*, 1998; Chillaron and Haas, 2000; Mancini *et al*, 2000; Walter *et al*, 2001). Regardless of the mechanism by which ERAD substrates are extracted, it has been difficult to determine whether specific chaperones, polyubiquitination, or the proteasome are sufficient to support retro-translocation, and more specifically whether polypeptide export and degradation can be uncoupled. As reported here, we find that the proteasome is sufficient to retro-translocate and degrade an ERAD substrate *in vitro* and that these activities can be uncoupled: that is, the 19S subunit drives ATP-dependent retro-translocation of the substrate, and addition of the 20S subunit catalyzes its subsequent degradation.

Results

Yeast cytoplasmic Hsp70 and Hsp90 chaperones are dispensable for pαF degradation

ERAD can be recapitulated *in vitro* using ER-derived microsomes and cytosol prepared from the yeast *Saccharomyces cerevisiae* (McCracken and Brodsky, 1996). This assay utilizes a mutated form of pre-pro α factor (ppαF), a precursor of the secreted α factor mating pheromone. After ppαF translocation into microsomes, signal sequence cleavage and core glycosylation of wild-type ppαF result in triply glycosylated pαF (3GpαF). In contrast, a precursor in which the core glycosylation sites have been mutated (ΔGppαF) is converted into pαF. When washed microsomes containing 3GpαF or pαF are resuspended in a 'chase' reaction containing cytosol and ATP, pαF is selectively degraded after its retro-translocation to the cytosol, whereas 3GpαF is stable in microsomes (McCracken and Brodsky, 1996). This assay was employed to demonstrate that pαF is degraded by proteasomes after its retro-translocation through the Sec61p channel (Werner *et al*, 1996; Pilon *et al*, 1997; Zhou and Schekman, 1999) and that pαF degradation requires the action of specific ER lumenal chaperones (McCracken and Brodsky, 1996; Brodsky *et al*, 1999; Gillece *et al*, 1999). These features are conserved for all soluble ERAD substrates, suggesting the validity of using pαF as a model protein with which to examine fundamental aspects of this pathway.

As Hsp70 chaperones can drive polypeptide transport across membranes, we examined whether the Ssa1-4p cytoplasmic Hsp70s in yeast facilitate pαF degradation. We found that *ssa* mutant cytosol, which was defective for the refolding of heat-denatured firefly luciferase, supported the degradation of pαF with wild-type efficiency (Brodsky *et al*, 1999). However, yeast contains a second family of cytoplasmic Hsp70s encoded by the *SSB1* and *SSB2* genes; strains deleted for both genes are cold-sensitive and display defects in protein translation (Nelson *et al*, 1992). To examine whether Ssb1/2p facilitates pαF retro-translocation and degradation, we prepared microsomes and cytosol from an isogenic wild-type and the *ssb1ssb2*-deleted strain and examined pαF degradation *in vitro*. However, the extent of degradation

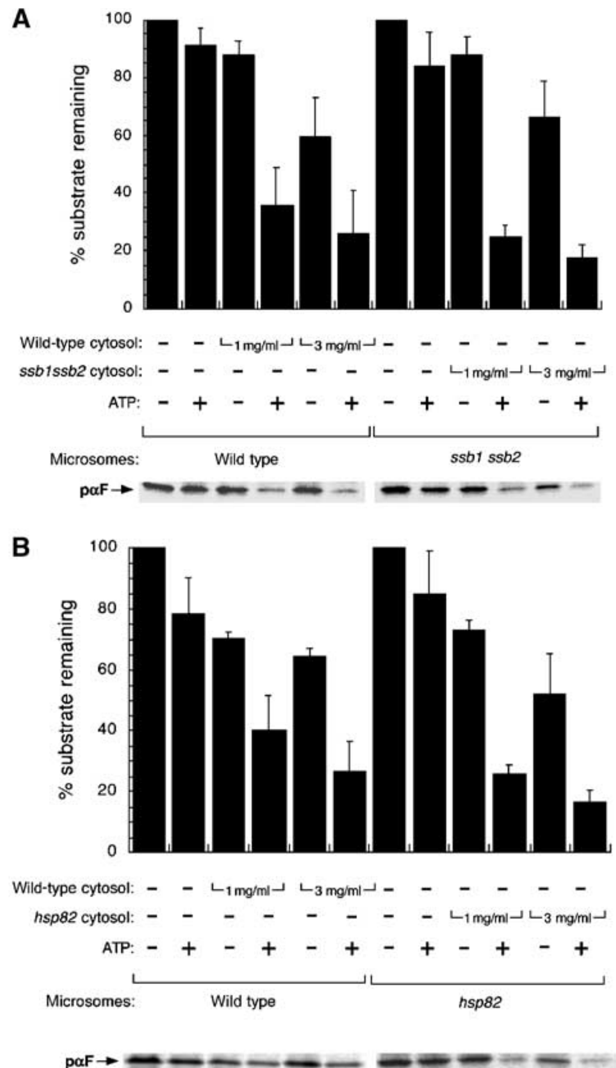

Figure 1 Cytosolic Hsp70 and Hsp90 are not required for pαf degradation *in vitro*. (**A**) Microsomes and cytosol were prepared from the wild-type and *ssb1ssb2* mutant strains grown at 30°C and analyzed in the *in vitro* ERAD assay at the indicated cytosol concentrations or lacking cytosol. ATPγS at a final concentration of 1 mM was included in reactions lacking ATP. A representative image demonstrating the pαF signal in one experiment is also shown. (**B**) Microsomes and cytosol were prepared from the wild-type and *hsp82* temperature-sensitive yeast strains grown at 26°C and then shifted to 37°C for 1 h, and were assayed as in part A. Data represent the means of 3–5 independent experiments, each performed in duplicate, ±s.d.

was similar when reagents from mutant and wild-type strain were compared (Figure 1A), suggesting that the Ssb proteins do not play a role in pαF proteolysis.

The most abundant molecular chaperone in the eucaryotic cytosol is Hsp90, which in yeast is encoded by the *HSC82* (constitutive form) and *HSP82* (heat-inducible form) genes. As Hsp90 facilitates the degradation of some ERAD substrates (Imamura *et al*, 1998; Fuller and Cuthbert, 2000; Gusarova *et al*, 2001), we examined whether microsomes and cytosol from a strain lacking functional Hsp90 supported pαF degradation. And, because yeast requires one isoform for survival, a strain lacking both chromosomal *HSP82* and *HSC82* but containing a wild-type or temperature-sensitive allele of

HSP82 on a plasmid (G313N; Bohen and Yamamoto, 1993) was utilized. Although cytosol prepared from the mutant was unable to degrade ApoB, a mammalian ERAD substrate (Gusarova *et al*, 2001), pαF degradation was robust when the *hsp82* microsomes and cytosol were employed (Figure 1B).

Yeast and mammalian 26S proteasomes augment ERAD

These data suggested that pαF is exported independently of Hsp70 and Hsp90 function. Another scenario is that pαF is driven from the ER by cytosolic polyubiquitination. However, pαF does not acquire ubiquitin side chains *in vitro*, and its degradation is unaffected by mutations in the ubiquitin conjugation machinery or by the addition of compounds that inhibit de-ubiquitination (Werner *et al*, 1996). Therefore, we reasoned that proteasomes might be sufficient to drive pαF retro-translocation and degradation.

To begin to address this hypothesis, we first examined whether proteasome-depleted cytosol supported pαF degradation. An ~3-fold depletion of yeast 26S particles was accomplished by affinity chromatography of cytosol prepared from a strain containing an epitope-tagged 20S resident protein (Figure 2A, 'Pre1p'; Verma *et al*, 2000); components of the 19S particle were also depleted ('Cim3p' and 'Cim5p'). In contrast, Hsp70, Hsp90, Cdc48p, and Arf1p levels were unaffected in the depleted cytosol (Figure 2A and data not shown). Proteasome-depleted cytosol was unable to degrade pαF *in vitro*, but ~60% of the substrate was degraded upon the addition of purified yeast or mammalian 26S proteasomes (Figure 2B). By comparison, ~70% of the pαF was degraded in cytosol at a final concentration of 5 mg/ml, but only ~25% was degraded when the proteasome inhibitor lactacystin was present (see below; Werner *et al*, 1996). We also noted that the yeast 26S particles were less active than those prepared from mammalian cells (data not shown); thus, 30 and 15 μg of yeast and mammalian proteasomes, respectively, were used in this experiment.

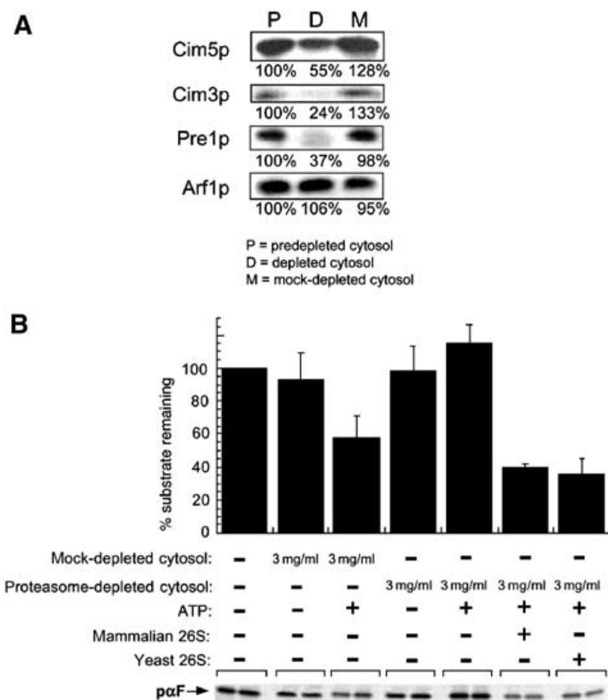

Figure 2 Yeast and mammalian 26S proteasomes augment ERAD in proteasome-depleted cytosol. (**A**) Yeast cytosol was depleted for the 26S proteasome or mock-depleted and 75 μg of the cytosols were immunoblotted using anti-FLAG-M2 antibody to detect 20S particles (Pre1p), anti-Cim3p and Cim5p antibodies to detect 19S particles, and anti-Arf1p as a loading control. (**B**) The mock and depleted cytosols were assayed for pαF degradation using wild-type (RSY607) microsomes. Either 15 μg (mammalian) or 30 μg (yeast) of 26S proteasomes were added to the ERAD reactions, and reactions were performed either in the presence (+) of an ATP-regeneration buffer or in the presence of 1 mM ATPγS (−). Data represent the means of three independent experiments, each performed in duplicate, ±s.d., and a representative image of the pαF signal from the reaction is shown.

Proteasomes replace the cytosolic requirement for pαF degradation

We next resuspended microsomes containing pαF in buffer, cytosol, or 15 μg of mammalian 26S proteasomes and in the presence of an ATP-regenerating system or ATPγS. We found that 15 and 150 μg of cytosol in a 30 μl reaction supported the degradation of 42 and 71%, respectively, of the translocated pαF (Figure 3A). When cytosol was replaced with 15 μg of 26S proteasomes, a similar degree of degradation was observed as when 150 μg of cytosol was used; supplementing the reaction with greater amounts of proteasomes failed to enhance pαF degradation (data not shown). Proteasomes constitute only ~1% of cytosolic protein; hence, the relatively low activity of our 26S proteasomes likely arises from the inefficient re-assembly of 19S and 20S particles (see Materials and methods; Chu-Ping *et al*, 1994); although this regimen reduces the efficiency of 26S-mediated ERAD, we chose to reconstitute the 26S particle so that the contributions of the 19S and 20S components could be examined individually (see below). We also found that degradation was reduced ~2-fold when the ATP-regenerating system was replaced with ATPγS (which prevents the assembly of the 26S particle) or if 20S proteasomes were added (Figure 3B), suggesting that the presence of the 19S cap is important for maximal degradation. The reduced degradation of pαF observed in the presence of 20S particles and ATPγS might result from the fact that purified 20S particles can degrade non-ubiquitinated, unfolded polypeptides (Voges *et al*, 1999), like pαF, which might also be extracted from the membrane. As a negative control, wild-type, glycosylated pαF was not subjected to ERAD (3GpαF; Figure 3A). Finally, MG132 and lactacystin, well-characterized proteasome inhibitors (Fenteany *et al*, 1995; Lee and Goldberg, 1996), blocked pαF degradation to the same degree as inclusion of ATPγS (compare Figure 3A and B).

We then examined whether epitope-tagged 26S proteasomes purified from yeast substituted for cytosol in the *in vitro* assay. As observed with mammalian proteasomes, yeast 26S particles supported pαF degradation in the presence of ATP (Figure 3C), but degradation was inhibited in the presence of lactacystin, MG132, or ATPγS.

The 19S (PA700) particle is sufficient to retro-translocate pαF

We next performed ERAD reactions in which only the mammalian 19S particle in the presence of ATP or ATPγS was added to microsomes harboring pαF. After a 20-min incubation, the microsomal (pellet) and cytosolic (supernatant)

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fractions were isolated by centrifugation, and 52% of the translocated pαF was exported from microsomes when ATP and 19S were present, whereas only 12–15% was exported when ATPγS was added; reactions lacking 19S were equally defective for export (Figure 4A). As *N*-ethylmaleimide (NEM) inhibits the activity of the AAA proteins in the 19S particle (DeMartino *et al*, 1994), NEM-treated 19S was incubated with microsomes containing pαF and again <15% of the translocated pαF was retro-translocated. Interestingly, we found that ppαF was also extracted from microsomes when 19S and ATP were present. This material in the supernatant arises most likely from ΔGppαF that was untranslocated but associated with the microsome exterior. 19S-mediated liberation of ppαF does not result from the loss of microsome integrity (see Materials and methods), a result that is supported by our inability to observe 3GpαF export from microsomes incubated with 19S/ATP (Figure 4B).

In an attempt to resolve the retro-translocation and degradation steps, we next exported pαF from microsomes using 19S and ATP (as in lane 6, Figure 4A), removed the microsomes by centrifugation, and incubated the supernatants (which contained retro-translocated pαF) with buffer, the 20S subunits, the 20S subunits plus MG132, or trypsin (lanes 1–4, Figure 4C). The extracted pαF was degraded only upon addition of 20S or trypsin, and MG132 prevented proteasome-dependent degradation. The same efficiency of export was observed in reactions containing ATP and either 10 or 1 μg of 19S particles (data not shown). These results indicate first that the polypeptide is solvent-exposed and second that pαF export and degradation can be uncoupled.

When yeast 19S subunits were examined in this assay, we again observed ATP-dependent pαF extraction from microsomes (Figure 4D, lane 1). This material was degraded upon the addition of 20S particles (lane 2) or trypsin (lane 4); addition of MG132 to reactions chased with the 20S particle compromised pαF degradation (lane 3). Moreover, we were able to immunoprecipitate 19S-p/pαF complexes in an ATP- and antibody-dependent manner, suggesting physical interaction between 19S and the polypeptides (Figure 4E). Complex formation between ppαF and the 19S particle is consistent with the observed proteasome-dependent degradation of ppαF in reactions lacking microsomes (KR, unpublished observations).

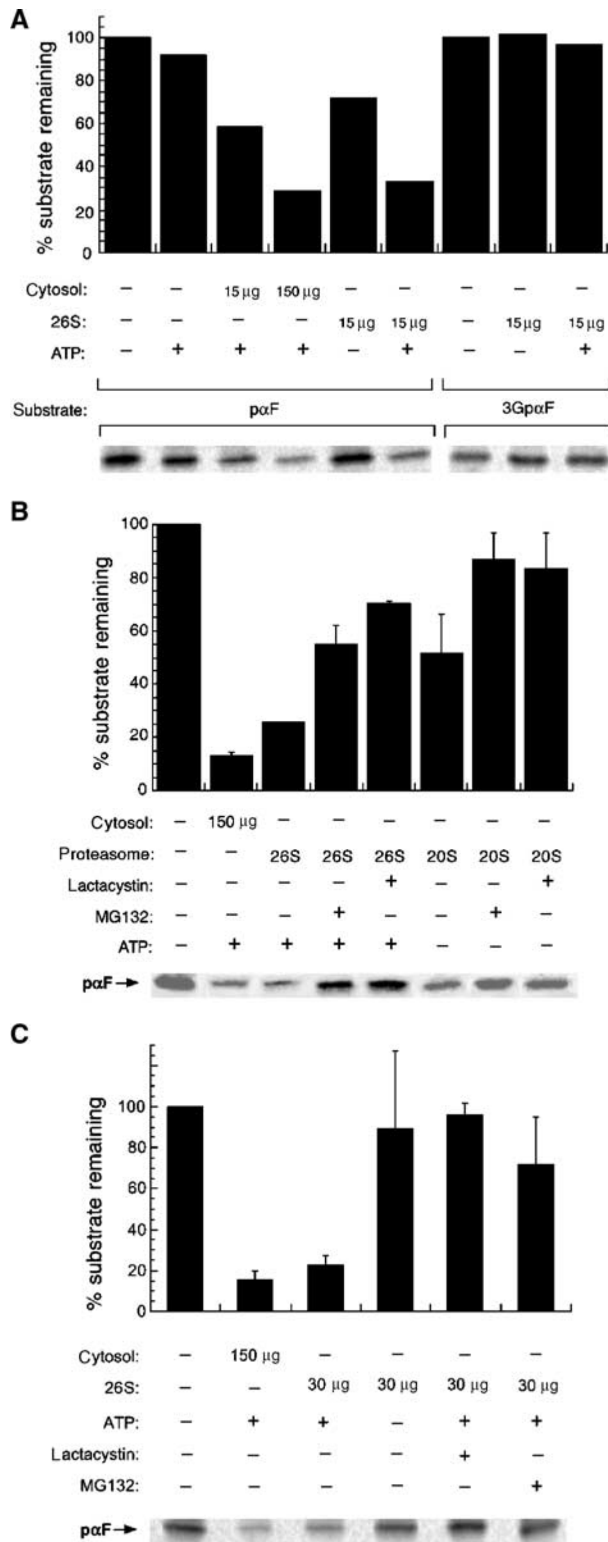

Figure 3 Yeast and mammalian 26S proteasomes are sufficient to support the degradation of pαF *in vitro*. (**A**) Microsomes containing either wild-type precursor protein (triply glycosylated pαF, 3GpαF) or the ERAD substrate pαF were resuspended in 30 μl reactions containing the indicated amounts of wild-type (RSY607) yeast cytosol or re-assembled mammalian 26S proteasomes (see Materials and methods) in the presence of an ATP-regenerating system (+) or ATPγS (−). A representative image from an experiment is shown, and it should be noted that the molecular mass of pαF is ∼18 kDa, whereas the mass of GpαF is ∼27 kDa. Data represent the means of two independent experiments, each performed in duplicate, and the ranges were <10% of the means. (**B**) Reactions were performed as in part A, except that only pαF was examined. Where indicated, either 26 or 20S proteasomes and lactacystin or MG132 were present. Data represent the means of two independent experiments, each performed in duplicate, ±range. (**C**) The degradation of pαF was assayed using the indicated amount of cytosol or yeast 26S proteasomes, in either the presence of an ATP-regenerating system or ATPγS, and lactacystin or MG132. Data represent the means of two independent experiments, each performed in duplicate, ±range.

Proteasome-mediated PαF degradation in vitro is Cdc48p-independent and is not enhanced by additional Hsp70/Hsp90

Mammalian valosin-containing protein (VCP, also known as p97 in mammals or Cdc48p in yeast) is a poly-ubiquitin/

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polypeptide-binding protein required for the ubiquitin-dependent degradation of some ERAD substrates and associates substoichiometrically with the 19S particle; Cdc48p also participates in the proteolytic maturation of ER-bound transcription factors in yeast (Verma *et al*, 2000; Dai and Li, 2001; Pickart, 2001; Bays and Hampton, 2002; Tsai *et al*, 2002; Ye *et al*, 2003). Quantitative immunoblotting of the yeast and mammalian 19S particles used in this study either failed to detect VCP/p97/Cdc48p or led to estimates that the protein represented <0.1% of the 19S preparations. VCP/p97 was also undetected in the purified bovine proteasome preparations as assessed by Edman degradation (G DeMartino and C Slaughter, unpublished data). Nevertheless, to examine whether proteasome-mediated degradation of pαF is Cdc48p-dependent, we prepared cytosol and microsomes from a *cdc48-3* and wild-type strain that were shifted to 37°C for 5 h, conditions that led to a Cdc48p-dependent defect in homotypic ER membrane fusion (Latterich *et al*, 1995). When ERAD reactions with pαF were performed at 30°C using these reagents, no difference in the extent of degradation was observed (Figure 5A). There was also no difference in the extent of degradation using materials prepared from wild-type and *cdc48* strains when the *in vitro* assay was performed at 37°C or when wild-type and mutant microsomes were assayed for pαF degradation upon addition of purified 26S proteasomes (data not shown). Purified Cdc48p added at the same molar ratio as 19S failed to extract pαF (Figure 5B) and did not affect 19S/ATP-mediated export (data not shown). The same batches of *cdc48* mutant membranes and cytosol used in the *in vitro* degradation assay exhibited the previously observed ER membrane fusion defect *in vitro* at 37°C (Supplementary Figure 1; Latterich *et al*, 1995), indicating that these reagents are defective for Cdc48p function.

Hsp70 and Hsp90 associate substoichiometrically with yeast 19S and might deliver substrates to the proteasome (Lüders *et al*, 2000; Verma *et al*, 2000). As it is possible that substoichiometric amounts of Hsp70 and Hsp90 augment 19S-dependent pαF extraction, we determined whether the addition of Hsp70 or Hsp90 to proteasomes in the presence of microsome-occluded pαF enhances ERAD. To this end, we performed pαF degradation assays using mammalian 26S proteasomes supplemented at a 1:1 stoichiometry with yeast or mammalian Hsp70 or Hsp90, but found that this was without effect or inhibited pαF degradation slightly (data not shown).

Mutations in the 19S particle reduce pαF degradation in vivo

To begin to define the requirements for pαF degradation *in vivo*, we introduced a plasmid that expressed ΔGppαF into recipient yeast (see Materials and methods). We first showed that proteasome inhibitors delayed pαF degradation in yeast strain SM4375, which lacks several drug efflux pumps that reside in the plasma membrane (see Table I; data not shown). Next, we examined the degradation of pαF and of CPY*, another well-characterized ERAD substrate (Finger *et al*,

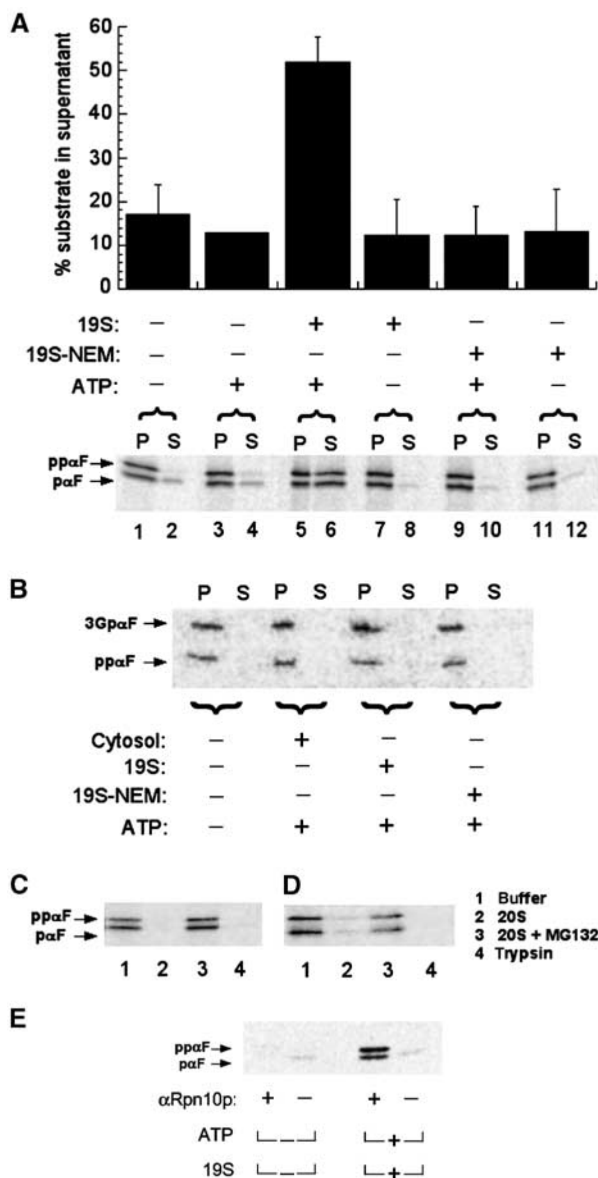

Figure 4 Retro-translocation and degradation of pαF can be uncoupled. (**A**) Microsomes containing p/pαF were resuspended in 30 μl reactions either lacking or containing 10 μg of untreated or NEM-treated mammalian 19S particles and in the presence of an ATP-regenerating system (+) or ATPγS (−). Top: data represent the means of three experiments in which the percent of pαF in the supernatant was calculated, ±s.d. Bottom: a representative imaged gel from one experiment. P and S represent the pellets and supernatants after centrifugation of the export reaction. (**B**) Washed wild-type (RSY607) microsomes incubated with wild-type ppαF and thus containing membrane-associated ppαF and triply glycosylated pαF ('3GpαF') were resuspended in 30 μl reactions either lacking or containing 10 μg of untreated or NEM-treated mammalian 19S particles and in the presence of an ATP-regenerating system (+) or ATPγS (−). P and S represent the pellets and supernatants after centrifugation of the export reaction. (**C**) The supernatant from a 60 μl pαF export reaction, as in panel (A), in the presence of 19S and ATP was quartered and then incubated with (1) buffer, (2) a one-half molar equivalent of mammalian 20S, (3) 20S in the presence of MG132, or (4) trypsin at a final concentration of 0.6 mg/ml. The reactions were incubated for 20 min at 30°C before proteins were precipitated and analyzed by SDS–PAGE and autoradiography. (**D**) Same as (C), except pαF was retro-translocated from wild-type microsomes using yeast 19S particles. (**E**) Supernatants from an experiment in the presence or absence of mammalian 19S/ATP were incubated in buffer or in the presence of anti-Rpn10. Immune complexes were collected with protein A-sepharose, washed with buffer, and resolved by SDS–PAGE and phosphorimager analysis.

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1993), in wild-type yeast and in those containing the *cim3-1* mutation. Cim3p is identical to Rpt6, an AAA subunit in the base of the cap (Ghislain *et al*, 1993), and stabilization of CPY* and pαF was observed in the mutant relative to the wild-type strain (Figure 6A). Next, to determine whether pαF retro-translocation was blocked in *cim3-1* mutant yeast, we used a protocol developed by Sommer and colleagues (Jarosch *et al*, 2002), in which retro-translocated, but stable CPY* was found in some strains containing mutations in genes encoding 19S components. We first noted stabilization of pαF in the *cim3-1* mutant (compare untreated samples in *CIM3* and the *cim3-1* mutant at 0 and 30 min chase; Figure 6B). Second, when comparing the intensities of the

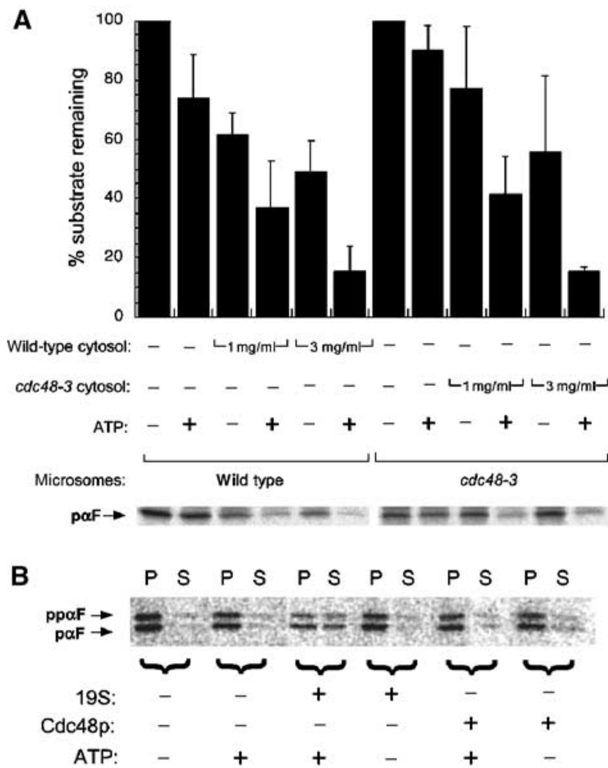

Figure 5 Microsomes and cytosol prepared from *cdc48* mutant yeast are proficient for pαf degradation. (**A**) Microsomes and cytosol were prepared from wild-type and *cdc48-3* mutant yeast grown at 26°C but shifted for 5 h to 37°C, and assayed at the indicated concentrations of cytosol containing ATP (+) or ATPγS (−). Data represent the means of 3–5 experiments, each performed in duplicate, ±s.d. (**B**) Equimolar amounts of yeast-purified hexameric Cdc48p and/or 19S particles were added to the pαF export reaction (see Figure 4) in the presence of either ATP (+) or ATPγS (−), and processed as described in Materials and methods.

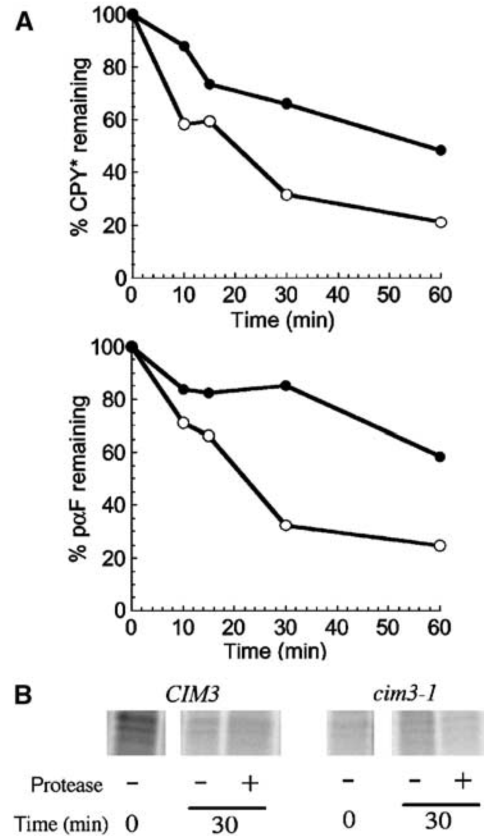

Figure 6 Degradation of CPY* and pαF *in vivo*. Results from a pulse-chase analysis of CPY* and pαF in (**A**) wild-type (○) and *cim3-1* mutant (MHY813:●) yeast at 30°C are shown. Data represent the averages of two independent experiments performed with unique transformants. (**B**) Wild-type and *cim3-1* mutant yeast expressing ΔGppαF were metabolically labeled and chased for the indicated times before the cells were gently lysed, and the extracts were treated with protease as described in Jarosch *et al* (2002). PαF was precipitated with polyclonal antiserum and the results were quantified by phosphorimager analysis.

Table I *S. cerevisiae* strains

Strain	Genotype	Source or reference
RSY607	*MATα ura3-52 leu2-3,112 pep4::URA3*	R Schekman
JN54	*MAT**a** his3-11,15 leu2-3,112 lys2 trp1-Δ1 ura3-52*	Nelson *et al* (1992)
JN212	*MAT**a** his3-11,15 leu2-3,112 lys2 trp1-Δ1 ura3-52 ssb1-1 ssb2-1*	Nelson *et al* (1992)
p82a	*MAT**a** ade2-1 can1-100 leu2-3,112 his3-11,15 trp1-1ura3-1 hsc82::LEU2 hsp82::LEU2* (pTGPD*HSP82* (*TRP1*)))	Gusarova *et al* (2001)
G313N	*MAT**a** ade2-1 can1-100 leu2-3,112 his3-11,15 trp1-1ura3-1 hsc82::LEU2 hsp82::LEU2* (pTGPD*hsp82*G313N (*TRP1*)))	Gusarova *et al* (2001)
RJD1144	*MAT**a** leu2-3,112 his3-Δ200 trp1-Δ63 lys2-801 ura3-52 PRE1^{FH}::YIplac211(URA3)*	Verma *et al* (2000)
RJD1171	*MAT**a** leu2-3,112 his3-Δ200 trp1-Δ63 lys2-801 ura3-52 RPT1^{FH}::YIplac211(URA3)*	Verma *et al* (2000)
MLY1600	*MATα ura3-52 leu2-3,112 pep4::URA3*	Latterich *et al* (1995)
MLY1640	*MATα ura3-52 leu2-3,112 pep4::URA3 cdc48-3*	Latterich *et al* (1995)
SM4375	*MATα ura3 his1 pdr1Δ::hisG yor1Δ::hisG snq2Δ::hisG pdr10Δ::hisG pdr11Δ::hisG ycf1Δ::hisG pdr3Δ::hisG pdr5Δ::hisG pdr15Δ::hisG*	S Michaelis
MHY810	*MAT**a** ura3-1::TRP1 trp1-Δ1 his3-Δ200 leu2-Δ1 lys2-801 lys1-1 met14*	M Hochstrasser
MHY898	*MAT**a** ura3-1::TRP1 trp1-Δ1 his3-Δ200 leu2-Δ1 lys2-801 lys1-1 met14 sen3-1*	M Hochstrasser
MHY813	*MATα ura3-52 his3-Δ200 leu2-Δ1 ade2-101 cim3-1*	M Hochstrasser

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anti-ppαF precipitable material that was obtained in the absence and presence of protease, we found that ~40% more pαF was digested by protease in the mutant strain compared to the wild-type strain. These data suggest that retro-translocation can occur in this mutant, but that functionally redundant components in the 19S particle and/or the Cdc48-containing complex facilitate retro-translocation *in vivo* (see Discussion).

Discussion

We demonstrate that purified yeast or mammalian proteasomes can replace the requirement for cytosol in the cell-free ERAD assay and that by supplying the 19S cap and the 20S proteasome separately, retro-translocation and degradation could be uncoupled. As NEM treatment of 19S abrogates retro-translocation, polypeptide extraction requires 19S-resident ATPases. More generally, these results suggest that the 19S particle can exert 'force' to drive or ratchet polypeptides from the ER, an effect consistent with the fact that the 19S particle conformationally re-models proteins *in vitro* and binds non-ubiquitinated substrates (Braun *et al*, 1999; Strickland *et al*, 2000; Liu *et al*, 2002).

The concept that the 19S cap exerts directional force on a polypeptide is consistent with observations that mitochondrial AAA ATPases and bacterial 'caps' on proteasome-like particles unfold and feed substrates into proteolytic core particles (Leonhard *et al*, 1999; Weber-Ban *et al*, 1999; Benaroudj and Goldberg, 2000; Hoskins *et al*, 2000; Kim *et al*, 2000; Singh *et al*, 2000). The degree to which 19S-mediated unfolding is required during the export and degradation of an ERAD substrate remains unclear. As the 26S proteasome sequentially unfolds a model protein as it is being degraded (Lee *et al*, 2001), the coupling of extraction and degradation by the 19S and 20S particles, respectively, may be sufficient to unwind and destroy folded ERAD substrates. Alternatively, 19S particles may have to remove any remaining secondary structure from proteins after passage through the confines of the translocation channel (Navon and Goldberg, 2001). Other studies (Fiebiger *et al*, 2002) suggest that an ERAD substrate can be exported without significant unfolding and that transport to the cytosol, unfolding, and proteolytic degradation may not have to be coupled. Our results support this model.

The export of diverse ERAD substrates from the ER may be driven by a variety of cellular factors. The Cdc48/p97 complex, for example, plays a role in the proteolysis of several ERAD substrates and ER membrane proteins, and it has been proposed that the complex may couple retro-translocation and degradation *in vivo*, or even ratchet ER-resident polypeptides (reviewed in Bays and Hampton, 2002; Tsai *et al*, 2002). p97 binds polypeptides prior to ubiquitination, but the extraction of these substrates from the ER requires both polyubiquitination and p97-mediated ATP hydrolysis (Flierman *et al*, 2003; Ye *et al*, 2003). Consistent with these data, we were unable to establish a role for Cdc48p in the *in vitro* retro-translocation of pαF, which lacks polyubiquitination, although it might assist in the retro-translocation of pαF *in vivo*. Our results also suggest that individual ERAD substrates might exhibit distinct requirements for their degradation, and are consistent with the fact that to date no two ERAD substrates possess identical requirements for their degradation.

19S-mediated extraction of pαF might be relevant to the mechanism by which several bacterial and plant toxins are exported to the cytoplasm after their retrograde transport through the secretory pathway from the cell surface. These toxins, which include ricin, *Pseudomonas* exotoxin A, and pertussis, cholera, and Shiga toxin, co-opt the ERAD pathway and many of the same factors required for ERAD augment toxin action (reviewed in Tsai *et al*, 2002). However, repeated attempts to measure ricin export from yeast ER-derived microsomes using purified 19S particles have been unsuccessful (data not shown), suggesting that toxin export requires additional factors, such as Cdc48p, or post-translational modification.

Another phenomenon in which the participation of the 19S particle might be sufficient is aggresome formation. The extraction but inefficient degradation of the cystic fibrosis transmembrane conductance regulator protein (CFTR) results in the deposition of aggregated protein at a peri-centriolar locus in the cell (Johnston *et al*, 1998; Wigley *et al*, 1999). Aggregation may be a result of 19S-mediated CFTR extraction without sufficient coupling to degradation by the 20S core proteasome.

The ability of both yeast and mammalian proteasomes to support pαF degradation *in vitro* suggests either that a conserved proteasome-binding site resides on the yeast ER membrane, and/or that retro-translocating polypeptides bind to free yeast or mammalian proteasomes in solution and bring them to the ER membrane. Although a specific proteasome-binding component at the membrane has not been identified, we note that a large fraction of proteasomes resides on the ER-nuclear membrane network in yeast at steady state, and it is difficult to imagine that these particles are all actively engaging ERAD substrates (Enenkel *et al*, 1998). We therefore favor the first scenario.

Finally, the degradation of most soluble ERAD substrates requires ER-resident but not cytoplasmic chaperones, whereas the proteolysis of most ER membrane proteins requires cytoplasmic but not lumenal chaperones (Hill and Cooper, 2000; Fewell *et al*, 2001; Zhang *et al*, 2001). One hypothesis to explain this phenomenon is that the cytoplasmic chaperones prevent the aggregation of cytoplasmic polypeptide loops on integral membrane ERAD substrates. In contrast, lumenal chaperones might retain soluble ERAD substrates in a retro-translocation-competent (i.e. aggregation-free) conformation, so they can transit through the Sec61p translocation channel (Nishikawa *et al*, 2001). The ERAD of at least some soluble lumenal proteins may be cytoplasmic chaperone-independent because of a post-translational modification, such as poly-ubiquitination (Biederer *et al*, 1997; de Virgilio *et al*, 1998; Shamu *et al*, 2001), or because of polypeptide interaction with the proteasome cap (this study). Thus, one future goal is to examine the relative contributions of polyubiquitination and 19S on the extraction of a more complex ERAD substrate.

Materials and methods

Strains and reagents

The strains used in this study are listed in Table I. Lactacystin and MG132 were purchased from EJ Corey (Harvard University) and Calbiochem, respectively. Anti-FLAG-M2 antibody, ATPγS, leupeptin, and pepstatin were obtained from Sigma, and anti-HA antibody was obtained from Roche. Purified bovine Hsc70 and human Hsp90

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were obtained from StressGen, Cdc48p was purified as described (Latterich *et al*, 1995), and purified yeast Ssa1p and Hsp90 were provided by S Fewell and R Youker (University of Pittsburgh). Antiserum against yeast Cdc48p was prepared as described (Latterich *et al*, 1995), anti-Cim3p and Cim5p were kindly provided by C Mann (Saclay, Gif-sur-Yvette, France), and anti-Arf1p antiserum was contributed by R Duden (Cambridge University). Anti-Rpn10 (human S5a) was prepared in rabbits against a peptide to the C-terminal 16 amino acids of the protein conjugated to KLH.

ERAD assays

Cytosol and ER-derived microsomes for ERAD assays were prepared, and the degradation of unglycosylated pro-alpha factor (pαF) in microsomes was measured essentially as described (McCracken and Brodsky, 1996). In brief, ^{35}S-labeled pre-pαF (ppαF) lacking the core consensus glycosylation sites was translocated into microsomes and the microsomes were harvested and washed twice in buffer88 (20 mM HEPES, pH 6.8, 150 mM KOAc, 250 mM sorbitol, 5 mM MgOAc) before being resuspended in a 60 μl chase reaction at 30°C either containing an ATP-regenerating mix or ATPγS and/or yeast cytosol. Reactions were quenched after 20 min with trichloroacetic acid and samples were resolved by SDS–PAGE. The percent pαF remaining was calculated after phosphorimager analysis. The integrity of all microsome preparations was assessed by translocation assays with ppαF and subsequent incubation in the presence or absence of the microsomes in 0.3 mg/ml trypsin at 4°C for 20 min. In all, >96% of the pαF was protected from degradation in every microsome preparation used, whereas 34–51% of the ppαF was degraded.

In vitro ERAD reactions with purified proteasomes were set up as above, but with the following modifications: pre-assembled reactions containing either 19S or 20S mammalian particles, or both, in proteasome reassembly buffer (PRB: 45 mM Tris–Cl, pH 8, 5 mM DTT, 10 mM $MgCl_2$) and in the presence of an ATP-regenerating system or ATPγS (at a final concentration of 1 mM) were incubated for 30 min at 37°C. Yeast 26S proteasomes were also subjected to the pre-assembly reaction. Washed microsomes were added and mixed gently into the reaction and the temperature was reduced to 30°C. MG132 and lactacystin were included where indicated in reactions at final concentrations of 100 and 10 μM, respectively. PRB lacked DTT in reactions assembled with NEM-treated 19S particles, as the reductant reversed NEM modification. Reactions to which only 20S particles were added were performed with microsomes prepared from strain RJD1171, which expresses a tagged form of a 19S component that compromises 19S association with 20S (Verma *et al*, 2000).

Reactions in which the export of pαF was assayed were set up as above, using 20 or 40 μg of mammalian and yeast 19S particles, respectively, and the ATP-regenerating system in a 60 μl reaction volume assembled in PRB. Leupeptin, pepstatin (at final concentrations of 20 and 70 μM, respectively), and MG132 were included to minimize the contaminating protease activity, but MG132 was absent when 20S particles were subsequently added. After 20 min at 30°C, the reaction was centrifuged for 2 min in a microfuge at 4°C and the supernatant and pellet fractions were either analyzed directly or the supernatant was quartered into reactions containing buffer, a 1:2 molar ratio of mammalian 20S particles, 20S subunits in the presence of 50 μM MG132, or trypsin at a final concentration of 0.6 mg/ml. Reactions were incubated for another 20 min at 30°C before being quenched and analyzed.

Degradation of CPY* was measured by pulse-chase analysis using an HA-tagged version of the protein (provided by D Ng, Pennsylvania State University) as previously described (Zhang *et al*, 2001). Degradation of pαF was assessed after production *in vivo* by expression of ΔGppαF from the *MET25* promoter (p416; Mumberg *et al*, 1994) or by using an HA-tagged form of this protein expressed from the CPY promoter (also provided by D Ng).

Preparation of proteasome-depleted yeast cytosol

Cytosol from *S. cerevisiae* strain RJD1144 was prepared as above and ATP and DTT were added to final concentrations of 1 mM. To deplete the 20S subunit from the clarified cytosol, 50 mg of cytosol was incubated in batch with 600 μl of packed anti-FLAG-M2 agarose (Sigma) or Sepharose G-75 ('mock-depleted') for 3 h at 4°C before the resin was sedimented. This incubation was repeated with fresh beads, DTT was added to a final concentration of 1 mM, and after removal of the resin the cytosol was snap-frozen in liquid nitrogen.

Proteasome purification

19S particles and latent 20S proteasomes were purified from bovine red blood cells as described (McGuire *et al*, 1989; DeMartino *et al*, 1994). Quantitative immunoblot analyses were used to assess Hsp70, Hsp90, and VCP in the PA700 preparation. There was 0.5–1% (w/w) of Hsc/p70 in five different PA700 preps using purified bovine Hsc70 (StressGen) as a positive control; Hsp90 was undetected in 30 μg of five PA700 preps (the detection limit is 600 ng), indicating that there is less than 2% (w/w) of Hsp90 in the PA700 preps; VCP was undetected in 30 μg of five different PA700 preps, indicating <0.1% contamination. NEM modification of PA700 was accomplished with a 1000-fold excess of NEM in 20 mM Tris–HCl, pH 7.5, 20 mM NaCl, 1 mM EDTA, 10% glycerol at 4°C for 6 h. NEM was removed by dialysis at 4°C against the same buffer.

Yeast 26S proteasomes were purified as described (Verma *et al*, 2000). In brief, cytosol from strain RJD1144 was mixed with an equal volume of buffer A (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10% w/v glycerol, 5 mM $MgCl_2$) and the extract was centrifuged at 37 000 *g* for 20 min. The supernatant (15 ml) was incubated with 700 μl of packed anti-FLAG-M2 agarose for 4 h at 4°C. The beads were washed in buffer A/2 mM ATP, 0.2% Triton X-100, and twice in buffer88/2 mM ATP. The proteasomes were eluted in 1.3 ml buffer88/2 mM ATP and 150 μg/ml FLAG peptide for 3 h at 4°C, and the supernatant was concentrated in Centricon YM-100 cartridges (Amicon) to ∼1 mg/ml and snap-frozen.

Yeast 19S and 20S particles were purified as for 26S proteasomes, but with several modifications. The particles were eluted in the absence of ATP and the eluate was dialyzed against buffer88 lacking MgOAc but including 350 mM NaCl and 2 mM EDTA overnight at 4°C to dissociate 19S and 20S particles and to remove the FLAG peptide and ATP, and then against buffer88 for 1 h at 4°C to remove NaCl and EDTA. The dialysate was then incubated with anti-FLAG-M2 agarose beads for 4 h at 4°C and the supernatant containing 19S was concentrated to ∼1 mg/ml and snap-frozen. The 20S particles were eluted from beads in buffer88/150 μg/ml FLAG peptide for 3 h at 4°C, concentrated to ∼1 mg/ml and snap-frozen.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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