Assembly of ER-Associated Protein Degradation In Vitro: Dependence on Cytosol, Calnexin, and ATP

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Abstract. To investigate the mechanisms of ER-associated protein degradation (ERAD), this process was reconstituted in vitro. Established procedures for post-translational translocation of radiolabeled prepro-alpha factor into isolated yeast microsomes were modified to inhibit glycosylation and to include a posttranslocation "chase" incubation period to monitor degradation. Glycosylation was inhibited with a glyco-acceptor peptide to compete for core carbohydrates, or by using a radiolabeled alpha factor precursor that had been genetically engineered to eliminate all three glycosylation sites. Inhibition of glycosylation led to the production of unglycosylated pro-alpha factor (pαF), a processed form of the alpha factor precursor shown to be a substrate of ERAD in vivo. With this system, both glycosylated and unglycosylated forms of pro-alpha factor were stable throughout a 90-min chase incubation. However, the addition of cytosol to the chase incubation reaction induced a selective and rapid degradation of pαF. These results directly reflect the behavior of alpha factor precursor in vivo; i.e., pαF is a substrate for ERAD, while glycosylated pro-alpha factor is not. Heat inactivation and trypsin treatment of cytosol, as well as addition of ATPγS to the chase incubations, led to a stabilization of pαF. ERAD was observed in *sec12* microsomes, indicating that export of pαF via transport vesicles was not required. Furthermore, pαF but not glycosylated pro-alpha factor was found in the supernatant of the chase incubation reactions, suggesting a specific transport system for this ERAD substrate. Finally, the degradation of pαF was inhibited when microsomes from a yeast strain containing a disrupted calnexin gene were examined. Together, these results indicate that cytosolic protein factor(s), ATP hydrolysis, and calnexin are required for ER-associated protein degradation in yeast, and suggest the cytosol as the site for degradation.

THE presence of aberrant proteins in the cell has made necessary the evolution of efficient mechanisms for their removal. Indeed, protein degradation is known to occur in different cell compartments, most notably in the lysosome and the cytoplasm. Recently, the results from many studies have clearly established the existence of a selective intracellular protein degradation process that removes aberrant and unassembled proteins from the ER (for review see Bonifacino and Klausner, 1994). This ER-associated protein degradation process (ERAD)[1] operates as an essential step in the quality control of newly synthesized proteins in the secretory pathway.

Analyses of various protein substrates in untreated and permeabilized cells have indicated that proteases of the mammalian lysosome and the yeast vacuole are not involved in ERAD, suggesting a novel proteolytic pathway (Chen et al., 1988; Amara et al., 1989; Finger et al., 1993; McCracken and Kruse, 1993; Hampton and Rine, 1994; and references therein). Experiments with protease inhibitors have shown that both cysteine and serine proteases may be involved (Wileman et al., 1991; Inoue and Simoni, 1992; Tsao et al., 1992; Wikstrom and Lodish, 1992), but the identity of the responsible protease(s) and the site of degradation are unknown.

ER-associated protein degradation is highly selective for specific soluble and integral membrane proteins, while the majority of ER resident and secreted proteins are quite stable (Lippincott-Schwartz et al., 1988; Wileman et al., 1990). Although the nature of this remarkable substrate selectivity is not yet known, it is likely that structural features of the protein function as degradation motifs (reviewed in Bonifacino and Klausner, 1994; Schmitz et al., 1995).

One major success in cell biology has been the achievement of cell-free systems to study protein translocation across cell membranes. Such in vitro systems have been used to identify targeting signals and receptors, and to ex-

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1. *Abbreviations used in this paper*: ERAD, ER-associated protein degradation; NYT, *n*-acetyl-asparaginyl-tyrosyl-threonyl-amide; pαF, unglycosylated pro-alpha factor; ppαF, prepro-alpha factor; ΔGppαF, genetically engineered ppαF with all three glycosylation sites removed.

amine the transport machinery (reviewed by Brodsky and Schekman, 1994). A cell-free system for the study of ERAD would provide the means to dissect this pathway at the molecular level. Experiments with permeabilized mammalian cells have demonstrated that the selectivity and regulation of ERAD was influenced by the redox conditions of the ER (Young et al., 1993), and that transport out of the ER was not an absolute requirement for degradation (Leonard and Chen, 1987; Stafford and Bonifacino, 1991; Megis and Simoni, 1992; Wikstrom and Lodish, 1992). We sought to assemble a cell-free yeast system to study ERAD in which the specific contributions of both membrane and soluble components of the degradative process could be analyzed in detail.

Established procedures for in vitro studies with ER-derived microsomes from yeast (Wuestehube and Schekman, 1992; Brodsky and Schekman, 1993; Brodsky et al., 1993) were modified to inhibit glycosylation of translocated prepro-alpha factor, a yeast mating pheromone precursor. In its unglycosylated form, pro-alpha factor (pαF) is a substrate for ERAD in vivo (Caplan et al., 1991). In our in vitro system, pαF is selectively and rapidly degraded via a cytosol-dependent mechanism. Furthermore, this cell-free system mimics the in vivo process of ER-associated protein degradation, as judged by similar rates of degradation, vacuolar protease independence, and substrate selectivity. We used this in vitro system to demonstrate that protein component(s) of the cytosol, ATP hydrolysis, and the molecular chaperone calnexin, are required for ER-associated protein degradation in yeast.

Materials and Methods

Materials

Strains used were RSY607 (*Matα, ura3*-52, *leu2*-3-112, *pep4*::*URA3*) provided by R. Schekman (University of California, Berkeley); AB122 (*Mata, prc1*-407, *prb1*-1122, *pep4*-3, *leu2, ura3*-52) provided by A. Brake (University of California, San Francisco); W303-1a (*Mata, ade2*-1, *can1*-100, *ura3*-1, *leu2-3*,112, *trp1*-1, *his3*-11,15) and W303-1a, *CNE1* delete (*Mata, ade2*-1, *can1*-100, *ura3*-1, *leu2-3*,112, *trp1*-1, *his3*-11,15 (*cne1*::*LEU2*) (Parlati et al., 1995); W303-1B (*Matα, ade2*-1, *can1*-100, *ura3-1, leu2*-3,112, *trp1*-1, *his3*-11,15) and W303-1B, *EUG1* delete (*Matα, ade2*-1, *can1*-100, ura3-1, *leu2*-3,112, *trp1*-1, *his3*-11,15, (*eug1*::*LEU2*) (Tachibana and Stevens, 1992); pSY103 (*Matα, ura3*-52, *leu2*-3,112, *ade2*) and pSY105, *SCJ1* delete (*Matα, ura 3-52, leu2*-3,112, *(scj*::*URA3)* (Blumberg and Silver, 1991); and RSY263 (*Matα, sec12-4, ura3-52*) and RSY250 (*Matα, ura3-52*) provided by C. Barlowe (Dartmouth Medical School). The pGem2(36-3Q expression plasmid that contains a ppαF gene with all three glycosylation sites altered to specify glutamine instead of asparagine (ΔGppαF) was provided by D. Meyer (University of California, Los Angeles). Radiolabeled prepro-alpha factor, both wild-type (ppαF) and mutant (ΔGppαF), was synthesized in a yeast cell-free transcription/translation reaction essentially as described (Rothblatt and Meyer, 1986). Glyco-acceptor peptide, *n*-acetyl-asparaginyl-tyrosyl-threonyl-amide (NYT), was provided by W. Hansen (University of California, San Francisco) (Hansen and Walter, 1988).

Preparation of Yeast Microsomes and Cytosol

Yeast cells were grown at 20°C in YPD (1% Bacto-yeast extract, 2% Bactopeptone, 2% dextrose) to a final optical density of 2–4 at 600 nm. Yeast microsomes were prepared from RSY607 cells by homogenization, essentially as described (Brodsky et al., 1993), washed, and diluted with buffer-88 (20 mM Hepes, pH 6.8, 150 mM KOAc, 250 mM sorbitol, 5 mM MgOAc) to a final concentration of ~10 mg protein/ml ($OD_{280} \approx 40$ in 1% SDS), quick-frozen in liquid nitrogen, and stored at −70°C. A yeast cytosol S100 fraction (23 mg protein/ml) was obtained from an AB122 cell lysate prepared by liquid nitrogen lysis (by centrifugation at 100,000 *g*) as previously described (Sorger and Pelham, 1987). To produce heat inactivated cytosol, aliquots were subjected to two rounds of heating to 85°C for 5 min, each followed by cooling on ice. To produce trypsin treated cytosol, 420 μl of cytosol (9.7 mg protein) was incubated with 100 μl (2.8 units) of trypsin-linked agarose beads (Sigma Chemical Co., St. Louis, MO) for 30 min at 37°C and the beads were subsequently removed by centrifugation according to the manufacturer's specifications.

Translocation and In Vitro ERAD Reactions

Reactions typically contained 60 μl, which included an ATP regenerating system (1 mM ATP, 50 μM GDP-mannose, 40 nM creatine phosphate, 0.2 mg/ml creatine phosphokinase), 6 μl of microsomes, and buffer 88. Mixtures were prepared and held on ice unless otherwise indicated. Reactions were commenced by the addition of 200,000-300,000 cpm of wild-type ^{35}S-prepro-alpha factor or mutant ^{35}S-prepro-alpha factor lacking the three consensus glycosylation acceptor sites (~4–6 μl of radiolabeled protein), and conducted for 50 min at 20°C. Microsomes were pelleted from the translocation reaction by micro-centrifugation (12,000 rpm) at 4°C for 4 min, gently resuspended and washed with buffer 88 and then resuspended in 60 μl buffer-88 containing the ATP regenerating system. The posttranslocation incubation was at 30°C for the indicated duration, and was commenced by addition of cytosol (5 μg protein per μl of reaction), and/or 1.8 mM Ca^{2+} and 0.5 mM DTT. After incubations were complete, 20-μl aliquots were removed and placed on ice, or mixed with trypsin (0.25 g/ml) or trypsin and 1% Triton X-100 and incubated at 0°C for 25 min. Proteolysis was quenched by the addition of TCA to a final concentration of 10–20%, and the reactions were left on ice for another 15 min, after which they were microcentrifuged (10,000 rpm) at 4°C for 10 min. The pellets were washed once with 200 μl of ice-cold acetone and the final pellet fraction was resuspended in 30 μl of Laemmli sample buffer and heated to 95°C for 3 min. The samples were resolved on an 18% polyacrylamide gel containing SDS and 4 M urea. Gels were fixed in 10% acetic acid containing 25% isopropyl alcohol, dried and exposed to a Phosphorimaging screen for 1–2 d. Results were visualized using a BioRad PhosphorImager and quantified by BioRad phosphor analyses software (Bio Rad Labs., Richmond, CA). First order decay curves, generated using Cricket Graph software (Cricket Software, Malvern, PA) were used to determine the half-life of pαF.

Results

The yeast pheromone precursor, prepro-alpha factor (ppαF), was chosen as an ERAD reporter substrate because it is competent for posttranslational translocation across yeast microsomes in vitro (Hansen et al., 1986; Rothblatt and Meyer 1986; Waters and Blobel, 1986) and in its unglycosylated form, pro-alpha factor (pαF) is a substrate for ERAD in vivo (Caplan et al., 1991). In the ER, the signal sequence is cleaved from ppαF, pαF receives three core carbohydrate chains, and the glycosylated species is transported to the Golgi complex for further processing and alpha factor is ultimately secreted (reviewed in Fuller et al., 1988). However, when glycosylation is inhibited, alpha factor is inefficiently secreted, and pro-alpha factor accumulates in the ER and is degraded (Julius et al., 1984; Caplan et al., 1991). Thus, if ERAD was reconstituted in vitro, glycosylated pro-alpha factor would be stable while pαF would be degraded.

Acceptor Peptide Inhibits Pro-Alpha Factor Glycosylation

To generate a substrate for ERAD, translocation of radiolabeled prepro-alpha factor into isolated yeast microsomes was performed in the presence of a competitor for glycosylation, NYT (see Materials and Methods). This peptide freely enters the ER where it becomes glycosylated and competes for available core carbohydrate chains (Hansen

and Walter, 1988). After the translocation reaction, untranslocated ppαF was removed by centrifugation, and the microsomes were incubated at 30°C to allow for degradation of the translocated protein. The identity of translocated protein was determined by differential migration during SDS-PAGE, based on the acquisition of N-linked core carbohydrate and by the cleavage of the presequence (Deshaies and Schekman, 1989). Cleavage of the signal sequence resulted in a species (pαF) that migrated further than the untranslocated ppαF, and glycosylation resulted in a form (3GpαF) that migrated slower than ppαF. As expected, signal sequence cleavage and glycosylation occurred in the absence of NYT peptide (Fig. 1, *3GpαF*) and glycosylation was inhibited in the presence of NYT peptide (Fig. 1, *pαF*). Both forms of pαF were stable and protease protected during the 90-min chase incubation, demonstrating the integrity of the microsomes. However, based on these results we concluded that ERAD had not been reconstituted.

Degradation of Unglycosylated Pro-Alpha Factor Requires the Addition of Cytosol

We reasoned that a component or condition for ERAD had not been met in this cell-free system. To identify the missing component, we first examined whether yeast cytosol would provide the absent factor(s). Translocation was carried out in the presence of NYT peptide, and chase incubations were supplemented with yeast cytosol. The addition of cytosol resulted in the selective degradation of pαF (Fig. 2). The presence of NYT peptide incompletely inhibited glycosylation in these experiments, and multiple forms of glycosylated pre-sequence cleaved pro-α factor species with one, two, or three attached core carbohydrate chains were present (Fig. 2, *1pαF*, *2pαF*, and *3pαF*). Significantly, these core-glycosylated forms of pro-alpha factor were stable during the chase with cytosol. Furthermore, the stability of glycosylated pro-alpha factor in the protease treated samples (Fig. 2, lane *b*) showed that the addition of cytosol did not permeabilize the microsomes. ERAD was absent when BSA instead of cytosol was added to the chase reaction (data not shown). We concluded from these results that cytosolic factor(s) were necessary and sufficient to reconstitute ERAD.

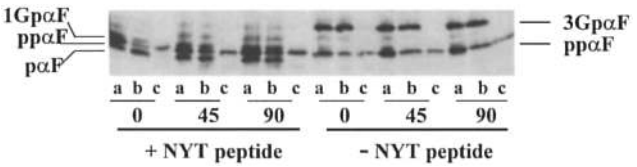

Figure 1. Glycosylation acceptor peptide inhibits glycosylation of pαF; both glycosylated and unglycosylated pro-alpha factor are stable in yeast microsomes. Translocation reactions containing wild-type yeast microsomes, radiolabeled ppαF, and in the presence or absence of 0.33 μM NYT glycosylation acceptor peptide, were followed by 0-, 40-, and 90-min chase incubations of washed microsomes resuspended in buffer with or without NYT glycosylation acceptor peptide. Reactions were processed as described, analyzed by SDS-PAGE, and visualized using a PhosphorImager (see Materials and Methods). Lane *a*, total TCA precipitable material from untreated samples; lane *b*, samples treated with trypsin to demonstrate translocated protease-protected protein; lane *c*, samples treated with trypsin and Triton X-100, showing depletion of translocated protein upon loss of membrane integrity. The protease protected ppαF seen in lanes *b* and *c*, is an untranslocated aggregated species that remains associated with the microsomes and is resistant to trypsin treatment even in the presence of Triton-X 100 (Brodsky et al., 1993). The species migrating slower than ppαF in lanes *a* and *b* are differentially glycosylated forms of protease-protected translocated pαF (*1GpαF*, *2GpαF*, *3GpαF*) that are degraded upon addition of detergent as in lanes c. The presence of glycosylated pαF indicates that the competition for carbohydrate by the glyco-acceptor peptide was incomplete in this experiment. Note the stability of glycosylated and unglycosylated pro-alpha factor (*pαF*) in the 40- and 90-min samples.

To eliminate the possibility that addition of cytosol stimulated glycosylation of pαF, thus reducing the pool of pαF in the microsomes, a second approach was used to generate unglycosylated pαF. A mutant prepro-alpha factor gene was transcribed and translated in a yeast lysate to produce radiolabeled prepro-alpha factor with all three glycosylation sites removed (ΔGppαF, see Materials and Methods). To show that ΔGppαF was translocation-competent, we incubated yeast microsomes with the radiolabeled precursor in the presence and absence of ATP. The formation of protease protected unglycosylated pαF was dependent on hydrolyzable ATP and yeast microsomes (Fig. 3). Furthermore, while the signal sequence of ΔGppαF was cleaved to yield pαF, this product was not glycosylated. These results demonstrated that ΔGppαF, like wild-type ppαF, was translocation competent and could be used as a substrate in our in vitro ERAD assay.

To test whether pαF generated from ΔGppαF was degraded in the presence of cytosol, radiolabeled ΔGppαF was translocated into yeast microsomes and cytosol was added or omitted from the microsomes during the chase incubations. In these experiments, pαF was degraded in the incubation reaction that contained cytosol but was stable in the absence of cytosol (Fig. 4 *a*), confirming the cytosol-dependent nature of in vitro ERAD.

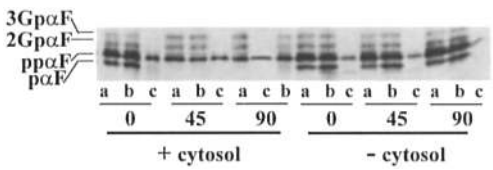

Figure 2. Unglycosylated pro-alpha factor but not glycosylated pro-alpha factor is degraded by the addition of cytosol. Translocation reactions containing wild-type yeast microsomes, radiolabeled ppαF, and 0.02 μM NYT peptide were followed by 0-, 45-, and 90-min chase incubations in the presence of NYT peptide and with or without cytosol (5 mg cytosolic protein/ml of reaction). Samples were processed as described in Materials and Methods. Lane *a*, untreated samples; lane *b*, trypsin-treated samples; lane *c*, samples treated with trypsin and Triton X-100. Note the disappearance of pαF in the 45- and 90-min incubation samples that contain cytosol (+ *cytosol*) and the presence of pαF in the 45 and 90 min samples without cytosol (− *cytosol*). Also, note that the glycosylated forms of pro-alpha factor are stable regardless of the presence or absence of cytosol, demonstrating substrate selectivity.

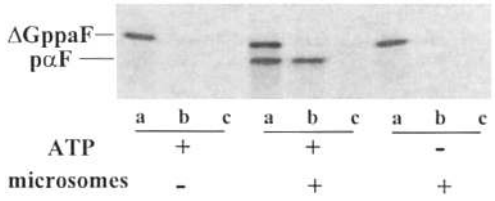

Figure 3. ATP and microsome-dependent translocation of ΔGppαF. Reactions containing the 35S-methionine labeled species of ppαF lacking glycosylation acceptor sites (*ΔGppαF*) were assembled in the presence or absence of wild-type microsomes and an ATP-regenerating system. After a 50-min incubation at 20°C, the reactions were distributed to determine: (*a*) total material, (*b*) protease protected material, and (*c*) species remaining after treatment with protease in the presence of Triton-X 100. Reactions were processed and visualized as described in Materials and Methods.

Rapid Degradation of Unglycosylated Pro-Alpha Factor in the Cell-free System

To determine the rate of degradation of pαF in this system, the amounts of pαF remaining over the duration of the chase incubation were quantified, averaged, and plots displaying the rate of decay were generated (Fig. 4 *b*). Results from experiments using both approaches to generate pαF indicated that in vitro degradation of pαF was very rapid. By fitting these data to an exponential curve, a half life of 13 min was calculated for pαF. This degradation rate is similar to that of pαF in vivo, as examined by Kurjan and colleagues (Caplan et al., 1991). Based on the decay curve and secretion data presented by these investigators, we calculated the in vivo degradation half-life of pαF to be ~15 min.

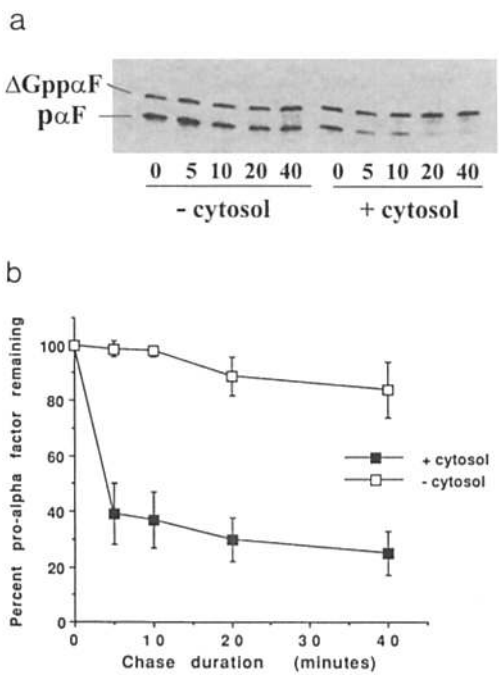

Figure 4. Cytosol-dependent degradation of pro-alpha factor produced by the translocation and processing of ΔGppαF. (*a*) Translocation reactions containing wild-type yeast microsomes and radiolabeled ΔGppαF were followed by 0-, 5-, 10-, 20-, and 40-min chase incubations of washed microsomes resuspended in buffer or buffer containing cytosol (+ *cytosol*). All lanes represent total TCA precipitated material from complete reactions (i.e., as in Fig. 3, lane *1*). Note the disappearance of pαF in the samples that contain cytosol and the stable presence of pαF in the samples without cytosol supplement (− *cytosol*). (*b*) Relative amounts of pαF were determined by volume analyses of the phosphorimages using the BioRad Phosphor Analyses program. Results from five experiments were averaged and presented with standard error bars.

Characterization of Factors Required for ERAD

Because ERAD might require the activity of calcium-activated proteases and since the normally elevated concentrations of lumenal calcium might have been lost upon microsome formation, we examined whether calcium could replace the cytosol-dependence of ERAD in vitro. In addition, the ability of DTT to support cytosol-free ERAD in microsomes was examined because it was previously observed that DTT enhanced the rate of ERAD in permeabilized cells (Stafford and Bonifacino, 1991). Thus, experiments were performed in which 1.8 mM Ca^{2+}, 0.5 mM DTT, and/or cytosol were added to the chase incubation samples. Neither Ca^{2+} nor DTT alone were able to replace the cytosol dependence of ERAD, and the addition of DTT or Ca^{2+} did not have an enhancing effect on the cytosol dependent degradation of pαF (data not shown). However, it is noteworthy that our cytosol preparations were performed in the presence of DTT (see Materials and Methods).

To determine if a protein factor of the cytosol was required for ERAD, three experiments were performed in which heat-inactivated or trypsin-treated cytosol was used to analyze ERAD (see Materials and Methods). Results from these experiments showed that 94 ± 6% of the pαF remained after a 20-min chase when supplemented with heat inactivated cytosol, 66 ± 9% of pαF remained after a 20-min incubation with trypsin treated cytosol, while 20 ± 6% of pαF was recovered when untreated cytosol was used. The fact that residual ERAD occurred with trypsin-treated cytosol might indicate that the required cytosolic component(s) is partially protease-resistant. We concluded from these results that at least one protein component in the cytosol was required for ERAD in vitro.

We also evaluated in vitro ERAD in the presence of ATPγS, a nonhydrolyzable analogue of ATP. Studies in permeabilized cells have produced conflicting results regarding the role of ATP in this process (Leonard and Chen, 1988; Megis and Simoni, 1992; Stafford and Bonifacino, 1992). We found that the addition of 1 mM ATPγS to the chase incubations efficiently inhibited the degradation of pαF; 91 ± 3% of pαF remained in ATPγS treated microsomes after a 20-min chase compared to 20 ± 6% remaining when an ATP regeneration mix was added to the chase incubation (also see Fig. 7). These results suggested a requirement for ATP hydrolysis, a property not tested in the permeabilized cell system.

Unglycosylated Pro-Alpha Factor Is Extruded from Microsomes

Neither the responsible protease(s) nor the precise intra-

cellular location for ERAD have been identified. To test the possibility that pαF was exported to the cytosol for proteolysis, we included a centrifugation step after the chase reaction to separate microsomes from cytosol. A small amount of pαF was observed in the supernatant after a 25-min chase in the absence of cytosol or in the presence of heat inactivated cytosol and ATP (Fig. 5 and Table I). However, the percent of pαF in the cytosol fraction increased approximately threefold when the chase incubation reaction included cytosol and ATP. Glycosylated pro-alpha factor, generated using wild-type ppαF in the translocation reaction, was not observed in the supernatant fraction in the presence or absence of cytosol (Fig. 5).

Because our ERAD chase conditions may support vesicular budding from the ER (Wuestehube and Schekman, 1992), pαF could be exported by vesicular transport. To test this possibility we analyzed ERAD in microsomes prepared from a budding-defective *sec12* strain (C. Barlowe, personal communication). In a 30-min ERAD assay, 30 ± 8% of pαF remained in microsomes prepared from an isogenic wild-type strain and 42 ± 8% of pαF was evident in the *sec12* microsomes. These results indicated that ERAD occurs independent of vesicle budding. Furthermore, microsomes from both strains exhibited cytosol/ATP-enhanced release of pαF into the supernatant during the chase period (Table I). We also observed that the pαF in the supernatant was trypsin sensitive (Fig. 5), indicating further that the substrate was not sequestered in transport vesicles. Together, these results suggest that ER-associated degradation of pαF occurs in the cytoplasm.

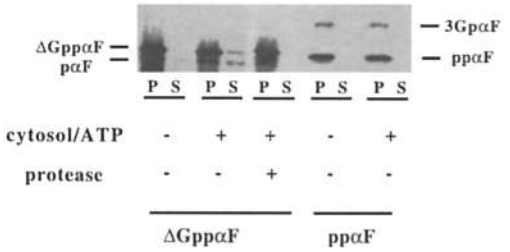

Figure 5. Unglycosylated pro-alpha factor is extruded from yeast microsomes. Either wild-type prepro-alpha factor (*ppαF*) or prepro-alpha factor lacking its core glycosylation consensus sites (*ΔGppαF*) was translocated into yeast microsomes and the membranes were isolated as described (Materials and Methods). After a 25-min chase reaction either containing or lacking cytosol and an ATP regeneration system, microsomes were again harvested and the pellets (*P*) and supernatants (*S*) were analyzed as described (Materials and Methods). For the protease-treated reactions, the final pellet was first resuspended in buffer and the resulting pellet solution and supernatant were treated with 0.2 mg/ml trypsin for 30 min on ice. In this experiment, only 13% of the pαF was found in the supernatant in the absence of cytosol/ATP, while 36% of the pαF was present in the supernatant in the presence of cytosol/ATP. Triply glycosylated pαF (*3GppαF*) was absent from supernatant fractions even upon over-exposure of the gel (data not shown). As displayed in this figure, the migrations of ΔGppαF and wild-type ppαF differ slightly in 18% polyacrylamide/4M urea gels. The appearance of ppαF in the supernatant fraction in this experiment represents adhering untranslocated precursor and was not reproducibly observed.

Calnexin Plays a Role in ERAD

We next sought to discover that microsomal factors might be required for ERAD in our assay. A likely class of proteins is the molecular chaperones, since they have been implicated in maturation and quality control of proteins in the ER (reviewed in Gething and Sambrook, 1992) and are known to mediate selective targeting of protein substrates for degradation in both mitochondria and *E. coli* (Gottesman and Maurizi, 1992; Craig et al., 1993; Sherman and Goldberg, 1993; Kandror et al., 1994; Wagner et al., 1994). To test the possibility that ER-localized molecular chaperones may be required for ERAD, we studied three mutant yeast strains (*Δeug1*, *Δscj1*, and *Δcne1*), each with a gene disruption of an ER molecular chaperone.

The *EUG1* gene in yeast encodes a soluble ER resident protein with some sequence identity to protein disulfide isomerase (PDI). In *S. cerevisiae*, both PDI and Eug1p have been shown to be functionally related and are thought to interact with nascent proteins in the yeast ER (Tachibana and Stevens, 1992). Scj1p, a yeast homologue of the *E. coli* DnaJ chaperone, resides in the ER and might modulate the activity of BiP, the ER luminal Hsp70 homologue (Blumberg and Silver, 1991; Schlenstedt et al., 1995). We discovered that translocation of ΔGppαF into microsomes prepared from wild-type and null strains was equally efficient, and that there was no difference in the rate or extent of degradation of pαF (Fig. 6). These results indicated that neither Eug1p nor Scj1p was required for ERAD.

We next examined ERAD in microsomes from a mutant strain with a disruption of the yeast calnexin gene, *CNE1* (Parlati et al., 1995). Calnexin plays a quality control role in the ER, assisting during initial folding of nascent proteins and retaining transport-incompetent misfolded proteins and incompletely assembled protein complexes in the ER (reviewed in Bergeron et al., 1994). Although calnexin has not previously been shown to participate in ERAD, this chaperone is suitably positioned to target abnormal proteins for degradation. We discovered that degradation of pαF in *Δcne1* microsomes was slower and less complete than in wild-type *CNE1* microsomes (Fig. 7). These results indicated a role for calnexin in ERAD.

Discussion

We have developed a cell-free assay to study ERAD using components from the yeast *S. cerevisiae*, and have demonstrated that a protein factor(s) of the cytosol and ATP hydrolysis are essential for ERAD. The ability of cytosol to activate a process that occurs within the lumen of the ER is not without precedent. For example, a peptide transporter in the yeast microsomal membrane is activated by the addition of exogenous cytosol (Römisch and Schekman, 1992). However, in permeabilized cell assays that measured ERAD, neither exogenous cytosol nor ATP was required for the degradation of Tac-TCRα chimera (Stafford and Bonifacino, 1991), asialoglycoprotein receptor (Wikstrom and Lodish, 1992), nor HMG CoA reductase (Megis and Simoni, 1992), although, an ATP-dependent degradation of HMG CoA reductase in permeabilized cells has been reported (Leonard and Chen, 1987). The finding that

Table I. ERAD Conditions Support Extrusion of pαF from Microsomes

	Chase Conditions*							
	Buffer‡		Cytosol/ATP		Buffer/ATP		HI cytosol/ATP	
Membranes	P	S	P	S	P	S	P	S
RSY607 wt	88 ± 0	12 ± 0	63 ± 5	37 ± 5	81 ± 6	19 ± 6	90 ± 4	10 ± 4
SEC12 wt	89 ± 3	11 ± 3	66 ± 2	34 ± 2	ND		ND	
sec12	87 ± 6	13 ± 6	74 ± 3	26 ± 3	ND		ND	

*Chase conditions included: buffer, buffer 88 without ATP regeneration system; cytosol/ATP, 5 mg/ml cytosol in buffer 88 plus ATP regeneration system; buffer/ATP, buffer 88 plus ATP regeneration system; HI cytosol/ATP, heat-inactivated cytosol plus ATP regeneration system.

‡Values are expressed as mean percent ± SD of total paF present in the pellet (*P*) and supernatant (*S*) after a 25-min chase incubation, for at least two independent experiments (each performed in duplicate). Microsomes were prepared from the indicated strains, assayed for in vitro ERAD, and micro-centrifuged (12,000 rpm) at 4°C for 2 min after the chase incubation to separate the microsomes from the cytosol. Supernatant and pellet samples were each TCA precipitated, resolved by SDS-PAGE, and quantified as described in Materials and Methods.

ERAD occurs in permeabilized cells in the absence of exogenous ATP and cytosol should not be interpreted as evidence that ERAD does not require these components, since residual ATP and cytosolic factors may remain in permeabilized cells but become lost during microsome preparation. A more accurate assessment of the potential role of ATP and cytosolic factors in the degradative process itself may be possible in a cell-free system. The development of this in vitro assay thus provides a method for the molecular dissection of the ERAD process.

How might cytosolic factors function in ERAD? It could be argued that the cytosol preparation contains proteases that selectively degrade pαF but not glycosylated pro-alpha factor. This hypothesis requires that the microsomes become permeable to proteases during the chase incubation, or that translocated, protease-protected pαF is selectively pumped out of the microsomes for degradation in the cytosol. To minimize vacuolar protease contamination, cytosol was prepared from a strain containing mutations in three vacuolar protease genes (see Materials and Methods). To directly address the possibility that microsomes are permeable to proteases, we measured the integrity of the microsomal membrane by determining whether pαF or glycosylated pro-alpha factor became accessible to exogenously added trypsin. As shown in Fig. 1 (lane *b*), these products are not degraded by trypsin even after the 90-min chase period. In addition, microsomes supplemented with cytosol are not permeable to proteases as demonstrated by the stability of glycosylated pαF in trypsin treated samples (Fig. 2, lane *b*).

The second possibility, that pαF is released from microsomes for degradation in the cytosol, is supported by our results that show pαF is exported to the cytosol via a selective transport process that is activated by conditions that support ERAD and glycopeptide export from the ER, i.e., cytosol and ATP (see Table I and Fig. 5; Römisch and Schekman, 1992). Furthermore, we have evidence that mutations in genes encoding a chymotrypsin-like subunit of the yeast proteasome complex (Heinemeyer et al., 1991) inhibit degradation of ERAD substrates in vivo and in vitro (Werner and McCracken, manuscript in preparation). Together, these data implicate the cytosol as the site of degradation.

Our results also demonstrate that yeast calnexin has a

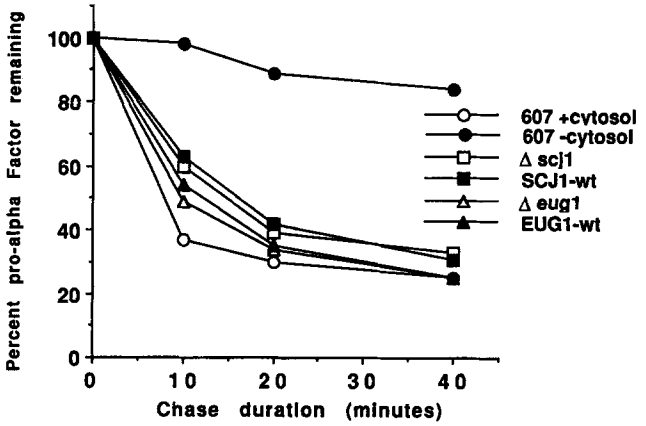

Figure 6. Decay curve of pro-alpha factor in microsomes prepared from wild-type and mutant strains with disrupted *SCJ1* or *EUG1* genes. Translocation reactions with either wild-type (*SCJ1-wt*, *EUG1-wt*) or mutant microsomes containing gene disruptions (*Δscj1*, *Δeug1*) were followed by chase incubations supplemented with cytosol. Pro-alpha factor was visualized and quantified by phosphorimage analyses as described in Materials and Methods. Averaged data of three or more experiments are presented and the standard error ranged from ±4 to ±10%.

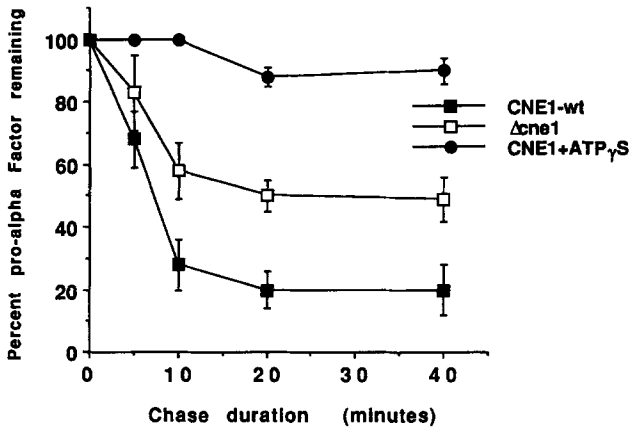

Figure 7. Decay curve of pro-alpha factor in microsomes prepared from wild-type and mutant strains with a disrupted *CNE1* gene. An isogenic wild-type strain (*CNE1-wt*) and a mutant strain containing a disrupted *CNE1* gene (*Δcne1*) were analyzed for ERAD as previously described in Materials and Methods. Cytosol was added to all chase incubation samples containing either the ATP regeneration system or 1 mM ATPγS (*CNE1 + ATPγS*). Averaged data of three or more experiments is presented ±SE.

role in ERAD. How might calnexin function in ERAD? Thomas and colleagues previously demonstrated that calnexin acts as a constituent of the ER quality control apparatus in yeast (Parlati et al., 1995). Perhaps the calnexin mediated retention of misfolded substrates in the ER facilitates their targeting for rapid degradation. This hypothesis is supported by the evidence that two mutant proteins known to be degraded by ERAD are among those retained in the ER by interaction with calnexin: alpha-1 protease inhibitor (McCracken and Kruse, 1993; Le et al., 1994) and ΔF508 CFTR (Pind et al., 1994).

Much evidence indicates that molecular chaperones interact with specific partner proteins that specialize their functions for protein trafficking, protein translocation, gene expression, and proteolysis (reviewed in Rassow et al., 1995). It is conceivable that such a partner may specialize calnexin function for a role in ERAD, or that calnexin may act as a partner for another chaperone. An example of such coordinate interactions leading to degradation of misfolded proteins is seen in the mitochondria. The ATP-dependent proteolytic degradation of misfolded proteins in the mitochondrial matrix by the PIM1 protease is mediated by the mitochondrial molecular chaperone mt-hsp70 and one of its partner proteins, Mdj1p. In the absence of Mdj1p, misfolded proteins either remain associated with mt-hsp70 or form aggregates that are not substrates for PIM1p (Wagner et al., 1994). In addition, complexes of calnexin with misfolded proteins and BiP/Grp78, an ER luminal Hsp70 family chaperone, have been observed (Hammond and Helenius, 1994). Perhaps calnexin and BiP cooperate to prevent the aggregation of aberrant proteins and mediate their degradation. The membrane location of calnexin could facilitate targeting of the aberrant proteins to a transporter for release to the cytoplasm.

It is important to note that calnexin has been shown to serve primarily as a lectin, binding specifically to glycosylated proteins (Ou et al., 1993; Hammond et al., 1994; Hebert et al., 1995). However, calnexin also binds to proteins that lack N-linked glycans (Rajagoplan et al., 1994; Kim and Arvan, 1995), and there is evidence that calnexin interacts with unfolded proteins by binding to polypeptides rather than glycan determinants (Zhang et al., 1995). Since no evidence is currently available in yeast to assess the nature of calnexin binding, the possibility exists that calnexin interacts directly with unglycosylated pαF. Studies to probe the interaction of calnexin with pαF will be required to support this premise.

In conclusion, we have reconstituted ER-associated protein degradation in vitro, and have demonstrated that protein components of the cytoplasm are required for this process. This cell-free system reveals a requirement for ATP hydrolysis and a role for calnexin in ERAD, and implicates the cytosol as the site for proteolysis of ERAD substrates. Further application of this in vitro system will allow the biochemical fractionation of cytosol to identify relevant factors, studies with protease-specific inhibitors and proteosome defective mutants to define the proteolytic activity, experiments to probe the interaction of calnexin and BiP with ERAD substrates, and the testing of ERAD-deficient mutants to characterize their defects. The research challenge is to identify additional components of ERAD, to understand how this process works, and particularly how it is controlled. We expect that answers to these questions will provide information of broad applicability, which will illuminate other areas of cellular control and intracellular trafficking of biomedically important proteins.

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