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The Use of *In Vitro* Assays to Measure Endoplasmic Reticulum-Associated Degradation

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Abstract

Approximately one-third of all newly translated polypeptides interact with the endoplasmic reticulum (ER), an event that is essential to target these nascent proteins to distinct compartments within the cell or to the extracellular milieu. Thus, the ER houses molecular chaperones that augment the folding of this diverse group of macromolecules. The ER also houses the enzymes that catalyze a multitude of posttranslational modifications. If, however, proteins misfold or are improperly modified in the ER they are proteolyzed via a process known as ER-associated degradation (ERAD). During ERAD, substrates are selected by molecular chaperones and chaperone-like proteins. They are then delivered to the cytoplasmic proteasome and hydrolyzed. In most cases, delivery and proteasome-targeting require the covalent attachment of ubiquitin. The discovery and underlying mechanisms of the ERAD pathway have been aided by the development of *in vitro* assays that employ components derived from the yeast, *Saccharomyces cerevisiae*. These assays recapitulate the selection of ERAD substrates, the “retrotranslocation” of selected polypeptides from the ER into the cytoplasm, and the proteasome-mediated degradation of the substrate. The ubiquitination of integral membrane ERAD substrates has also been reconstituted.

1. Introduction

Cells are continuously faced with various forms of stress, including altered temperature, limited nutrient availability, changes in osmotic pressure, and the presence of toxic agents. To surmount such challenges, adaptive pathways are triggered that induce the synthesis of proteins that lessen the effects of cell stress. In model eukaryotes, such as the yeast *Saccharomyces cerevisiae*, many of the stress-induced adaptive pathways have been defined, thanks to the multitude of available genetic, genomic, and biochemical tools.

Stress-responsive pathways can also be triggered from within intracellular compartments. One compartment in which this has been examined in detail is the endoplasmic reticulum (ER), which is the first organelle encountered by newly synthesized secreted proteins. In *S. cerevisiae*, nascent secreted proteins can translocate into the ER either during or soon after translation (Cross *et al.*, 2009; Rapoport *et al.*, 1999). Translocation is facilitated by a multiprotein complex that resides at the ER membrane. The key component of this complex

is an aqueous translocation pore, and the pore and its associated partners have been termed the “translocon” (Schnell and Hebert, 2003).

Concomitant with translocation, most secreted proteins are posttranslationally modified and begin to fold, which explains why the ER is stocked with molecular chaperones and enzymes that catalyze protein folding (Vembar and Brodsky, 2008). Because the acquisition of the native or near-native state is a prerequisite for the subsequent delivery of secreted proteins to their ultimate destinations, the ER also contains a rich variety of factors that monitor protein folding. Many of these “quality control” factors are molecular chaperones. In the event that proteins fail to fold, either as a result of the stresses noted above or due to genetic or stochastic errors, an ER stress response, known as the unfolded protein response (UPR), is initiated. One outcome of the UPR is an increase in the machinery that destroys aberrant secreted proteins, thus clearing the ER of potentially toxic protein conformers (Jonikas *et al.*, 2009; Travers *et al.*, 2000).

For many years, the existence of a quality control protease was sought (Vembar and Brodsky, 2008). Early data from mammalian cell systems suggested that the protease resided within the ER, and a candidate protease was eventually purified (Otsu *et al.*, 1995). However, parallel studies suggested the existence of an alternate system to degrade aberrant proteins that had entered the ER. In short, evidence emerged that misfolded ER proteins might employ the services performed by the cytoplasmic ubiquitin–proteasome system (UPS). The proteasome is a large (26S), multi-catalytic enzyme that binds and unfolds proteins and then processively degrades substrates to short peptides (Hanna and Finley, 2007; Pickart and Cohen, 2004). Nearly all proteasome-targeted substrates are modified with poly-ubiquitin, which facilitates proteasome-capture. Early evidence indicated that the UPS proteolyzes misfolded, integral membrane proteins in the ER of yeast (Hampton *et al.*, 1996; Sommer and Jentsch, 1993) and mammalian (Jensen *et al.*, 1995; Ward *et al.*, 1995) cells. These substrates were multispinning membrane proteins, which by definition contained cytoplasmic polypeptide loops; therefore, it made sense that the cytoplasmically localized UPS might recognize and destroy misfolded membrane proteins. These data were also consistent with the established function of the UPS in mediating cytoplasmic protein quality control (Sherman and Goldberg, 2001). What remained unknown was how soluble misfolded proteins within the ER lumen were destroyed.

To answer this question, we developed an *in vitro* system that monitored the fates of an ER-localized wild-type secreted protein and a secreted protein that was unable to acquire N-linked oligosaccharides (McCracken and Brodsky, 1996; Werner *et al.*, 1996). Our establishment of this assay built-upon the pioneering *in vitro* yeast systems developed in the Walter, Blobel, Meyer, and Schekman labs that had been co-opted to follow the translocation of nascent secreted proteins into the yeast ER (Deshaies and Schekman, 1989; Hansen *et al.*, 1986; Rothblatt and Meyer, 1986; Waters and Blobel, 1986). In our system, the wild-type substrate was the alpha mating type prepheromone, pre-pro-alpha factor (ppαF). Upon translocating into the ER, ppαF is processed by the signal sequence peptidase, generating pro-alpha factor (pαF). PαF is then triply glycosylated, which generates GpαF (Julius *et al.*, 1984). This substrate is competent for ER-exit through the action of Golgi-targeted COPII vesicles (Belden and Barlowe, 2001). In contrast, the mutated substrate

cannot be *N*-glycosylated so that p α F is the terminal species that forms within the ER (Fig. 27.1). These substrates were chosen for the following reasons. First, wild type and mutant forms of p α F posttranslationally translocate into the ER *in vitro* (Hansen *et al.*, 1986; Rothblatt and Meyer, 1986; Waters and Blobel, 1986). This feature allowed for the large-scale synthesis and isolation of radiolabeled substrate, which could then be aliquotted and stored prior to use. Second, p α F appeared to be degraded within the yeast secretory pathway (Caplan *et al.*, 1991), indicating the existence of a secretory protein quality control system for soluble proteins. Finally, p α F-derivatives were also degraded in mammalian cell lines (Su *et al.*, 1993). Thus, a dissection of the p α F biogenic pathway might lead to the elucidation of a conserved quality control machinery.

In 1996, we reported that p α F could be selectively exported—or retrotranslocated—from ER-derived vesicles back to the cytoplasm (McCracken and Brodsky, 1996). Once in the cytoplasm, p α F was destroyed by the proteasome (Werner *et al.*, 1996). However, Gp α F, which derived from the wild-type precursor (Fig. 27.1), was stable. Based on our results, we named this process ER-associated degradation (ERAD) (McCracken and Brodsky, 1996). In parallel to our efforts, Wolf and colleagues established that another mutated secreted protein, CPY*, was also degraded by the proteasome in yeast (Hiller *et al.*, 1996). Collectively, these data indicated that integral membrane and soluble proteins in the ER were both handled by the UPS (Vembar and Brodsky, 2008). In fact, a subsequent modification of our assay established that the proteasome was necessary and sufficient to retrotranslocate and degrade p α F (Lee *et al.*, 2004). Moreover, the use of ER-derived vesicles, or “microsomes” from mutant strains allowed our laboratory and others to identify the ER luminal chaperones required for p α F degradation (Brodsky *et al.*, 1999; Gillece *et al.*, 1999; Kabani *et al.*, 2003; Lee *et al.*, 2004; McCracken and Brodsky, 1996; Nishikawa *et al.*, 2001). The *in vitro* system was also employed by Römisch and Schekman to provide evidence suggesting that the translocon might serve as the conduit for retrotranslocation (Pilon *et al.*, 1997). Each of these *in vitro* assays is described in detail, in Section 2.

Do integral membrane proteins also retrotranslocate and become solubilized from the ER membrane prior to proteasome-mediated destruction? Data obtained from mammalian cell systems suggested that this might be the case. First, Kopito and colleagues reported that cytoplasmic “aggresomes” accumulated in mammalian cells expressing high levels of a misfolded membrane protein that were simultaneously challenged with proteasome inhibitors (Johnston *et al.*, 1998). The aggresomes contained the misfolded substrate and components of the UPS (Johnston *et al.*, 1998; Wigley *et al.*, 1999). Second, Ploegh and coworkers reported that a human cytomegalovirus gene product catalyzed the “dislocation” of the major histocompatibility class I molecule into cytoplasmic fractions in transfected cells (Wiertz *et al.*, 1996). Because these phenomena were only evident in mammalian cells, further attempts to elucidate the mechanism of membrane protein retrotranslocation have had to rely on pharmacological and RNAi-related technologies.

To better define the pathway by which integral membrane proteins are selected and retrotranslocated for ERAD, we developed an *in vitro* assay in which each step in the degradation pathway could be dissected (Nakatsukasa *et al.*, 2008). The components for this assay—ER-derived microsomes and concentrated cytosol—were again isolated from *S.*

cerevisiae. The use of this assay led to the following discoveries. First, we observed that Hsp70 and Hsp40 molecular chaperones help link ERAD substrates to E3 ubiquitin ligases. Second, we found that the E3s required for ERAD exhibit functional redundancy. Third, we were able to evoke the ATP- and cytosol-dependent retrotranslocation of a polytopic integral membrane protein into the cytosolic fraction. Fourth, we determined that membrane protein extraction required the Cdc48p complex, which was previously found to play an important role in ERAD (Jentsch and Rumpf, 2007). Fifth, we established that a cytoplasmic polyubiquitin extension enzyme, or “E4,” elongated the polyubiquitin chain on the ERAD substrate and was required for maximal rates of substrate degradation. And sixth, we confirmed that the solubilized substrate was competent for proteasome-dependent degradation. These discoveries were made possible through the use of ER-derived microsomes and cytosol that were prepared from yeast containing specific loss-of-function and thermosensitive mutant alleles. The assays that led to these discoveries are described in Section 3.

2. *In Vitro* ERAD Assays Using a Soluble Substrate, p α F

In this section, first, the isolation of the materials required to monitor the degradation of p α F is described. Next, the assays for p α F retrotranslocation and ERAD are detailed. Throughout the section, comments are added to note how reagents prepared from mutant strains have been used to better define the ERAD pathway.

2.1. Materials

2.1.1. Microsome preparation—The preparation of ER-derived microsomes from *S. cerevisiae* has been well documented (Deshaies and Schekman, 1989; Rothblatt and Meyer, 1986) but is described in outline form with minor revisions, below. When temperature sensitive mutants are employed, the cultures are grown at a permissive temperature and are then shifted to the restrictive temperature (i.e., 37 °C). Although thermosensitive phenotypes can be recapitulated *in vitro*, the growth period at the nonpermissive temperature needed to obtain the mutant defect in the assay must be determined empirically and can range from <20 min to 5 h (Becker *et al.*, 1996; Brodsky and Schekman, 1993; Brodsky *et al.*, 1993; Latterich *et al.*, 1995; Nakatsukasa *et al.*, 2008). Smaller scale isolations of microsomes have also been used in ERAD studies (Nakatsukasa *et al.*, 2008), but the translocation efficiency is somewhat lower (our unpublished data).

1. Yeast are grown at the desired temperature in rich medium and with vigorous shaking until the culture reaches mid-log to late-log phase (optical density at 600 nm [OD₆₀₀] of 2.0–3.0). We typically grow 1–2 l of yeast for a microsome preparation.
2. The cell walls are digested with a β -1,3-glucanase hydrolyzing enzyme that either can be purified from recombinant *Escherichia coli* that express the enzyme (Shen *et al.*, 1991) or purchased commercially (e.g., Zymolyase, from MP BioMedicals).
3. The resulting spheroplasts are collected by centrifugation through 20 mM HEPES, pH 7.4, 0.8 M sucrose, 1.5% Ficoll 400 at 6000 rpm, in an HB-6 swinging bucket rotor. It is critical that each of the following steps is performed at 4 °C.

4. The spheroplasts are resuspended to a final OD₆₀₀ of 100/ml in 20 mM HEPES, pH 7.4, 0.1 M sorbitol, 50 mM KOAc, 2 mM EDTA, and a protease inhibitor cocktail, and the solution is transferred to a tight-fitting, Teflon-glass homogenizer that can be driven with a motor.
5. The plasma membrane is then broken by 10 strokes with the motor running at the highest setting.
6. The broken cellular material is layered onto a cushion that contains 20 mM HEPES, pH 7.4, 1.0 M sucrose, 50 mM KOAc, 1 mM DTT.
7. After centrifugation at 6500 rpm in an HB-6 swinging bucket rotor, the crude microsomal fraction is resuspended in an equal volume of B88 (20 mM HEPES, pH 6.8, 250 mM sorbitol, 150 mM KOAc, 5 mM MgOAc).
8. The microsomes are collected by centrifugation at ~15,000×g for 10 min, resuspended in B88, and recentrifuged.
9. After final resuspension in a small volume of B88, the microsome concentration is adjusted such that the OD₂₈₀ should equal ~40 when a small aliquot of a 1:10 dilution of the resuspended material is assessed in 2% SDS.
10. Microsomes aliquots (~50 µl) are frozen and stored at -80 °C.

2.1.2. Isolation of GppaF, the precursor of a soluble ERAD substrate, and ppaF, the wild-type control—The substrates required for the *in vitro* ERAD assay are the wild-type ppaF control (which is encoded by the MFa1 locus; Kurjan and Herskowitz, 1982) and a form of ppaF that contains site-directed mutations in the three sites required for the addition of *N*-linked oligosaccharides (Caplan *et al.*, 1991). We denote this mutant as

GppaF. As described above and in Fig. 27.1, the signal sequence of ppaF is removed in the ER and the resulting species becomes triply glycosylated, ultimately forming 3GppaF in the microsomes. In contrast, GppaF is converted to paF, which is an ERAD substrate in the yeast microsome-based system (McCracken and Brodsky, 1996; Werner *et al.*, 1996). PaF has also been prepared with a fluorescent tag and is retrotranslocation competent after its entrapment in dog pancreas microsomes (Wahlman *et al.*, 2007).

Using SP6 polymerase, wild-type ppaF is transcribed from plasmid pDJ100 and GppaF is transcribed from plasmid pGEM2alpha36. We next isolate the messages encoding ppaF and

GppaF and perform an *in vitro* translation reaction in the presence of ³⁵S-methionine and concentrated, gel-purified yeast lysate to obtain radiolabeled ppaF and GppaF (Lee *et al.*, 2004; McCracken and Brodsky, 1996; Werner *et al.*, 1996). The logic underlying this protocol is that maximal ppaF translocation efficiency requires factors in the yeast lysate—presumably binding stably to ppaF—that are absent in other translation-competent lysates (Chirico *et al.*, 1988; Deshaies *et al.*, 1988).

More recently, we have employed the Promega TnT SP6 Coupled Reticulocyte Lysate System to synthesize radiolabeled ppaF and GppaF and other substrates (Hrizo *et al.*, 2007), and have discovered that the resulting products translocate efficiently into yeast microsomes. In brief, each plasmid template is mixed on ice with the commercial buffer,

ribonuclease inhibitor, SP6 polymerase, an amino acid mixture (lacking met), and the supplied rabbit reticulocyte lysate. The reaction is then supplemented with 20 μ Ci of 35 S-labeled amino acid (PerkinElmer EXPRE 35 S 35 S Protein Labeling Mix). A 50- μ l (total volume) reaction is typically performed according to the manufacturer's instructions at 30 °C for 90 min. Single-use aliquots are then flash frozen and stored at –80 °C.

2.1.3. Yeast cytosol—The preparation of concentrated yeast cytosol using liquid nitrogen was first described by Sorger and Pelham (1987) and modified for the ERAD assay as described (McCracken and Brodsky, 1996). Strains containing temperature sensitive mutants can be used to prepare cytosol but again the conditions required to recapitulate temperature sensitive defects *in vitro* must be determined empirically.

1. Yeast cells are grown with vigorous shaking in rich medium to log phase ($OD_{600} = \sim 2.0$) at 30 °C or at the desired alternate temperature(s). In our experience, at least 6 l of culture are needed for efficient lysis.
2. The cells are collected by centrifugation, resuspended in water, recentrifuged, and resuspended in a minimal amount of B88. Typically, we use <5 ml of B88 for the number of cells obtained from a 6 l yeast culture.
3. The cells are added slowly to 500 ml of liquid nitrogen in a tripour plastic beaker. After the yeast are frozen, the liquid nitrogen is decanted and the cells are stored at –80 °C.
4. Approximately 500 ml of liquid nitrogen is added to a stainless-steel blender, followed by the frozen yeast. The blender is initially turned-on at the lowest setting, but the blade speed is soon increased to the highest setting. The volume must be maintained above the rotating blades by the periodic addition of liquid nitrogen. To prevent spills, the blender must be covered as often as possible with a lid that can withstand low temperatures (e.g., a thick Styrofoam slab).
5. After 10 min, the blender is turned-off, the liquid nitrogen is evaporated, and the resulting powder is transferred to a 50-ml plastic tube, which can be stored –at 80 °C.
6. A minimal amount of B88 (e.g., ~0.5 ml/40 ml of broken yeast) is added as the lysate begins to thaw at room temperature or on ice, and freshly prepared DTT is added to a final concentration of 1 mM.
7. The lysate is centrifuged at 10,000 $\times g$ for 10 min at 4 °C, and the supernatant is collected and recentrifuged.
8. The second supernatant is centrifuged at 300,000 $\times g$ for 1 h at 4 °C and is aliquoted, frozen in liquid nitrogen, and stored at –80 °C. A Bio-Rad protein assay is used to determine the protein concentration. In our experience, cytosols at >20 mg/ml work best in ERAD assays as long as they are not thawed and refrozen.

2.1.4 ATP regenerating system—Optimal ERAD efficiency requires an ATP regenerating system. To this end, a 10 $\times$ stock is made up as follows:

- 10 mM ATP
- 500 mM creatine phosphate
- 2 mg/ml of creatine phosphokinase
- B88 to volume

The solution is then distributed into single-use aliquots, frozen in liquid nitrogen, and stored at -80°C . Reactions lacking the addition of the ATP regenerating system may support a low level of retrotranslocation/ERAD and ubiquitination. Thus, to fully decipher the ATP-dependence of the following reactions, we have performed incubations with either ATP γ S (Lee *et al.*, 2004) or apyrase (Nakatsukasa *et al.*, 2008) in place of the regenerating system.

2.2 The *in vitro* degradation assay for p α F

Prior to examining the retrotranslocation and degradation efficiencies of p α F, the precursor to this substrate and the precursor to the wild-type control, 3Gp α F, must be introduced into ER-derived microsomes through an *in vitro* translocation assay. The resulting microsomes are then reisolated to examine the degradation efficiency and retrotranslocation steps during the ERAD of a soluble substrate.

2.2.1. Translocation of p α F and Gpp α F into yeast microsomes

1. A translocation reaction is set up in a microcentrifuge tube on ice. Most commonly, the 60 μl reaction contains 45 μl of B88, 6 μl of the 10 $\times$ ATP regenerating system, 5 μl of yeast microsomes, and 4 μl of ^{35}S -labeled Gpp α F or p α F (or the appropriate volume to obtain $\sim 300,000$ cpm per reaction). The reaction is mixed gently and then incubated for 1 h in a 20°C water bath.
2. Following the incubation, the solution is centrifuged at $\sim 16,000\times g$ for 3 min at 4°C .
3. After the tubes are placed on ice, the supernatant is removed with a gel-loading tip. Care must be taken not to disturb the pellet.
4. The pellet is gently resuspended in 60 μl of ice-cold B88 and the solution is recentrifuged, as above.
5. The supernatant is again removed and the pellet is taken up in 5 μl of ice-cold B88.

2.2.2. Reconstitution of cytosol- and ATP-dependent degradation—The following assay has been adapted to assess the contributions of a number of ER lumenal and integral membrane components on the ERAD of a soluble substrate (Brodsky *et al.*, 1999; Gillece *et al.*, 1999; Kabani *et al.*, 2003; Lee *et al.*, 2004; McCracken and Brodsky, 1996; Nishikawa *et al.*, 2001; Pilon *et al.*, 1997). The role of the proteasome during this process can be assessed either through the use of cytosol from a proteasome mutant or through the addition of proteasome inhibitors (Werner *et al.*, 1996). Moreover, the cytosol requirement can be circumvented through the addition of purified 26S proteasomes isolated from yeast or mammals (Lee *et al.*, 2004). Negative controls for this experiment include reactions

supplemented with ATP γ S or apyrase, and/or reactions lacking cytosol (Lee *et al.*, 2004; McCracken and Brodsky, 1996; Werner *et al.*, 1996).

To a microcentrifuge tube on ice, with the appropriate amount of ice-cold B88 for a final volume of 60 μ l, the following reagents are added (in order):

- 5 μ l of microsomes containing 35 S-labeled paF (a product of the translocation reaction with GppaF) or 3GpaF (a product of the translocation reaction with ppaF), prepared as described above
 - 6 μ l of the 10 $\times$ ATP regenerating system
 - An appropriate amount of yeast cytosol to obtain a final concentration of 1–3 mg/ml
1. The reaction is incubated at 30 $^{\circ}$ C for 20 min or at higher temperatures if the contributions of some temperature-sensitive mutations will be monitored (Brodsky *et al.*, 1999; Gillece *et al.*, 1999; Kabani *et al.*, 2003; Lee *et al.*, 2004; Nishikawa *et al.*, 2001; Pilon *et al.*, 1997). Multiple reactions can also be set up if a time-course will be conducted.
 2. The reaction tubes are placed on ice and 12 μ l of an ice-cold 100% TCA stock solution is added.
 3. The quenched reactions are agitated vigorously on a Vortex mixer for $\sim$ 3 s and incubated on ice for 15 min.
 4. The solutions are centrifuged at 16,000 $\times$ g for 5 min at 4 $^{\circ}$ C, and the supernatant is removed with a gel-loading tip.
 5. Sufficient ice-cold acetone is added to cover each pellet, and the mixture is again briefly agitated on a Vortex mixer and immediately recentrifuged.
 6. The acetone is removed with a gel-loading tip and the pellet is air-dried for 2–3 min.
 7. The final pellets are resuspended in SDS–PAGE sample buffer by repetitive pipetting, and the mixture is incubated for 10 min at $\sim$ 70 $^{\circ}$ C.
 8. The radiolabeled proteins are best resolved through the use of an 18% denaturing polyacrylamide gel that also contains 6 M urea. To further maximize the separation between the signal sequence-containing (i.e., GppaF, $\sim$ 18 kDa) and signal sequence-cleaved (i.e., paF, $\sim$ 16 kDa) species, which is an ERAD substrate (Fig. 27.2), we use 6 cm gels. Once the dye front is near the bottom of the gel, the plates are disassembled, the gels are fixed and dried, and the radioactivity is visualized using a phosphorimager. A 2- to 3-day exposure is usually sufficient.
 9. To quantify the amount of degradation, we consider only the translocated paF (i.e., the paF species in the “-cytosol” control at $t = 0$ min; Fig. 27.2). Thus, the amount of paF remaining under conditions that promote degradation should be averaged and compared to the average amount of

material in control reactions that lack ATP and/or cytosol. The wild-type substrate (i.e., 3GpaF, ~28 kDa) should be stable regardless of the assembled reaction conditions.

2.3. The paF retrotranslocation assay

The degradation assay, described above, can be modified to monitor the retrotranslocation of paF from yeast ER microsomes (Fig. 27.3). The translocation of wild-type ppaF, which forms 3GpaF, serves as a negative control (i.e., 3GpaF should remain in the microsome fraction). The inclusion of ATP γ S instead of the ATP-regenerating system also serves as a negative control. In addition, the assay can be used to assess retrotranslocation efficiency upon the addition of purified cytoplasmic proteins, such as the proteasome, the 19S proteasome “cap,” Cdc48p, and purified chaperones (Lee *et al.*, 2004). Of note, the retrotranslocated paF is protease sensitive, indicating that it is not encapsulated in ER-derived vesicles (Fig. 27.3) (McCracken and Brodsky, 1996).

1. Translocation reactions (60 μ l) are assembled as described above.
2. The microsomes are harvested, washed, and used in the degradation assay containing B88, the ATP-regenerating system, and yeast cytosol, as presented in the preceding section.
3. The reaction is incubated for 20 min at 30 °C or higher temperatures in the event that reagents from temperature sensitive mutant strains are being examined. The initial rate of retrotranslocation can also be examined by taking earlier time points (e.g., 5 and 10 min).
4. The microsomes are pelleted in a refrigerated microcentrifuge at 16,000 $\times$ *g* for 3 min.
5. The tubes are returned to ice and the supernatant is quickly removed with a gel-loading tip and placed in a prechilled fresh tube. The supernatant will contain the retrotranslocated paF. Care must be taken not to disturb the pellet.
6. Twelve microliters of ice-cold 100% TCA is immediately added to the supernatant.
7. This solution is agitated on a Vortex mixer for ~3 s and then incubated at 4 °C for 15–20 min.
8. During this time, the pellet is resuspended in 60 μ l B88 and 12 μ l of 100% ice-cold TCA is added. The solution, which contains micro-some-retained paF, is also agitated and incubated at 4 °C for 15 min.
9. After 15–20 min, each of the samples (the supernatant/cytosol and pellet/microsomes) is centrifuged at 16,000 $\times$ *g* in a refrigerated micro-centrifuge, and the supernatants are removed with a gel-loading tip.
10. Sufficient ice-cold acetone is added to cover the pellets, and the solution is briefly agitated on a Vortex mixer.
11. The samples are immediately recentrifuged, as above, and the supernatant is removed with a gel loading tip.

12. The pellets are air-dried for 2–3 min and resuspended in SDS–PAGE sample buffer by repetitive pipetting.
13. The solution is incubated for 10 min at ~70 °C, and the products are resolved on 6 M urea–18% denaturing polyacrylamide gels, as described above.
14. After the gels are fixed and dried, and the radioactive species are visualized using a phosphorimager, the amount of pαF in the supernatant and pellet are summed to establish the total pαF in the reaction. Then, the amount of pαF in the supernatant is divided by the total pαF to calculate the percentage of pαF retrotranslocated to the supernatant. These values are averaged among triplicate experiments. Most commonly, the amount of pαF exported in the negative controls is <10%. For the data shown in Fig. 27.3, 36% of pαF was retrotranslocated in the presence of cytosol/ATP.

3. *In Vitro* Assays for Integral Membrane Proteins that are ERAD Substrates

To better define the ERAD pathway taken by integral membrane proteins in the yeast ER, we developed a system in which the selection, ubiquitination, retrotranslocation, and degradation of Ste6p*, a mutated form of the Ste6p *a*-mating factor transporter, could be followed. Ste6p* was chosen because the genetic requirements for the degradation of this integral membrane protein were relatively well defined (Huyer *et al.*, 2004; Loayza *et al.*, 1998; Vashist and Ng, 2004). In addition, the mutation in STE6 (Q1249X) results in a C-terminally truncated form of the protein; therefore, the quality control “decisions” that trigger the destruction of Ste6p* are made posttranslationally. In principle, this simplifies the machinery required for the ERAD of the substrate, and excludes the contribution of cotranslational (i.e., ribosome-associated) factors. Finally, Ste6p* is a member of the large ABC family of transporters, which includes the cystic fibrosis transmembrane conductance regulator (CFTR): The topologies of Ste6p* and CFTR are identical and the proteins share domain-restricted sequence homology. Previous work had also established many of the genetic requirements for the ERAD of CFTR after its heterologous expression in yeast (Ahner *et al.*, 2007; Gnann *et al.*, 2004; Youker *et al.*, 2004; Zhang *et al.*, 2001). Consequently, microsomes prepared from yeast strains expressing either Ste6p* or CFTR can be employed for many of the assays described below (Nakatsukasa *et al.*, 2008).

3.1. *In vitro* ubiquitination assay

3.1.1. Isolation of yeast microsomes containing integral membrane ERAD substrates—Yeast expressing HA-tagged versions of Ste6p* (Huyer *et al.*, 2004) or CFTR (Zhang *et al.*, 2001, 2002) have been described previously, and ER-derived microsomes are prepared from these strains as presented above. The only difference is that microsomes are isolated from strains expressing the ERAD substrate; therefore, yeast are grown in selective media.

When one wishes to recapitulate a temperature-sensitive mutant defect in these assays, a more rapid, smaller scale microsome preparation should be used (Nakatsukasa *et al.*, 2008). The small-scale preparation minimizes the elapsed time between the *in vivo* temperature shift and the use of the isolated reagents in the following assays. An appropriate negative

control for each reaction is the preparation of yeast microsomes from a strain lacking the plasmid for the expression of the ERAD substrate, but that instead contains the expression plasmid without an insert.

3.1.2. Yeast cytosol—The cytosol required for the following experiments is prepared as described in Section 2.1. When the phenotypes associated with temperature-sensitive mutants are to be recapitulated, the *in vivo* shift to the nonpermissive temperature must be determined empirically, and can range substantially (also see above).

3.1.3. Preparation of ^{125}I -labeled ubiquitin—Bovine ubiquitin (Sigma) is dissolved in phosphate-buffered saline at a final concentration of 10 $\mu\text{g}/\text{ml}$. The protein is then labeled with ^{125}I (NEN Research, BioRad) using the ICL method (Helmkamp *et al.*, 1960; McFarlane, 1958). The labeled ubiquitin is enriched and unincorporated ^{125}I is removed with a *D*-salt Excellulose Desalting column (Pierce). The final, isolated product is stored in 20 μl aliquots at -80°C at a final concentration of 0.2 $\mu\text{g}/\text{ml}$ ($\sim 1.0 \times 10^6$ cpm/ μl). The reagent must be used within 2 months after its preparation.

3.1.4. The ubiquitination reaction—The following reagents are combined into the appropriate volume of B88 (total volume, 18 μl) on ice:

- 2 μl of yeast microsomes containing the HA-epitope-tagged integral membrane ERAD substrate
- 2 μl of the 10 ATP regenerating system (see Section 2.1)
- Sufficient yeast cytosol to achieve a final concentration of 1–4 mg/ml

The reaction can also be supplemented with apyrase, which serves as a “-ATP” control, or methylated ubiquitin, which inhibits ubiquitin chain extension (Hershko and Heller, 1985) (Fig. 27.4). As noted above, micro-somes lacking the ERAD substrate serve as another negative control, as can reactions lacking cytosol.

1. The mixture is preincubated in a 23°C water bath for 10 min before 2 μl of ^{125}I -labeled ubiquitin are added.
2. The incubation is then continued for up to 1 h at 23°C .
3. At the desired time point, 80 μl of an SDS stop solution (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1.25% SDS, 1 mM PMSF, 1 $\mu\text{g}/\text{ml}$ leupeptin, 0.5 $\mu\text{g}/\text{ml}$ pepstatin A, 10 mM NEM) are added.
4. The solution is briefly agitated on a Vortex mixer and then incubated at 37°C for 30 min.
5. In preparation for an immunoprecipitation, 400 μl of 50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 2% Triton X-100 is added and the solution is mixed gently and placed on ice.
6. A 2 μl aliquot ($\sim 10 \mu\text{g}$) of anti-HA antibody (Roche) is added and the immunoprecipitation reaction is incubated overnight at 4°C with mild agitation.

7. A 30 μ l, 50% (v/v) suspension of Protein A-Sepharose (GE Health-care), equilibrated in 50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM azide, is added and the mixture is incubated at 4 °C for another 2–3 h.
8. The beads are harvested by a 10 s, low-speed centrifugation at 4 °C and are then washed four times with 800 μ l of ice-cold 50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.2% SDS, 10 mM NEM.
9. After the final wash, the residual buffer is removed with a gel-loading tip and 30 μ l of 2 $\times$ SDS-PAGE sample buffer are added.
10. The bound proteins are eluted by a 37 °C, 30 min incubation, and the supernatant is split after a brief centrifugation.
11. One-half of the supernatant (to detect 125 I-ubiquitin-modified protein) is analyzed using a 6 cm, 6% (denaturing) SDS-polyacrylamide gel. After fixation and drying, the gel is exposed to a phosphorimager plate.
12. The other half of the sample is analyzed first by SDS-PAGE but the gel is then blotted and used to detect the amount of unmodified Ste6p* with anti-HA antibody and the appropriate secondary antibody. We use the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) to visualize the signal.

3.2 Analysis of integral membrane protein retrotranslocation

The Cdc48p complex-dependent retrotranslocation of ubiquitinated Ste6p* can be followed by inserting a centrifugation step into the protocol described above. The involvement of the Cdc48p complex in this event was established through the use of cytosols prepared from a *cdc48-3* strain that had been shifted to 37 °C for 5 h and from an *npl4* strain (Nakatsukasa *et al.*, 2008). Therefore, these materials serve as a negative control in the following experiments. A TAP-tagged version of Cdc48p was also shown to coprecipitate the retrotranslocated species (Nakatsukasa *et al.*, 2008), further implicating the Cdc48p complex in Ste6p* retrotranslocation.

1. Ubiquitination reactions are set up as described in Section 3.1, except that a final volume of 25 μ l is achieved.
2. At the completion of the 1 h 23 °C incubation, the microsomes are pelleted in a refrigerated microcentrifuge at 18,000 $\times$ g for 10 min.
3. The reaction tube is returned to ice and the supernatant (~20 ml), which contains the retrotranslocated, ubiquitinated Ste6p*, is removed with a gel-loading tip and placed in a new microcentrifuge tube on ice.
4. The pelleted microsomes are resuspended in 25 μ l of ice-cold B88, and 20 μ l of this suspension is placed in a new tube.
5. To analyze the amount of ubiquitinated Ste6p* in the supernatant (cytosol) and pellet (microsome) fractions, 80 μ l of the SDS stop solution (see above) is added to the supernatant and resuspended microsomes, and the mixtures are briefly agitated on a Vortex mixer.

6. As above, the solution is incubated at 37 °C for 30 min, and then 400 μ l of 50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 2% Triton X-100 is added and placed on ice.
7. The Ste6p* in both fractions is immunoprecipitated with anti-HA antibody/Protein A-Sepharose and analyzed under denaturing conditions via SDS-PAGE.

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Fig. 27.1.

The early biogenic pathways utilized by wild-type ppaF and GppaF, a soluble ERAD substrate. Upon translocation into the ER, the signal sequence in ppaF is liberated and the protein becomes triply glycosylated. The resulting species, 3GpaF, is stable in yeast ER-derived microsomes. In contrast, the three sites required for the addition of *N*-linked glycans in GppaF have been mutated. Thus, GppaF is converted into paF, which is an ERAD substrate.

Fig. 27.2.

pαF is a soluble ERAD substrate. The cytosol- and time-dependent degradation of pαF is shown. Values represent the time (in min) that microsomes containing pαF were incubated in the presence or absence of cytosol and an ATP-regenerating system at 30 °C. GppαF is membrane associated, untranslocated precursor. Figure taken from (McCracken and Brodsky, 1996). © McCracken and Brodsky (1996). Originally published in *The Journal of Cell Biology*. 132: 291–298.

Fig. 27.4.

Ste6p* is polyubiquitinated *in vitro*. Microsomes containing an HA-tagged form of Ste6p* were incubated with 2 mg/ml cytosol, an ATP-regenerating system, and ¹²⁵I-labeled ubiquitin at 23 °C or on ice for the indicated times (lanes 1–3) or for 60 min (lanes 4–11). The reactions were then quenched and Ste6p* was immunoprecipitated with an anti-HA antibody. Samples were processed as described in the text. Where indicated, reactions either lacked cytosol, or were treated with apyrase (final concentration of 0.02 μg/ml), 0.5 mg/ml methylated-ubiquitin, or 100 μM MG132, a proteasome inhibitor. “-anti-HA” denotes a precipitation performed in the absence of antibody and “-Ste6p*” denotes that microsomes were prepared from cells lacking the substrate. Figure taken from Nakatsukasa *et al.* (2008).