Degradation of Mutated Bovine Pancreatic Trypsin Inhibitor (BPTI) in the Yeast Vacuole Suggests Post-Endoplasmic Reticulum Protein Quality Control

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Running title: Quality control of an unstable protein

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SUMMARY

The rate-limiting step in protein secretion is folding, which occurs in the ER lumen, and almost all secreted proteins contain disulfide bonds that form in the ER and stabilize the native state. Secreted proteins unable to fold may aggregate or they may be subject to ER associated protein degradation (ERAD). To examine the fate of aberrant forms of a well-characterized, disulfide-bonded secreted protein, we expressed bovine pancreatic trypsin inhibitor (BPTI) in yeast. BPTI is a single domain, 58 amino acid polypeptide containing three disulfide bonds, and yeast cells secrete the wild type protein. In contrast, the Y35L mutant, which folds rapidly but is unstable, remains soluble and is not secreted. Surprisingly, the proteolysis of Y35L is unaffected in yeast containing mutations in genes encoding factors required for ERAD and is stable if artificially retained in the ER. Rather, Y35L is diverted from the Golgi to the vacuole and degraded. Because only the mutant protein is quantitatively proteolyzed these data suggest that a post-ER quality control check-point diverts unstable proteins to the vacuole for degradation.
INTRODUCTION

Mis-sense mutations or small in-frame deletions or insertions might not only affect protein function but might compromise the ability of a protein to fold into its native conformation. On occasion, non-native proteins exhibit gain-of-function conformations and are cytotoxic. In fact, defects in protein folding leading to toxic protein conformations have been implicated in the pathogenesis of several diseases, including antitrypsin deficiency, Alzheimer’s Disease, and the prion-related diseases (1-5). However, mis-folded proteins do not necessarily harm the cell as they might instead be degraded. For example, ER associated protein degradation (ERAD) is a quality control mechanism in which aberrant or mis-folded secreted proteins are detected in ER, retro-translocated to the cytoplasm, and degraded by the proteasome (4,6-9). ER lumenal chaperones, such as BiP and calnexin facilitate the ERAD of soluble, lumenal polypeptides (10-16). It is thought that these chaperones—and recently characterized lectins (17-19)—select mis-folded proteins and then target them for retro-translocation. The accumulation of mis-folded proteins in the ER can also induce the Unfolded Protein Response (UPR), an intracellular signaling pathway that leads to ER expansion and induces the expression of chaperones and other factors required for protein biogenesis and transport (20,21). Consequently, the UPR, like ERAD, reduces ER stress when the concentration of aberrant, secreted proteins rises.

The rate-limiting step in protein secretion is folding in the lumen of the ER, and the efficiencies of disulfide bond formation and protein folding are linked (22-24). Loss of a native disulfide affects protein secretion in unpredictable ways, ranging from ER luminal protein aggregation and retention, to intracellular protein degradation, to a dramatic
acceleration of secretion (25-32). As a result, one cannot yet predict how a mutation will alter the fate of a secreted protein.

To begin to address this question, we expressed wild type and mutant forms of bovine pancreatic trypsin inhibitor (BPTI) in yeast. BPTI is a 58 amino acid polypeptide and the native structure contains three disulfide bonds, formed by C14-C38, C5-C55 and C30-C51, and distinct disulfide-bonded intermediates in the folding pathway are N* (C5-C55, C14-C38), N' (C30-C51, C14-C38) and N_{SH,SH} (C30-C51, C5-C55). Two of the most highly populated intermediates are N* and N', and the conversion of N' to N_{SH,SH} represents the rate-limiting step during BPTI folding in vitro (33,34).

In native BPTI a Tyr at position 35 is buried and immobile (35,36). Although the final conformation of Y35L BPTI is not radically altered, some of the normally buried contacts become solvent-exposed and the protein is unstable relative to wild type BPTI (37-40), however the rate of disulfide bond formation and overall folding is enhanced in this mutant (40). The conformation of C5A BPTI, in which a disulfide bond in the final and intermediate states cannot form, is also thought to be quite unstable because the C5-C51 bond is buried in the hydrophobic core of the protein. Not surprisingly, significant conformational deviations between wild type and C5A BPTI have been noted (38).

The fates of Y35L and C5A BPTI in the yeast secretory pathway were examined previously, and although neither mutant protein was secreted, Y35L was degraded and C5A accumulated (27,28). Based on these and other data (41,42) the thermodynamic stability of a secreted protein—but not the rate of folding—appears to determine secretion-competence. In this study, we set-out to investigate whether mutant BPTI derivatives were handled by ERAD and/or aggregated. We found that Y35L escaped ERAD and was degraded in the yeast
vacuole. These data suggest a “second line” of protein quality control in the yeast secretory pathway.
EXPERIMENTAL PROCEDURES

Yeast and molecular methods

The yeast strains used for this study are listed in Table I. Plasmids for the constitutive expression of Wild Type, Y35L and C5A BPTI were described previously (43) and DNA inserts corresponding to these genes and promoter elements in YE112-GPD-BPTI (27) were sub-cloned into PRS426 (44). When the resulting plasmids are introduced into yeast cells, BPTI is expressed from the glyceraldehyde-3-phosphate dehydrogenase promoter. The N-terminus of the protein contains a synthetic pre-pro sequence derived from pre-pro alpha factor but lacks the core oligosaccharyl consensus sites (“G” BPTI). The pro region is cleaved in the Golgi by the Kex2p protease, and an added glutamate-alanine C-terminal to the existing cleavage site improves Kex2p processing. Similar levels of BPTI expression and secretion in yeast are evident if the bona fide pre-pro alpha factor sequence is fused to BPTI or if the synthetic pre-pro sequence contains the glycosylation site (data not shown). The individual BPTI expression vectors were transformed into the desired strains using lithium acetate (45) and transformants were selected for growth on Synthetic Complete (SC) medium lacking uracil but containing glucose at a final concentration of 2%. Cells expressing BPTI were grown to mid-log phase and glycerol stocks were made and stored at –70°C. All experiments were performed using cells freshly broken-out onto solid selective medium.

Cycloheximide chase assay to monitor BPTI maturation and degradation

Yeast were grown to mid-log phase in selective liquid medium at 26°C and were collected and resuspended to a final OD (Optical Density at 600 nm) of 4/ml in the same medium. The cells were incubated at 37°C for 10 min before cycloheximide was added to a final
concentration of 200 µg/ml. At the indicated times, 1.6 ODs of cells were collected and harvested. The supernatant (~500 µl) from this centrifugation step, which represents the secreted pool of BPTI, was concentrated using Centricon 3s microconcentrators (Amicon) to a final volume of 15 µl and assayed as described below. The pellet, which represented intracellular material, was resuspended in 100 µl of Stop Buffer (20 mM Tris-HCl pH 8, 50 mM ammonium acetate, 500 mM iodoacetamide, 20 mM sodium azide, 0.45 mM cycloheximide, 5 mM EDTA) and 100 µl of Cell Extract Buffer (10 mM Tris HCl pH 8, 25 mM ammonium acetate, 500 mM iodoacetamide, 20 mM sodium azide, 20 mM EDTA, 10% Trichloroacetic acid [TCA], 0.45 mM cycloheximide, 1 mM phenylmethylsulfonylfluoride [PMSF], 1 µg/ml pepstatin A, 3 µg/ml leupeptin) was added. A total of 1.5 g of Zirconia/silica beads (0.5 mm diameter, Biospec Products, Bartlesville, OK) and 20 µl of 20% TCA were added and the cells were disrupted with one 60 sec burst in a mini-Bead Beater (Biospec Products, Bartlesville, OK) followed by cooling on ice for 60 sec. Two 500 µl washes with Wash Buffer (10 mM Tris HCl pH 8, 25 mM ammonium acetate, 2 mM EDTA, 10% TCA) were combined with the original lysate. Each wash included a 60 sec burst in the mini-Bead Beater with cooling on ice for 60 sec between bursts. This mixture was centrifuged (16,000g for 5 min at 4°C), the supernatant was aspirated and the resulting pellet was resuspended in 40 µl of Resuspension Buffer (100 mM Tris pH 11, 10 mM DTT, 3% SDS). The samples were heated at 72°C for 5 min before 160 µl of Dilution buffer (60 mM Tris pH 7.4, 190 mM NaCl, 6 mM EDTA, 1.25% TritonX-100) and 4 µl of a 0.5 M stock of iodoacetamide were added. The solution was mixed vigorously, 10µl of Omnisorb (Calbiochem) was added and the samples were incubated at 4°C for 30 min. The samples were centrifuged at 16,000g for 5 min at 4°C and the resulting
supernatant, which represented clarified, cellular lysate, was used for gel samples. NuPage 4x LDS sample buffer (Invitrogen) was added to the lysate, and to the concentrated extracellular material (see above), to a final dilution of 1x LDS sample buffer and supplemented with a final concentration of 0.06M DTT and the samples were heated at 72°C for 10 min. Proteins in the samples were resolved on 4-12% Bis-Tris NuPAGE gradient gels (Invitrogen) using NuPage SDS-MES Running Buffer (Invitrogen). The resolved proteins were transferred to PROTRAN nitrocellulose membranes (Pore size = 0.2µ ; Bioscience) and incubated for 30 min at room temperature in TBS-T (20 mM Tris HCl pH7.5, 0.5 M NaCl, 0.05% Tween 20) containing 5% nonfat dry milk and sodium azide at a final concentration of 5 mM. The filters were then incubated with polyclonal anti-BPTI antiserum (8371T) diluted 1:1,000 in TBS-T/5% nonfat dry milk/5 mM sodium azide for 16 h at 4°C. Filters were washed three times for 10 min with TBS-T and then incubated at room temperature with secondary antibody (horseradish peroxidase conjugated donkey anti-rabbit IgG; Amersham) diluted 1:5,000 in TBS-T/5% nonfat dry milk for 1 h. The filters were washed three times for 10 min with TBS-T and then treated with the Super Signal West Pico chemiluminescence reagent (Pierce Chemical), exposed to X-ray film and where indicated data were quantified using films exposed in the linear range and the Image Gauge software (Fuji Film).

Sucrose density gradient analysis

A total volume of 100 ml of exponentially growing cells (OD 0.5-0.8) were pelleted, washed, and concentrated to 0.5 ml in STED10 buffer (10% wt/vol sucrose, 10 mM Tris-Cl, pH 7.6, 10 mM EDTA) containing protease-inhibitors (1 µg/ml leupeptin, 0.5 µg/ml pepstatin A and 1 mM PMSF) and split into two microcentrifuge tubes, which were subsequently placed on
Zirconia/silica beads were added to the meniscus and the samples were lysed using a 60 sec burst in a mini Bead-Beater (as above) for 2 min. An additional 500 µl of STED10 containing protease inhibitors was added and the resulting solution was centrifuged at 400g for 10 min at 4°C. The resultant supernatant was transferred to a new tube and 0.3 ml of the extract was layered onto a 30-70% linear sucrose gradient, which was formed by layering 2.2 ml of STED buffer with decreasing amounts of sucrose (from the bottom): STED70 (70% w/v sucrose in STED buffer), STED60, STED 50, STED40 and STED30. The gradients were centrifuged for 18 h at 100,000g at 4°C in a Beckman SW41 rotor. Fractions of 800 µl were collected from the top (Fraction 1) to the bottom (Fraction 13) of the gradient and the pellet at the bottom of the tube was resuspended in the remaining 800 µl of STED30 and represented aggregated material (Fraction 14). A total of 600 µl of extraction buffer (10 mM Tris HCl pH8, 25 mM ammonium acetate, 500 mM iodoacetamide, 20 mM sodium azide, 20 mM EDTA, 10% TCA, 0.45 mM cycloheximide, 1 mM PMSF, 1 µg/ml pepstatin A, 3 µg/ml leupeptin) was added to each of the fractions and the samples were incubated on ice for 30 min. The mixture was centrifuged at 16,000g for 10 min at 4°C and the resultant pellet, containing precipitated protein, was resuspended in 10 µl of TCA resuspension buffer (3% SDS, 100mM Tris pH11, 10mM DTT). This sample was incubated at 72°C for 5 min before 40 µl of BPTI dilution buffer (60mM Tris pH 7.4, 190mM NaCl, 6mM EDTA, 1.25% Triton X-100) and 1 µl of 0.5 M iodoacetamide were added. Samples were prepared and proteins were separated on 4-12% Bis-Tris gradient gels as described above.
Indirect Immunofluorescence

The cellular distribution of BPTI and intracellular proteins in yeast were visualized using a protocol supplied by M. Snyder’s laboratory (Yale University; personal communication). Yeast were grown to mid-log phase at 26°C, a 1/10th the volume of 37% formaldehyde was added and the cells were incubated for 60 min at room temperature with gentle shaking. The cells were harvested, washed twice with 1.5 ml of Solution A (1.2 M sorbitol; 50 mM KPO₄ pH 7.0) and then resuspended in 0.5 ml of Solution A containing 1 µl/ml of 2 mg/ml Z100T zymolase (made in double-distilled water [ddH₂O]). The mixture was incubated for 30-40 min at 37°C. Polylysine slides were prepared during this incubation; in brief, 0.5 mg/ml of polylysine (Sigma) resuspended in ddH₂O was added to each well of a 25 x 75 mm Teflon coated immunofluorescence slide (Polysciences Inc.), incubated for 10 min at room temperature, and the slides were washed with ddH₂O and air dried for 20 min. The yeast cells, which had been incubating, were harvested and washed 1-2 times with 100 µl of solution A. The final cell pellet was resuspended in 0.2 ml of Solution A and 30 µl were added to a slide well and allowed to adhere for 30 min. Unbound cells were aspirated and the wells were rinsed gently with PBS/0.1% BSA and twice with PBS/0.1% NP40/0.1% BSA. Each sample was incubated with the primary antibody (BPTI: 1:50 and Vma2p: 1:25, each in PBS/0.1% BSA) for 2 h at room temperature, or overnight at 4°C. Cells were incubated for 5 min and washed one time each with PBS/0.1%BSA, PBS/0.1%BSA/0.1% NP40 and PBS/0.1% BSA. Alexa secondary antibodies (goat anti-rabbit, 488; goat anti-mouse, 594; Molecular Probes), which had been diluted 1:500 in PBS/0.1% BSA, were added to the cells for 1-2 h. Finally, the cells were washed for 5 min with PBS/0.1% BSA, twice with PBS/0.1% BSA/0.1% NP40, and once with PBS/0.1% BSA. DAPI
diamidino-2-phenylindole dihydrochloride; Sigma) was incubated with each sample for 5 min and the cells were washed once with PBS/0.1% BSA and then mounted in 4 µl of mount solution (70% glycerol/PBS containing 2% n-propyl gallate). The samples were sealed with a cover-slip and clear nail polish, allowed to dry, and immunofluorescence was either assessed immediately or the samples were stored at –20°C in the dark for several months. Cell fluorescence was visualized on an Olympus BX60 microscope fitted with a Hamamamatsu digital camera and images were analyzed using QED Imaging Software (Pittsburgh, PA).

**Concanavalin A immunoprecipitation**

A total of 300 µl of Concanavalin A Buffer (500 mM NaCl, 1% TritonX-100, 20 mM Tris pH 7.5, 2mM NaN₃) and 20 µl of Concanavalin beads (added from a 1:1 stock in Concanavalin A Buffer) were added to 15 µl of cellular lysate, prepared as described in the cycloheximide chase protocol (see above). This mixture was incubated for 16 h at 4°C. The beads and those proteins bound were harvested by centrifugation at 16,000g for 1 min at 4°C. The beads were washed three times with Concanavalin A Buffer and 1 x LDS sample buffer /0.06M DTT buffer was added to the pellet at a 1:1 bead:sample buffer ratio. The samples were heated to 72°C for 10 min, and proteins were separated on 4-12% Bis-Tris NuPage gels and analyzed by western blotting as described above for the cycloheximide chase protocol.
RESULTS

Y35L BPTI degradation is independent of BiP, calnexin and proteasome function

Previous studies indicated that wild type BPTI is secreted from yeast but the Y35L mutant is degraded intracellularly (28). To determine whether Y35L BPTI was degraded via the ERAD pathway, wild type and Y35L BPTI-expressing plasmids were transformed into cells containing the kar2-1 allele in the gene encoding BiP (KAR2; (46)), into a strain lacking calnexin (cne1Δ; (47)), and into the pre1-1pre2-1 mutant in which ~95% of the activity of the proteasome is abrogated (48). We and others have noted that the degradation of soluble ERAD substrates is defective in these strains or in ER-derived microsomes or cytosol derived from these mutants when ERAD is examined in vitro (12,15,16,49,50). Isogenic wild type yeast strains expressing these proteins were examined in parallel. As an additional control, yeast were transformed with a URA-marked 2 micron vector, PRS426 (44), lacking an insert. Cycloheximide chase experiments were performed as described in the Experimental Procedures and Bis-Tris polyacrylamide gels (4-12% acrylamide) were used to resolve the ER-resident, immature BPTI, which contains a pro region, from mature BPTI, which results from Kex2 protease-mediated removal of the pro region in the Golgi. The mature and pro-region-containing BPTI species were detected by immunoblot analysis of cellular extracts using anti-BPTI antiserum. A signal was absent when immunoblotted extracts from yeast transformed with the vector control were probed with anti-BPTI antiserum (data not shown).

As shown in Fig. 1., we noted a time-dependent decrease in the amount of the immature (“pro”) ER-resident form of the wild type and Y35L proteins, regardless of which strain was examined. In these experiments the level of the mature form either decreased or
remained constant, suggesting that the immature form is “chased” into the mature species, which in the case of wild type BPTI is then secreted (27; see below), and/or that a variable population of the mature protein is stable. To differentiate between these possibilities, total yeast protein was radiolabeled with $^{35}$S-methionine/cysteine, cell extracts and extracellular fluid/media were isolated (see Experimental Procedures), and BPTI was detected by immunoprecipitation and radiography as previously described (27, 28). As shown in Fig. 2, we observed precursor-product relationships between immature pro-BPTI and the mature intracellular and extracellular BPTI species. It is important to note that only wild type, mature BPTI was secreted, regardless of whether it was expressed in the wild or mutant strains (data not shown, but see Fig. 2 and 6 and 27, 28). These results suggest that the Y35L mutant protein is degraded intracellularly, independent of the ERAD pathway, or that it aggregates and cannot be resolved by immunoblot analysis.

**Y35L mutant BPTI is degraded in the vacuole**

Because the time-dependent disappearance of Y35L BPTI occurred in mutants defective for ERAD, and because the vacuole/lysosome has been found to play a distinct role or complementary role to ERAD in the degradation of some aberrant, soluble proteins in yeast (51-54) we investigated whether the vacuole was the site of Y35L BPTI degradation. To this end, plasmids expressing wild type and Y35L BPTI were transformed into wild type and pep4 mutant yeast, in which vacuolar protease activity is negligible (55). Cycloheximide chase reactions were again performed but in this case quantitative data for the levels of mature and pro-BPTI were obtained. In these experiments we found that the mature form of the Y35L BPTI protein, which was not secreted, was markedly stabilized (Fig. 3A).
Moreover, we noted significantly greater quantities of Y35L BPTI in the pep4 mutant strain compared to the wild type strain (compare “short” and “long” exposures; Fig. 3A), suggesting that the protein accumulated in yeast defective for vacuolar proteolytic processing. We also measured the stability of wild type BPTI in these strains. Although the wild type protein can be secreted (Fig. 2 and (27, 28)), we noted that the intracellular population (see Experimental Procedures) was stabilized in the pep4 mutant (Fig. 3B). These data are consistent with recent results suggesting that the vacuole can degrade proteins that arise via secretory pathway “overflow”, particularly when the cell is stressed (54). We surmise that wild type BPTI can escape degradation and be secreted but that the secretory pathway is overwhelmed, leading to vacuolar degradation of the intracellular pool. In contrast, Y35L mutant BPTI is quantitatively retained in the cell and degraded.

The results presented in Fig. 3 suggest that Y35L BPTI, and to some extent wild type BPTI, is targeted to and degraded by the vacuole. To obtain additional support for this hypothesis the residence of BPTI in wild type and pep4 mutant yeast was assessed using anti-BPTI antiserum and indirect immunofluorescence microscopy. A vacuolar-resident protein was identified using a monoclonal antibody against a subunit of the H+-ATPase, Vma2p (56). Primary anti-sera were visualized using Alexa goat anti-rabbit antibody for BPTI and goat anti-mouse antibody for Vma2p. Images were also merged to determine protein co-localization, and representative images of stained cells are shown in Fig. 4. Wild type BPTI (green) resided in discrete, punctate intracellular locations, consistent with its transit through the secretory pathway, and in the pep4 mutant somewhat increased co-localization of the protein with respect to the vacuolar marker was evident. A similar amount of intracellular Y35L BPTI co-localized with the vacuolar marker, but a substantial increase in the pep4
mutant was evident (\textit{pep4} Y35L “Merge”). Combined with the data presented in Fig. 3A we conclude that the Y35L mutant protein is quantitatively disposed of by vacuolar proteases. These combined data also indicate that the cell can distinguish between wild type and Y35L BPTI.

\textbf{Y35L mutant BPTI is not aggregation-prone}

Although the data presented above indicate that at least a portion of Y35L BPTI is degraded by vacuolar proteases, another explanation for the complete lack of secretion and disappearance of intracellular Y35L BPTI is that the protein aggregates, an effect that might be exacerbated when vacuolar protease activity is compromised. To test this hypothesis, wild type and \textit{pep4} yeast expressing wild type and Y35L BPTI were lysed and the extracts were analyzed by sucrose gradient density centrifugation (see Experimental Procedures). We also assessed the fractionation of the non-secreted C5A mutant form of BPTI that is more stable than Y35L (27). Gradients were fractionated from the top (fraction 1) to the bottom (fraction 13), and pelleted, potentially aggregated material was analyzed as “fraction 14”. The proteins were resolved on 4-12\% gradient gels and the migration of BPTI through the gradients was assessed by immunoblot analysis. In each of these extracts the vast majority of BPTI sedimented between fractions 1 and 8 (Fig. 5), which correspond to sucrose densities of 25-52\% wt/wt and in identical experiments represent intra-cellular organelles in the secretory pathway (57,58). Notably, low amounts of immature and mature forms of BPTI were observed in fraction 14 in gradients from cells expressing wild type and Y35L BPTI, regardless of whether Pep4p was active. In contrast, we observed a greater population of the
C5A mutant form of BPTI in fraction 14, suggesting that this intracellularly retained and stable form of BPTI aggregates to some extent.

**Y35L mutant BPTI is stabilized in pep3 mutant yeast**

Proteins can be targeted to the vacuole through multiple routes, including the endosomal, non-endosomal (direct), and cytoplasm-to-vacuole (CVT) pathway (59,60). Vacuolar degradation of some mis-folded soluble proteins through the endosomal route requires the Pep1/Vps10 protein (51,52), which functions as a receptor for the vacuole-targeted protease, carboxypeptidase Y [CPY;(61,62)]. However, the degradation of other substrates is Pep1/Vps10p-independent ((63,64) E. Spear and D. Ng, personal communication).

To begin to define how the mutant BPTI is targeted to the vacuole, we first expressed wild type and Y35L BPTI in yeast lacking **VPS10/PEP1**, but no impact on either the secretion or degradation of these proteins was noted; in contrast, Y35L BPTI was stabilized in yeast deleted for **PEP3** (data not shown), which is required for the endosomal, non-endosomal, and CVT pathways to the vacuole (65). These data further support vacuole-mediated degradation of Y35L BPTI, and suggest that the degradation of this protein is Pep1/Vps10p-independent.

**Kex2p-processing is not required for Y35L BPTI degradation, but is necessary for wild type BPTI secretion**

Mature wild type and Y35L BPTI are formed by the Kex2p-mediated cleavage of the synthetic pro sequence fused at the N-termini of these proteins, and previous work using Kex2p-over-expressing strains suggested that the Y35L BPTI secretion defect was not due to
a limiting amount of cellular Kex2p (43). However, Arvan and colleagues reported that a single-chain insulin derivative was diverted from vacuole-mediated degradation to the cell surface in \textit{kex2} mutant yeast (63). We therefore wished to investigate the behavior of the wild type and Y35L protein in a \textit{kex2} genetic background in order to determine whether Y35L could be secreted in the absence of Kex2p processing.

Wild type and a \textit{kex2} yeast strain were transformed with the wild type BPTI or Y35L expression constructs and cell lysates and extracellular fluid (medium) were collected during a cycloheximide chase assay. As shown in Fig. 6A, Y35L was not secreted from the \textit{kex2} mutant strain, and the extent of pro-Y35L formation was significantly reduced. Moreover, the level of the immature Y35L BPTI protein decreased over time, suggesting that the protein was degraded. We also found that the secretion of wild type BPTI was blocked in \textit{kex2} yeast (Fig. 6A), consistent with a requirement for this post-translational processing event during protein secretion.

Interestingly, little difference in the extent of processing of the wild type pro region was evident when the wild type and \textit{kex2} mutant were compared. This phenomenon could arise from spurious cleavage of the pro region via other endosomal proteases to which the Y35L protein is inaccessible. It is also important to note that in a cycloheximide chase a population of stable, slowly processed material can build-up. Therefore, to establish whether the rapid cleavage of the pro region requires Kex2p, wild type and \textit{kex2} yeast expressing BPTI were metabolically labeled (27, 28), cell extracts were prepared, and BPTI was immunoprecipitated using anti-BPTI antiserum and Protein A-Sepharose. As shown in Fig. 6B cleavage of the pro region was only evident in the wild type strain.
**ER-retained Y35L BPTI is stable and glycosylated**

The results presented thus far suggest that Y35L BPTI is not an ERAD substrate, even though the protein is unstable (see Introduction). One scenario to explain these results is that Y35L BPTI might escape ERAD because it traffics from the ER before it can be targeted for retro-translocation and proteasome-mediated degradation. Thus, if the mutant protein were “trapped” in the ER, it might now become an ERAD substrate. To begin to examine this hypothesis, we expressed wild type and Y35L mutant BPTI in a *sec12* mutant yeast strain, which at the non-permissive temperature exhibits a strong block in the budding of ER-derived COPII vesicles (66). Upon shifting cells to 37°C and performing a cycloheximide chase there was no change in the total amount of wild type or Y35L BPTI, as assessed by comparing all BPTI species in each panel in Fig. 7A. The Golgi-resident forms of BPTI and Y35L BPTI in this experiment are likely present because they matured prior to the 37°C shift, or because maturing Kex2p might have become trapped in the ER with the BPTI in the *sec12* mutant upon shift to the non-permissive temperature. Nevertheless, this result indicates that Y35L BPTI is not recognized as an ERAD substrate in the *sec12* mutant, and/or that proteasome-mediated degradation of this protein requires recycling from the Golgi apparatus, as has been observed for other soluble ERAD substrates (67-69). However, consistent with our results, Zhang et al. (63) reported that mutant forms of insulin that are targeted for vacuole-mediated degradation are stable in yeast compromised for ER-to-Golgi transport.

Interestingly, the presence of new immunoreactive wild type and Y35L BPTI species that migrated slower than immature (pro) BPTI during SDS-PAGE was observed in *sec12* cells (Fig. 7A). The slower migrating species most likely derived from ER-resident BPTI as
the level of immature BPTI decreased over time concomitant with the appearance of this species (denoted by an “*” in Fig. 6A). Because the targeting of some proteins to the vacuole/lysosome requires ubiquitination, we examined whether the higher molecular weight form of BPTI represented ubiquitinated protein. First, immunoblots were probed with anti-ubiquitin antiserum after proteins in concentrated lysates were resolved by SDS-PAGE and blotted onto membranes. Second, we examined immunoblots from lysates after co-transformation of dominant negative ubiquitin mutants (70); however, the results of both experiments suggested that BPTI was not ubiquitinated (data not shown).

Römisch and colleagues reported previously that an ERAD substrate retained in ER-derived microsomes acquired O-linked mannose side chains, and they suggested that this post-translational modification stabilized the protein (71). In addition, K. Nakatsukasa and T. Endo (personal communication) have provided data that O-mannosylation of an aggregation-prone substrate in the ER helps maintain its solubility and promotes secretion. To determine whether the higher molecular weight BPTI species observed in Fig. 7A represent a novel, glycosylated form of BPTI, we incubated extracts from wild type and sec12 yeast with Concanavalin A-Sepharose and examined for the presence of BPTI in the load (“-”) and bound (“+”) fractions using anti-BPTI antiserum (Fig. 7B). Although the three forms of BPTI were again evident in sec12-derived extracts, the highest molecular weight species bound preferentially to the lectin (“*” in Fig. 7B). As controls for this assay, we found that Gas1p—a glycosylated secreted protein—also bound to Concanavalin A-Sepharose, but that the cytoplasmic molecular chaperone, Ssa1p—which lacks glycans—did not (Fig. 7B). Because the form of BPTI used for these studies lacks a consensus site for N-linked glycosylation but contains 4 serine and 5 threonine residues, we suggest that BPTI
similarly acquires *O*-linked mannose side chains when trapped in the ER. Also in accordance with data obtained from other laboratories (see above), we suggest that this modification stabilizes BPTI in the ER.
DISCUSSION

Unstable secreted proteins, such as Y35L BPTI, exist in rapid equilibrium between folded and unfolded states, the latter of which transiently exposes normally buried amino acids that might be recognized by ER molecular chaperones. Therefore, we anticipated that Y35L BPTI would be targeted for ERAD, and hoped to begin to correlate the relative instability of a secreted protein with its propensity to become an ERAD substrate. Instead, we found that Y35L underwent Kex2p-dependent maturation in the Golgi and was diverted to the vacuole for degradation. Support for this conclusion comes from the observed stabilization of mature Y35L BPTI in pep4 yeast, from its increased residence in the vacuole when Pep4p is disabled, and from its stabilization in pep3 mutant cells. Although ERAD is probably involved in the degradation of most aberrant secreted proteins, other soluble proteins transit beyond the ER and are similarly degraded by the vacuole. In addition to Y35L BPTI, additional members of this class are mutated forms of a yeast invertase-lambda repressor hybrid (51), a fusion protein consisting of Hsp150 and β-lactamase (52) a CPY-proteinase A (PrA) hybrid protein (53) and a chimera containing the yeast pre-pro α factor leader sequence and insulin (63). There is no sequence similarity between these diverse substrates that are subjected to post-ER protein quality control, and at present it is not clear how they are targeted for degradation. Mis-folded human proteins associated with specific diseases are degraded in the vacuole equivalent—the lysosome—and here too it is unclear how they are selected (72).

Our study indicates that post-ER protein quality control exhibits substrate specificity because the Y35L mutant form of BPTI is quantitatively retained in the cell and degraded,
whereas ~10-20% of wild type BPTI is secreted based on the data in Fig. 2. We suggest that
the relative instability of the mutant compared to the wild type protein provides the
foundation for substrate selectivity. Specifically, the Y35L mutant has been estimated to be
>3 kcal/mol less stable than wild type BPTI, and although it folds faster than the wild type
protein (40), we suspect that during its transit through the secretory pathway it partitions on
average into an unfolded or partially folded species more frequently than the wild type
protein. The non-native state might be sufficient “tag” the protein for vacuole targeting and
degradation.

The recognition mechanism for post-ER protein quality control in yeast and mammals
is unclear (72), but in three cases Pep1/Vps10p has been suggested to play a role in targeting
soluble substrates to the vacuole in yeast (51-53). Pep1/Vps10p also recognizes vacuolar
hydrolases, such as CPY (61,62). To reconcile these data, Winther and colleagues have
suggested that Pep1/Vps10p contains two polypeptide-binding sites, one that is sequence-
specific (73) and one that may recognize mis-folded protein structure (53). However, we
failed to detect a difference in the stability of Y35L BPTI in wild type and pep1/vps10
mutant yeast, and other examples of Vps10/Pep1p-independent vacuolar targeting have been
reported (63,74). For example, Ng and colleagues have observed that the ERAD pathway is
saturable and that during stress aberrant proteins may be directed to the vacuole for
degradation (54). Under these conditions, Pep1/Vps10p does not appear to be essential for
vacuole-targeting (E. Spear and D. Ng, personal communication). Therefore, the
mechanisms by which aberrant proteins in the ER and post-ER compartment are recognized
might, in some cases, be similar.
Although it is unknown how Y35L BPTI is selected for degradation two scenarios can be envisaged. First, it is possible that a chaperone-like protein resides in the Golgi complex, endosomes, and/or pre-vacuolar compartment, recognizes unstable proteins, and facilitates their delivery to the vacuole. Consistent with this view, many unstable, integral membrane proteins transit through the endosome for vacuole-mediated degradation (72), in some cases after they first reside at the plasma membrane (75-78). Second, it is formally possible that the delivery of Y35L BPTI to the vacuole represents the default pathway, whereas the secretion of wild type BPTI is receptor-mediated. Because the Y35L mutant is more likely than the wild type protein to exist in a non-native state after leaving the ER (see above), we favor the first model and suggest that protein(s) in addition to Vps10/Pep1p might exhibit chaperone-like properties. In any event, the complete spectrum of mediators and pathway of protein quality control for this and other substrates post-ER remain mysterious, but is the focus of ongoing efforts.

Another question that arises from this study regards how an unstable protein escapes ERAD. Normally, ERAD substrates are recognized by ER resident molecular chaperones, such as BiP, which bind to amino acid segments that are enriched for hydrophobic residues; these segments are normally buried in the native structure. Because BiP has been found to be required for the degradation of every soluble ERAD substrate examined (7), Y35L BPTI might escape ERAD if it fails to interact with BiP. Several results are consistent with this hypothesis. First, the secretion of BPTI is unaffected in the kar2-1 strain, a mutant that exhibits both ERAD defects and compromises the folding of secreted proteins (15,16,52,79-81). Second, 5-fold over-expression of BiP has no effect on wild type BPTI secretion (27). Third, BPTI lacks BiP binding sites, as determined using an algorithm first developed by
Blond-Elguindi et al. (1993) and later modified by Stevens and Argon (82). Fourth, current models indicate that the UPR is induced when BiP is released from the UPR-sensor, Ire1p (83,84). When a plasmid that reports on UPR induction was transformed into wild type and Y35L-expressing BPTI strains we failed to observe significant UPR induction (data not shown), suggesting that Y35L does not recruit BiP from Ire1p. More trivially, Y35L might fail to interact with BiP because, as noted above, it folds rapidly. Therefore, we suggest that an unstable protein can transit from the ER to the Golgi and escape ERAD if it eludes BiP capture.
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| Strain     | Genotype                                                                 | Source     |
|------------|---------------------------------------------------------------------------|------------|
| RSY 801    | *Mata, ura 3-52 leu2-3,112 ade 2-101*                                    | 15         |
| MS 1111    | *Mata ura 3-52 leu2-3,112 ade 2-101 kar2-1*                              | 15         |
| W303-16    | *Mata ade2-1, can1-100, ura3-1, leu2-3,112, trp1-1, his3-11,15*           | 47         |
| YFP73      | *Mata ade2-1, can1-100, ura3-1, leu2-3,112, trp1-1, his3-11,15 cne1::LEU2*| 47         |
| WCG4       | *Mata his3-11,15 leu2-3,112 ura3 (D5)*                                   | 48         |
| WCG4/11-21 | *Mata his3-11,15 leu2-3,112 ura3 pre1-1 pre2-1*                          | 48         |
| RSY620     | *Mata ade2-1 trp1-1 leu2-3,112 ura3-1 his3-11,15 pep4::TRP1*              | R. Schekir|
| RSY367     | *Mata ade2-1 trp1-1 leu2-3,112 ura3-1 his3-11,15*                        | R. Schekir|
| BJ5146     | *Mata can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2 kex2::HIS3-5*     | E. Jones   |
| FY834      | *Mata his3 ura3 leu2 lys2 trp1*                                          | C. Barlow  |
| CBY557     | *Mata his3 ura3 leu2 trp1 sec12-4*                                       | C. Barlow  |
| BJ5558     | *Mata ura3-52 his1 trp1 leu2-D1*                                         | E. Jones   |
| BJ5557     | *Mata ura3-52 his1 trp1 leu2-D1 pep3::LEU2*                              | E. Jones   |
| BJ4785     | *Mata ura3-52 leu2 trp1 pep1-35                                          | E. Jones   |
| BJ4763     | *Mata ura3-52 leu2 trp1*                                                 | E. Jones   |
FIGURE LEGENDS

**Figure 1.** The disappearance of immature (“Pro”) BPTI is independent of BiP, calnexin, and proteasome function. Wild type (WT) and the Y35L mutant form of BPTI were expressed in (A) *kar2-1* (BiP), (B) *cne1Δ* (calnexin), and (C) *pre1,2* (proteasome) mutant yeast strains and isogenic wild type yeast (*KAR2*, *CNE*, *PRE1,2*, respectively), and cycloheximide chase experiments from 0 to 120 minutes were performed as described in the Experimental Procedures. Immature (ER localized) BPTI in the yeast secretory pathway is apparent by the presence of a pro region (denoted by a black square to the left of the gel) that is removed in the Golgi by the Kex2 protease. The mature region of BPTI, lacking the pro region, is denoted by a gray rectangle.

**Figure 2.** Wild type pro-BPTI matures into a secretion-competent BPTI species. Total protein in a wild type yeast strain expressing BPTI was radiolabeled and cell extracts and media were resolved. BPTI was detected in the extract by immunoprecipitation using a BPTI-specific polyclonal antibody and in the media BPTI was evident as the only radiolabeled protein at this molecular weight, as previously described (28). Duplicate sets of data were quantified and the relative amount of each species was calculated as a fraction of the maximal amount of that species over time. Open circles: pro-BPTI; closed circles: intracellular mature BPTI; open triangles: secreted, mature BPTI.
Figure 3. Y35L and wild type BPTI are stabilized in the absence of vacuolar degradation. (A) The Y35L mutant of BPTI was expressed in wild type (closed symbols) or pep4 mutant yeast strains (open symbols), and the mature (squares) and immature (triangles) species were visualized after a cycloheximide chase reaction. Data represent the means of 3 independent experiments, +/- SEM, and were standardized to the amount of protein at the beginning of the chase reaction (“100%”). (B) The fate of wild type BPTI was assessed in wild type and pep4 mutant yeast. Symbols are the same as those used in part “A”, and the data represent the means of 2 independent experiments. Shown at the bottom of both parts are two exposures of representative gels used for the quantified data.

Figure 4. Localization of wild type and Y35L BPTI and a vacuolar marker in wild type and pep4 mutant yeast. Indirect immunofluorescence microscopy on wild type (PEP4) or pep4 mutant (pep4Δ) yeast expressing either wild type (WT) or Y35L BPTI was performed as described in the Experimental Procedures. BPTI was visualized using polyclonal anti-BPTI antiserum and an Alexa goat anti-rabbit (488: green) secondary antibody. The vacuole was marked with a monoclonal anti-Vma2p antibody (13D11; Molecular Probes) followed by an Alexa goat anti-mouse secondary antibody (594: red). Co-localization (Merge) is suggested by yellow.

Figure 5. Aggregation-state of wild type, Y35L, and C5A BPTI. Cellular extracts were prepared from wild type (WT) BPTI-expressing, and Y35L and C5A mutant BPTI-expressing wild type (PEP4) and pep4 mutant yeast, and were analyzed by sucrose density centrifugation as described in the Experimental Procedures. “L” refers to an aliquot of the
material loaded onto the gradient and fractions 1-13 represent samples removed from the top (fraction 1) to the bottom (fraction 13) of the gradient. Fraction 14 is material resuspended from the bottom of the gradient, which is inferred to represent high molecular weight oligomeric species. The upper band in each panel is the immature form of BPTI, which still contains the pro region, and the lower band is mature BPTI, lacking the pro region.

**Figure 6.** The absence of Kex2p-mediated cleavage has no effect on the degradation of Y35L BPTI, but prevents the secretion of wild type BPTI. (A) Cycloheximide chase reactions were performed using wild type (KEX2) and kex2 mutant yeast expressing wild type BPTI (WT) or the Y35L mutant, and cell lysates were analyzed for the presence of BPTI as described in the Experimental Procedures. Supernatants (“Secreted”) 60 and 120 minutes after the initiation of the reaction were also collected and monitored for the appearance of mature BPTI. (B) Wild type and kex2 mutant yeast expressing wild type BPTI were metabolically labeled and BPTI was precipitated from cell extracts immediately after addition of cycloheximide.

**Figure 7.** Wild Type and Y35L BPTI acquire O-linked glycans in sec12 mutant yeast. A. Wild type (SEC12) and sec12-4 mutant yeast expressing wild type or Y35L BPTI were examined by cycloheximide chase at 37°C to induce the sec12 mutant phenotype. In addition to immature BPTI and a reduced amount of mature BPTI, a higher molecular weight form of BPTI was observed in extracts prepared from the sec12 mutants (*). B. Extracts from the 60 minute time point in part “A” were incubated with Concanavalin A-Sepharose and a portion of the load (-) and the bound material (+) was assayed for the presence of BPTI.
(molecular mass = ~6.3 kDa), Gas1p (molecular mass = ~115 kDa), and Ssa1p (molecular mass = ~70 kDa) by immunoblot analysis.
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| strain | BPTI | Vma2p | BPTI | merge |
|--------|------|-------|------|-------|
| PEP4   | WT   |       |      |       |
| PEP4   | Y35L |       |      |       |
| pep4   | WT   |       |      |       |
| pep4   | Y35L |       |      |       |
Degradation of mutated bovine pancreatic trypsin inhibitor (BPTI) in the yeast vacuole suggests post-endoplasmic reticulum protein quality control
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