Protein Quality Control Processes in the Yeast Golgi

by

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ABSTRACT

Quality control mechanisms, generally thought to be solely administered in the endoplasmic reticulum (ER) of eukaryotic cells, mediate the recognition, retention, and eventual degradation of misfolded or unassembled secretory proteins. To examine these processes, the secretory fates of thermally-unstable mutants of the N-terminal domain of λ repressor fused to the secretory protein invertase were evaluated in yeast. Our findings demonstrate that these mutant λ fusion proteins undergo at least two quality control mechanisms that are located in the Golgi compartments.
(1) In the cis-Golgi, the mutant fusions were bound by Kar2p and recycled back to the ER, most likely in COPI-coated vesicles, for re-exposure to the ER molecular chaperones and folding environment.
(2) In the trans-Golgi, the mutant fusions were delivered by a Vps10p receptor-mediated targeting process to the vacuole, where the mutant repressor moiety was degraded by vacuolar proteases.

In addition, invertase fusions to varying lengths of random peptides were constructed to test their secretory fates. Ten amino acid peptides that were enriched for bulky, hydrophobic residues caused retention of invertase in the ER by interacting with Kar2p. Invertase fused to longer random peptides, from 50 to 100 residues, exhibited a number of different fates, including retention of intact fusion in the ER, vacuole-dependent degradation of the random peptides, and a post-ER degradation process that functions independent of the vacuole. We propose that the yeast cell contains multiple sites for quality control along the secretory pathway that continually monitor for the presence of misfolded protein.

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Chapter 1

Cellular protein quality control and degradation processes

Introduction

In eukaryotic cells, newly-synthesized cargo proteins destined to be secreted to the cell surface enter the secretory pathway by translocation into the lumen of the ER. Once in the ER, secretory proteins become exposed to both an oxidizing environment, which facilitates the formation of disulfide bond linkages (Hwang et al., 1992), and an extensive folding machinery, in which molecular chaperones and modifying enzymes assist in the conversion of nascent polypeptide chains into correctly-folded, glycosylated secretory protein (for review, see Gething and Sambrook, 1992). Further transport of cargo to other organelles in the secretory pathway (i.e. the Golgi compartments and vacuole/lysosome) and to the cell surface is highly regulated and occurs only if cargo proteins have acquired their mature conformation and proper oligomeric state.

Proteins that do not fold into their native, transport-competent forms are retained in the cell, predominantly in the ER, and eventually degraded. Retention of misfolded protein is thought to be mediated by ER resident chaperone proteins that bind to some sequence or structural determinant exposed on misfolded protein, thus blocking the export of mutant cargo out of the ER. This ability of the cell to segregate misfolded protein from luminal resident proteins and the continuous flux of cargo traffic has been termed "quality control" and serves to prevent the secretion or cellular incorporation of defective proteins that might prove detrimental to cell metabolism or viability (de Silva et al., 1990).

This introduction summarizes the current thinking on how quality control is administered in the cell and is divided into two sections: (1) the unique ER environment and the role of chaperones in retaining misfolded protein in the ER lumen (2) the proteolytic systems in the cell that degrade non-functional protein.

I) Quality control in the ER

The ER provides a unique environment for the folding and oligomerization of protein into mature forms that are competent for transport through the secretory pathway. Nascent chains being translated via ribosomes attached to the ER membrane are inserted through a translocation pore into the ER lumen, where ER modifying enzymes and folding proteins immediately initiate the folding process even before the translocation of the entire protein is completed. The N-terminal signal peptide, which directed the nascent secretory protein to the ER membrane, eventually undergoes cleavage by the signal peptidase complex after protein translocation is completed.

The ER oxidizing environment and disulfide bond formation

Secretory proteins in the ER lumen become exposed to a much more oxidizing environment compared to the cytoplasm, primarily through maintenance of the redox buffer glutathione in an oxidized state (Hwang, et al., 1992). The ratio of reduced to oxidized glutathione (GSH/GSSH) within the ER ranged from 1:1 to 3:1, compared to 30:1 to 100:1 in the overall cell. As a result of the more oxidizing redox potential in the ER, covalent disulfide linkages are favored to form during the folding and maturation of secretory protein. Formation of disulfide linkages has been demonstrated to be required for the transport of many cargo proteins out of the ER. Treatment of cells with the reducing agent dithiothreitol (DTT) resulted in ER retention of the membrane proteins influenza hemagglutinin precursor (HA0) (Braakman et al., 1992), the vesicular stomatitis virus (VSV) G protein (Tatu et al., 1993) and the soluble yeast protein CPY (Jamsa et al., 1994). Interestingly, HA0 that had already oligomerized into the mature trimeric state were resistant to reduction by DTT, suggesting that disulfide bonds in trimeric HA0 were stably formed.

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The enzyme protein disulfide isomerase (PDI) is thought to catalyze the formation, isomerization, and reduction of disulfide bonds on cargo protein in the ER lumen. PDI is a soluble protein that has been isolated as a homodimer consisting of two 57kD subunits in mammalian cells (70kD in yeast). Originally PDI was purified as a factor that accelerated the in vitro reactivation of denatured pancreatic ribonuclease (Goldberger et al., 1963). More recent in vitro studies also support a role for PDI in disulfide bond formation. PDI dramatically increased both the yield and rate of folding of kinetically trapped disulfide bond intermediates of bovine pancreatic trypsin inhibitor (BPTI) (Weissman and Kim, 1993). In addition, translocation of the wheat storage protein γ-gliadin into PDI-deficient dog pancreas microsomes resulted in impaired γ-gliadin disulfide bond formation (Bulleid and Freedman, 1988). Reconstitution of these microsomes with purified PDI rescued this defect, implying a direct role for PDI in disulfide bond formation.

Sequence analysis of PDI revealed the presence of two active site sequences -CXXC-, which were previously identified in the small ubiquitous redox protein thioredoxin found in the cytoplasm. Direct evidence for the involvement of the CXXC sequences in disulfide bond formation came from studies in yeast in which the PDI1 gene is essential. Mutation of both CXXC active sites in yeast PDI to CXXS resulted in the loss of ability to catalyze folding of BPTI under oxidizing conditions (LaMantia and Lennarz, 1993). Interestingly, expression of Pdi1p active site mutants did not affect the cell's viability, calling into question whether the disulfide isomerase activity of PDI constituted the essential function of the protein. More recent work has demonstrated that the CXXS mutants retained partial function in that they were still capable of catalyzing the reshuffling of disulfide bonds that have already formed (Laboissiere et al., 1995). Alteration of both active sites to SXXC abolished both the dithiol oxidation and reshuffling functions and proved to be a lethal mutation. In studies examining the peptide-binding affinity of PDI, cysteine-containing peptides were 4-8 fold more likely

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to competitively inhibit PDI-catalyzed reduction of insulin by glutathione, suggesting that PDI specifically recognizes and interacts with cysteine residues (Morjana and Gilbert, 1991).

Although the primary role of PDI appears to be in the mediation of disulfide bond rearrangments, other functions for PDI have been proposed. PDI is identical to the β subunit of prolyl-4-hydroxylase, which catalyzes proline hydroxylation of collagen (Vuori et al., 1992). Chaperone function has also been ascribed to PDI by several criteria, including the ability to bind to a variety of peptides (Noiva et al., 1991), formation of crosslinks with immunoglobulins and misfolded lysozyme in the ER (Roth and Pierce, 1987; Otsu et al., 1994), and the presence of an unfolded protein response element (UPRE) in the promotor region, which elevates levels of PDI mRNA in response to accumulation of misfolded protein in the ER. This unfolded protein response has been described for several ER chaperones that play a role in ER protein folding and stability, including BiP, prolyl-peptidyl *cis/trans* isomerase (PPI), Eug1 (a PDI-like protein), and Grp94, and will be discussed in more detail in the next section. PDI has also been reported to play a dual role during the oxidative folding of reduced lysozyme depending on the refolding conditions, functioning either as a chaperone that catalyzes folding of the denatured substrate or as an "anti-chaperone" that promotes the formation of inactive lysozyme aggregates (Puig and Gilbert, 1994). Moreover, addition of PDI during refolding of D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which does not contain any disulfide bonds, increased the rate of GAPDH enzyme reactivation and prevented aggregate formation, implying that PDI possesses additional folding activities (Cai et al., 1994).

A second enzyme in the ER lumen that catalyzes a distinctly different isomerization reaction belongs to the protein family, prolyl-peptidyl *cis/trans* isomerase (PPI), whose members localize to different subcellular compartments. Newly-

synthesized secretory proteins primarily bear *trans* oriented peptidyl-proline bonds. PPI mediates the interconversion between the *cis* and *trans* conformers. Strains expressing mutant alleles of *ninaA*, a member of the PPI family in Drosophila, that contain lesions at the peptidyl-prolyl substrate binding site, compromised the transport of rhodopsin through the secretory pathway and exhibited a decreased level of functional rhodopsin, presumably through lack of active PPI required for protein maturation (Colley et al., 1991; Stamnes et al., 1991; Ondek et al., 1992). The immunosuppressive cyclic peptide cyclosporin A, which inhibits the cyclophilin sub-family of PPIs, was also shown to impair the folding of transferrin receptor and the triple helix of type I collagen in chicken embryo fibroblasts, most likely by inactivating an ER PPI (Davis et al., 1989; Lodish and Kong, 1991).

Interestingly, mRNA levels of *FKB2*, a member of the FK506/rapamycin-binding protein sub-family of PPIs in yeast, are elevated in response to the accumulation of unfolded proteins in the ER (Partaledis and Berlin, 1993). Regulation of *FKB2* by the unfolded protein response pathway suggests that PPIs may also mediate chaperone-like functions, in addition to the more specific enzymatic activity of proline peptidyl *cis/trans* isomerization.

Recognition and binding of misfolded protein by ER chaperones

The term "chaperone" has been used very generally to define an assortment of cellular proteins that recognizes and binds to immature polypeptides that are in the process of folding into a stable conformation. Many cellular chaperones were initially observed as proteins whose expression levels were greatly increased under the stress condition of heat shock. Hence, the three major families of chaperones, which have been highly conserved across species, were termed Hsp60, Hsp70, and Hsp90 (Craig et al., 1993).

Chaperones that have been identified in the ER include BiP or Kar2p (GRP78), calnexin, calreticulin, GRP94, ERp72, and GRP170. The GRP members (glucose-regulated proteins) were originally described as being induced under conditions of glucose deprivation. Through regulated binding and release, ER chaperones prevent immature protein from being accessed by degradative enzymes or ensnared in misfolded protein aggregates, while allowing the nascent chain to continue folding properly. ER chaperones also exhibit binding to single subunits of multisubunit membrane complexes to prevent their degradation, aggregation, or premature secretion before the entire oligomeric structure can be formed. One confusing aspect of using this definition in labeling proteins as ER chaperones is that often, these chaperones also function in the capacity of remaining complexed to misfolded or unassembled protein, and retaining them in the ER. Since misfolded protein most likely display the same immature determinants found on nascent chains, it is not surprising that chaperones would be able to bind to both types of unfolded substrate. Therefore, I will be using the term "chaperone" to describe proteins that have been demonstrated to perform both or either of these functions.

BiP (in mammalian cells) or Kar2p (in yeast)

The most abundant and best-characterized ER chaperone is BiP, a member of the evolutionarily conserved hsp70 family. BiP is a soluble protein that resides in the ER lumen and bears a C-terminal retrieval signal -KDEL (or -HDEL in yeast) that directs recycling of escaped BiP from the cis-Golgi back to the ER. BiP was originally identified independently as an induced protein under conditions of glucose deprivation (Pouyssegur et al., 1977) and through its transient interaction with immunoglobulin heavy chain subunits in the absence of light chain synthesis in myeloma cell lines (Haas and Wabl, 1983). A primary role for BiP is to stabilize partially-folded intermediates and prevent aggregate formation during protein folding

and assembly in the ER. Extensive studies in mammalian cells have demonstrated that BiP transiently associates with folding intermediates of immature protein and incompletely-assembled subunits of multimeric membrane proteins, including incompletely disulfide-bonded VSV G protein (Machamer et al., 1990), the influenza virus hemagglutinin precursor HA0 (Hurtley et al., 1989), monomeric thyroglobulin (Kim and Arvan, 1995), the α subunit of mouse muscle nicotinic receptor (Blount and Merlie, 1991), and the α subunit of the acetylcholine receptor (Forsayeth et al., 1992). Presumably, BiP specifically recognizes immature determinants on nascent polypeptide chains. Upon acquisition of a mature conformation or proper oligomerization, the folded protein no longer displays a target site for BiP binding and subsequently becomes packaged into secretory vesicles for transport from the ER.

This ability to recognize immature deteminants is critical to BiP's function in binding misfolded or partially-assembled cargo proteins and retaining them in the ER lumen. A small percentage of the protein population in the ER normally misfolds as a result of errors in protein translation or addition of modifications. Experimentally, the physical interactions between BiP and misfolded substrate have been studied by inducing the formation of mutant protein through incubation of cells with the glycosylation inhibitor tunicamycin (Marquardt and Helenius, 1992), addition of DTT to prevent disulfide bond formation (Braakman et al., 1992), expression of single subunits of a multi-subunit complex (Bole et al., 1986), or introduction of amino acid substitutions that are critical for native folding (Doms et al., 1988). All of these physiological stresses stimulate transcription of the BiP gene as a direct response to the increased levels of misfolded protein in the ER (Kozutsumi et al., 1988). BiP has been found to be complexed with many different mutant secretory proteins in the ER, including mutant VSV G protein (Machamer, et al., 1990), α subunit expression of the T-cell antigen receptor (Suzuki et al., 1991), and unfolded forms of hemagglutinin (HA)

(Gething et al., 1986). Misfolded cargo protein are unable to sequester all of their immature determinants, and thus remain a permanent target for BiP recognition.

In addition to recognition of single targets, BiP has also been found in complexes with misfolded protein aggregates. Aggregation results from the insolubility of partially-folded proteins and subsequent interaction between separate polypeptide chains to form a multi-protein structure. In the cytoplasm of bacteria, newly-synthesized or heterogeneously-expressed proteins often form dense protein aggregates termed inclusion bodies. Addition of tunicamycin to mammalian cells infected with Semliki Forest virus (SFV) led to the accumulation of the SFV spike glycoproteins E1 and p62 and subsequent formation of aggregates that were linked by aberrant interchain disulfide bonds (Marquardt and Helenius, 1992). When these cells were also transfected with HA, mixed complexes containing E1, p62 and HA were generated, implying that all available misfolded protein were incorporated into aggregates in a non-specific manner. BiP associated non-covalently with these aggregates, suggesting that unfolded determinants were exposed on the exterior face of the complexes.

The role of BiP as a mediator of ER retention is dependent on its ability to recognize a wide variety of unfolded determinants on misfolded protein that do not share any obvious sequence similarity. The optimal target site for BiP binding was identified from in vitro affinity panning studies of bacteriophage libraries that displayed random octapeptide and dodecapeptide sequences (Blond-Elguindi et al., 1993). BiP exhibited preferential binding for heptameric sequences that were enriched for bulky, hydrophobic amino acids arranged in alternating positions. The placement of hydrophobic residues in this consensus motif suggested that peptides bound to BiP in an extended conformation, with the bulky side chains of the alternating residues making contacts with a binding pocket on the BiP protein. Recognition by BiP of short hydrophobic sequences is consistent with its binding to nascent polypeptides and

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misfolded protein, both of which most likely display immature determinants that are normally buried within the hydrophobic core of a folded protein.

Chapter 4 will describe a screen which also identified short hydrophobic peptides that, when appended to the secretory protein invertase, retained the fusion in the ER. These retained fusions were found to be specifically associated with BiP, providing in vivo support to the hypothesis that BiP retains unfolded substrate in the ER by recognizing and binding hydrophobic-rich target sites.

The protein structure of BiP and other hsp70 family members consists of two major domains, a ~30kD C-terminal domain that contains the polypeptide binding site and a ~44kD N-terminal domain bearing an ATP binding/hydrolysis consensus sequence. A role for ATP hydrolysis in BiP recognition of misfolded protein was originally suggested by the finding that BiP could be partially released from interaction with the immunoglobulin heavy chain subunit in vitro upon incubation with exogenous ATP, whereas addition of ADP or nonhydralyzable ATP analogs had no effect (Munro and Pelham, 1986). In addition, BiP was shown to exhibit a low basal level of ATPase activity that was stimulated by the binding of synthetic peptides (Flynn et al., 1989).

Surprisingly, however, in binding studies examining several different point mutations in the ATPase domain of BiP, a mutant that abolished ATP hydrolysis still released bound peptide upon the addition of ATP, implying that ATP hydrolysis is not required for dissociation of substrate (Wei et al., 1995). In contrast, BiP mutants that affected either ATP binding or an ATP-induced conformational change remained complexed with the test peptide, suggesting that a conformational change in the BiP protein initiated by ATP binding is the trigger for peptide release. These results are consistent with in vitro experiments investigating the bacterial ATPase chaperones, DnaK and Hsc70, in which release of bound substrate occurred at a faster rate than ATP hydrolysis (Palleros et al., 1994). Furthermore, a DnaK mutant impaired for ATP hydrolysis nevertheless released unfolded substrate in the presence of ATP and KCl.

These observations promote a new model for BiP-mediated chaperoning and retention in which ATP binding induces a conformational change in BiP that causes the release of the bound substrate. Earlier results reporting the loss of BiP substrate release upon incubation with nonhydralyzable ATP analogs can be explained by proposing that BiP binding of these analogs inhibited the requisite conformational change rather than prevented ATP hydrolysis. Further work will have to be performed to clarify whether ATP hydrolysis does play a role in the cycles of BiP association/dissociation with immature and misfolded substrate. Perhaps ATP hydrolysis is coupled to a post-BiP conformational change event that somehow prepares BiP for the next round of substrate recognition and binding.

Analysis of Kar2p, the BiP homolog in yeast *Saccharomyces cerevisiae* (Normington et al., 1989; Rose et al., 1989), has contributed to our understanding of how the presence of misfolded proteins in the ER signals the nucleus to increase the levels of chaperone mRNA transcripts. The promotor region of *KAR2* (and other ER proteins presumed to possess a chaperone-like function, such as *PDI1*, *EUG1*, *GRP94* and *FKB2*) contains a cis-acting domain termed the unfolded protein response element (UPRE), which regulates the stimulation of *KAR2* transcription under conditions where mutant cargo accumulate in the ER, such as treatment with tunicamycin or 2-deoxyglucose (Kohno et al., 1993). Ire1p, which was identified in a screen to isolate mutants that failed to induce the unfolded protein response in response to tunicamycin, encodes a transmembrane protein whose cytosolic domain bears homology to a family of serine/threonine kinases (Cox et al., 1993). Ire1p is speculated to reside in the ER membrane and function as a sensor for the amount of misfolded protein retained in the ER. Upon accumulation of a threshold level of mutant cargo in the ER, Ire1p kinase activity would be activated through oligomerization of its luminal domains, leading to the transmittance of a signal to the nucleus for *KAR2*

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transcription (Shamu and Walter, 1996). A genetic screen also identified Hac1p, a transcription factor that specifically bound to DNA sequences bearing UPREs, implying that Hac1p played a regulatory role in the transcriptional control of *KAR2* (Cox and Walter, 1996). *HAC1* itself was demonstrated to undergo an unusual form of posttrancriptional regulation, in which stable Hac1p was expressed upon induction of the unfolded protein response, whereas in uninduced cells, an alternatively-spliced *HAC1* mRNA was produced and subsequently subjected to ubiquitin-dependent proteolysis.

Two roles for Kar2p that utilize its ability to bind unfolded determinants in the yeast ER have been elucidated. First, Kar2p directly facilitates efficient translocation of nascent polypeptides across the ER membrane in a mechanism most likely involving interaction with the DnaJ-like domain of Sec63p, an ER membrane protein component of the translocation machinery (Vogel et al., 1990; Sanders et al., 1992; Brodsky and Schekman, 1993; Scidmore et al., 1993). The Kar2p/Sec63p complex has been proposed to act as a molecular motor which couples ATP hydrolysis to the transport of nascent chains through the translocation pore into the ER lumen. In more recent studies, Kar2p was also demonstrated to play a role in an early stage of translocation, mediating the ATP-dependent release of nascent chains from association with the translocon subcomplex, the signal sequence receptor (consisting of Sec62p, Sec71p, and Sec72p) for posttranslationally-translocated proteins (Lyman and Schekman, 1997). Second, Kar2p was proposed to be required for the maturation and secretion of the vacuolar protease CPY (Simons et al., 1995). Conditional mutants of *KAR2* were incubated at the permissive temperature to allow for translocation of CPY into the ER lumen and in the presence of DTT to slow folding of the CPY protein. Shifting the mutant strains to the restrictive temperature to inactivate the chaperone activity of Kar2p while simultaneously diluting out the DTT resulted in the formation of

Kar2p/CPY aggregates that were retained in the ER, suggesting that Kar2p function was normally required for the proper folding of transport-competent CPY.

Although mammalian BiP has been found to be complexed with numerous examples of misfolded protein and unassembled subunits of multimeric proteins, the roles of yeast Kar2p that have been investigated so far have been limited to its requirement in the translocation of nascent chains and the single study described above in the folding pathway of CPY. Possibly, Kar2p function may not encompass long-term interactions with misfolded cargo in the yeast ER.

Calnexin and calreticulin

Calnexin, a non-glycosylated ER type I integral membrane phosphoprotein of 90 kD, was designated a chaperone after a number of studies isolated transient calnexin associations with newly-synthesized, unassembled polypeptides and more permanent interactions with misfolded protein in mammalian cells. Unlike BiP, calnexin expression is not induced in response to accumulation of misfolded protein in the ER or glucose deprivation. Sequence analysis of calnexin did not reveal any apparent nucleotide binding site, implying that association/dissociation with immature protein was not linked to ATP binding.

Calnexin was found to be associated with partially-assembled membrane proteins, such as T cell receptor (TcR) (Hochstenbach et al., 1992), membrane-bound immunoglobulin (mIg) (David et al., 1993), β_1 integrin (Lenter and Vestweber, 1994), and major histocompatibility classI (MHC) complexes (Degen and Williams, 1991), and with soluble secretory proteins in HepG2 cells, including α1-antitrypsin, transferrin, and complement 3 (Ou et al., 1993). Upon either complete assembly of the multimeric proteins or protein maturation, calnexin dissociated from the cargo. A correlation between the rate of calnexin dissociation and the rate at which folded cargo was transported to the Golgi compartments suggested that calnexin interactions

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in the ER may be the rate-limiting step in the maturation of some secretory proteins (Ou, et al., 1993). Analysis of cross-linked complexes between calnexin and HA folding intermediates isolated by sucrose velocity gradient centrifugation revealed that calnexin binds unfolded substrate with a 1:1 stoichiometry (Tatu and Helenius, 1997).

More prolonged associations between calnexin and misfolded or incompletely-assembled protein, including free subunits of the MHC class I complex (Jackson et al., 1994), TcR (David, et al., 1993), inactive mutants of the multidrug resistance protein P-glycoprotein (Loo and Clarke, 1994), the ΔF508 mutant of the cystic fibrosis conductance regulator (CFTR) (Pind et al., 1994), and the truncated null Hong Kong variant of human α-antitrypsin (Le et al., 1994) have been observed in the ER. In addition, both the incorporation of the proline analog azetidine-2-carboxylic acid to induce the formation of misfolded protein (Ou, et al., 1993) and prevention of proper disulfide bond formation with the addition of DTT (Wada et al., 1994) led to more stable complexes with calnexin. In yeast, deletion of the calnexin homolog *CNE1* resulted in an increase in the cell surface expression of both an ER retained temperature-sensitive mutant of the Ste2p α-factor receptor and heterologously-expressed mammalian α1-antitrypsin, implying that calnexin normally functioned to retain these proteins in the ER (Parlati et al., 1995).

Two studies have provided more direct evidence that calnexin mediates the retention of incompletely-assembled membrane proteins. First, expression of canine calnexin prevented the aberrant secretion that is seen in *Drosophila melanogaster* cells of both the MHC class I heavy chain and peptide-deficient heavy-chain β_2-microglobulin heterodimer to the cell surface. Presumably, calnexin formed a complex with the MHC class I subunits to block their transport out of the ER (Jackson, et al., 1994). In the second study, expression of a truncated form of human calnexin lacking the ER retention signal in the cytoplasmic tail resulted in the redistribution of the TcR ε subunit from the ER to the Golgi compartments and the cell surface, implying that full-

length calnexin normally retained the TcR ε subunit in the ER (Rajagopalan et al., 1994).

Investigation of what determinants on misfolded protein elicit calnexin recognition revealed a completely different target site from that of BiP binding to short sequences enriched for hydrophobic residues. Calnexin has been proposed to bind misfolded glycoproteins that bear N-linked core oligosaccharides marked with a single glucose residue, $GlcNAc_2Man_9Glc_1$. The first indication that calnexin specifically targeted glycoproteins was the observation that calnexin did not associate with albumin, the major non-glycosylated secretory protein in HepG2 cells, but did bind to a related glycosylated protein α-fetoprotein (Ou, et al., 1993). The interaction between calnexin and newly-synthesized influenza virus hemagglutinin (HA) and VSV G protein was shown to be disrupted upon incubating cells in tunicamycin, which blocks all N-linked glycosylation, and castanospermine and 1-deoxynojirimycin, which inhibit glucosidases I and II, enzymes that remove the the outermost and remaining two glucose residues, respectively, from the complete core oligosaccharide $GlcNAc_2Man_9Glu_3$ (Hammond et al., 1994). Calnexin binding was further abolished in two mutant cell lines, Lec23 and Pha^R27, that did not produce any glucosidases, further implicating a requirement for glucose trimming (Ora and Helenius, 1995). Finally, treatment of cells with castanospermine resulted in the accelerated degradation of the TcR α chain, suggesting that loss of subunit binding to calnexin led to protein instability (Kearse et al., 1994).

Calnexin was found to be specifically associated with monoglucosylated forms of HA folding intermediates and the conditional ts045 VSV G protein (Suh et al., 1989; Hebert et al., 1995). In vitro binding studies demonstrated that the luminal binding domain of calnexin associated solely with the oligosaccharide substrate $GlcNAc_2Man_9Glc_1$, and not with $GlcNAc_2Man_9,Glc_{2-3}$ (Ware et al., 1995). Coincidentally, a separate line of research had identified the UDP-

glucose:glycoprotein glucosyltransferase (UGGT) as an enzyme which catalyzed the addition of a single glucose residue specifically onto misfolded substrate (Sousa et al., 1992; Sousa and Parodi, 1995). UGGT recognizes a bipartite binding site that consists of the innermost GlcNAc of the core oligosaccharide and hydrophobic domains exposed on immature protein.

Taking into account all of these findings, a model for calnexin-mediated quality control has been put forth in which immature protein is subjected to successive cycles of reglucosylation/deglucosylation, regulated by UGGT and glucosidase II respectively, in a mechanism that links calnexin binding to the folded state of the cargo protein. A monoglucosylated state can be maintained by UGGT, which specifically tags misfolded protein in the ER with a single glucose residue to signal for calnexin association. Upon acquisition of a mature conformation, the protein undergoes deglucosylation by glucosidase II, but is no longer a substrate for UGGT and concomitant calnexin binding, resulting in its freedom to be transported from the ER. In in vitro binding experiments, the luminal domain of calnexin was observed to interact with both unfolded and folded conformations of bovine pancreatic ribonuclease B (RNase B), suggesting that calnexin binding is solely dependent on the presence of monoglucosylation rather than immature protein determinants (Zepun et al., 1997). This model provides a physiologically relevant explanation for why N-linked oligosaccharides are attached to secretory proteins in the ER and then subsequently trimmed by glucosidases.

Despite the extensive evidence implicating monoglucosylation in calnexin recognition, a number of studies have reported interactions between calnexin and non-glycosylated proteins, suggesting that binding can not be mediated exclusively through oligosaccharides. For example, two proteins mentioned earlier as being complexed with calnexin, the ε subunit of the TcR and thyroglobulin, do not bear N-linked glycosylation sites. Deletion of polypeptide regions, the transmembrane and

cytoplasmic domain, from the α chain of TcR eliminated calnexin binding although the truncated subunit retained its full complement of potential N-linked glycosylation sites. Either deletion of the transmembrane domain or replacement with a glycosylphosphatidylinositol (GPI-linked) anchor in the MHC class I heavy chain abolished calnexin binding, implying that the transmembrane domain interacted with calnexin (Margolese et al., 1993). Incubation of complexes containing calnexin bound to either the MHC class I heavy chain or α1-antitrypsin with the deglycosylating enzyme endo H failed to abolish calnexin binding although all of the N-linked oligosaccharide chains were removed by the enzyme (Ware, et al., 1995). Similarly, treatment of MHC class II α, β, and invariant chain with tunicamycin or endo H did not impair complex formation with calnexin (Arunachalam and Cresswell, 1995).

Thus, calnexin binding to immature substrate may also be mediated through protein-protein interactions, in addition to monoglucosylation. Most likely, both monoglucosylation and protein-protein interactions contribute to calnexin recognition and binding, with monoglucosylation being more crucial during initial recognition of unfolded substrate. However, once the complex has formed, protein-protein interactions may play a larger role in maintaining a tight association.

In addition to calnexin, recognition of glycoproteins in the ER lumen is also mediated by calreticulin, a 46kD soluble ER protein that shares 33% amino acid identity with human calnexin, including two unique sets of internal repeats. Calreticulin formed complexes with folding intermediates of HA and myeloperoxidase, a neutrophil lysosomal protein required for efficient oxygen-dependent microbicidal activity (Nauseef et al., 1995; Peterson et al., 1995). For both substrates, calreticulin binding was dependent on the presence of oligosaccharide. Similarly, transient interactions detected between a chimera fusing calreticulin to the transmembrane domain of calnexin and a set of nascent secretory proteins were sensitive to the

addition of castanospermine (Wada et al., 1995). A comparison between calreticulin and calnexin associations with different folding intermediates of HA in canine pancreas microsomes revealed an overlapping but not identical binding pattern, indicating that the two related chaperones also displayed their own unique binding specificities (Hebert et al., 1996). Nevertheless, the dependence of calnexin and calreticulin recognition of specific oligosaccharide modifications suggest that both chaperones comprise one branch of the quality control system that monitors the folded state of glycoproteins.

7B2, a specialized chaperone for prohormone convertase PC2

While molecular chaperones such as BiP and calnexin recognize general immature determinants exposed on nascent chains or misfolded protein, the pituitary gland neuroendocrine polypeptide 7B2 was found to specifically associate with the prohormone convertase PC2, a member of a family of endoproteases that cleaves prohormones at pairs of basic amino acids in secretory granules of endocrine cells maintaining a regulated secretory pathway (Braks and Martens, 1994). The amino-terminal half of 7B2 displays a 32% identity and 62% similarity to segments of human, wheat and *E. coli* members of the hsp60 family of chaperones. Kinetic analysis and co-immunoprecipiation studies revealed that the precursor form of 7B2 transiently binds to the newly-synthesized proform of PC2. However, proteolytic cleavage of 7B2 and PC2 into their processed forms in the trans-Golgi network and secretory granule compartments coincided with dissociation of the two proteins, suggesting that 7B2 acts as a chaperone to prevent the premature activation of proPC2 in the secretory pathway before transport to the secretory granules.

Sequential relays of chaperones / ER chaperone matrix

The fact that the two most well-characterized chaperones, BiP and calnexin, possess distinct binding specificities, suggests the possibility that several chaperones may assist nascent polypeptides during the course of folding and oligomerization, each recognizing a different type of unfolded determinant. In bacteria, evidence for a sequential relay of chaperones consisting of DnaK, DnaJ, and GroEL/GroES catalyzing the folding of rhodanese from bovine mitochondria has been reported (Langer et al., 1992). In a sequential model, unfolded substrate would be "passed" from one chaperone to the next as the initial chaperone completes its folding activity, and a different type of unfolded determinant becomes exposed. Possibly, a competition between different chaperones for binding might arise if several different types of unfolded determinants are displayed simultaneously, the outcome of which would depend on the relative abundance of the chaperones and their relative binding affinities.

For eukaryotic chaperones, studies have also described two separate relays of sequential action by two chaperones on a single folding substrate. First, the association of an unassembled, secretable form of immunoglobulin (Ig) light chain with the chaperones BiP and Grp94 were analyzed by pulse/chase experiments (Melnick et al., 1994). Protein folding of Ig light chain was delayed by the addition of the reducing agent β-mercaptoethanol (βME) to labeled cells, which yielded a reduced light chain intermediate that was almost exclusively bound by BiP. Upon dilution of the βME during the chase, BiP dissociated within a few minutes, while Grp94 binding increased with time, exhibiting a half-life of 50 min. Thus, the two chaperones exhibited different kinetic and structure-dependent interactions with the light chain, with BiP binding to nascent, reduced chains at the initial stages of folding for a short amount of time, while Grp94 maintained a longer association and preferred more mature, oxidized folding intermediates.

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A second chaperone relay comprised of BiP and calnexin was reported to mediate the folding of thyroglobulin (Tg), which matures through a series of intermediates from disulfide-linked aggregates, to monomers, and finally to the stable dimer (Kim and Arvan, 1995). Cells were incubated in the reducing agent DTT to form reduced aggregates of Tg. Dilution of the DTT led to dissolution of the reduced aggregates and subsequent maturation of transport-competent Tg, in which BiP and calnexin were found to be complexed with the folding intermediates at different stages of the folding pathway. Calnexin interaction with nascent Tg occurred primarily at the early stages of folding, with >92% of Tg dissociated from calnexin within 30 minutes of the DTT washout. In contrast, BiP exhibited a delayed interaction with Tg that culminated in ~40% of Tg binding at the 30 minute mark. This differential time lag demonstrated a "precursor-product" relationship in which Tg appeared to be transferred from calnexin to BiP during its folding pathway.

It is interesting to note that both of these studies utilized reducing agents to synchronize folding of the nascent chains and slow the maturation process for easier detection of folding intermediates. Although in both cases, the two chaperones bound to their respective substrates at markedly different times to suggest the action of a sequential chaperone relay, the addition of DTT and β-ME, in effect, created artificial folding conditions that may not be physiologically relevant.

The coordination of different chaperone activities on a single substrate would be facilitated by having protein folding take place where all the chaperones are easily accessible to nascent chains. From chemical crosslinking experiments, HA was shown to be weakly associated with large complexes consisting of an assortment of ER chaperones, including BiP, calnexin, calreticulin, Grp94, and other proteins during the initial stages of folding, but not upon formation of the mature trimer (Tatu and Helenius, 1997). These large complexes were speculated to form part of an ER matrix network of chaperones and luminal resident proteins, calculated to exceed a

concentration of 100 mg/ml, that act like a complex sieve through which nascent chains must pass, all the while interacting with the different chaperones and modifying ER enzymes that mediate protein folding. Electron micrographs have disclosed that a reticular, lace-like structure spanned the ER lumen, providing visual evidence for the presence of an ER matrix. Misfolded or unassembled protein would be retained in the ER through prolonged interaction with the chaperone matrix. However, upon acquiring a mature conformation, protein would no longer be bound by the matrix, and instead, be packaged into secretory vesicles for transport to the Golgi.

The effects of Ca2+ on retention of resident and misfolded protein in the ER

The ER lumen is the major intracellular reservoir of calcium ions in the cell with a total concentration estimated to be 3 mM (Sambrook, 1990). Many of the chaperones that have been discussed so far, including BiP, calnexin, calreticulin, and Grp94, have been found to weakly bind calcium, which may somehow modulate chaperone activity (Macer and Koch, 1988). The ER membrane in both yeast and mammalian cells is thought to possess at least one calcium-ATPase pump that mediates a net transfer of calcium ions from the cytoplasm into the ER (Burk et al., 1989; Rudolph et al., 1989). Sequestration of calcium in the ER lumen is most likely maintained through association with calcium binding proteins like the chaperones or with phospholipid head groups.

A number of studies have investigated how perturbation of calcium levels in the ER affect the transport of secretory proteins to the cell surface and the ER retention of resident luminal proteins. Conflicting results on the fate of ER resident proteins have been described upon incubation of cells with reagents that alter the concentration of calcium in the ER, such as the calcium ionophores A23187 and ionomycin or thapsigargin, an inhibitor of the ER calcium ATPase pump. In one group of studies,

lowering calcium levels blocked the maturation process and transport out of the ER of such cargo protein as immunoglobulin M in plasma cells (Shachar et al., 1994), α1-antitrypsin (Kuznetsov et al., 1993), the H1 subunit of the asialoglycoprotein receptor (Lodish et al., 1992), and rotavirus (Poruchynsky et al., 1991). ER degradation of the TcR β and CD3-δ subunits was also shown to be accelerated under these conditions (Wileman et al., 1991). These findings can be explained by proposing that proper levels of calcium in the ER are required for calcium-binding chaperones to function correctly. Otherwise, loss of chaperone folding activity leads to retention of unfolded protein in the ER and enhanced susceptibility to protein degradation. In addition, treatment of cells with ionophores resulted in an increase in the level of BiP, Grp94, ERp72 and calreticulin expression, suggesting that cargo proteins accumulated in the ER and activated the unfolded protein response pathway (Macer and Koch, 1988; Dorner et al., 1990; Lodish and Kong, 1990).

Other studies have demonstrated enhanced secretion of both resident luminal ER protein and cargo protein out to the cell surface upon incubation with ionophores or thapsigargin. Several luminal ER resident proteins were transported out into the extracellular medium after eight hours of ionophore treatment in murine fibroblast 3T3 cells (Booth and Koch, 1989). By immunofluorescence, these treated cells were visualized to contain dispersed ER membranes and a heightened accumulation of ER protein in the Golgi compartments. Transport of immunoglobulin M out to the cell surface in B lymphocytes was also shown to be accelerated (Shachar, et al., 1994). These opposing reports of ER cargo proteins being retained in the ER or secreted to the cell surface upon calcium perturbation are not easily reconciled but may be attributed to either the use of different cell lines or the amount of chaperone interaction required for maturation of individual proteins.

An alternative method to lowering calcium levels in the ER by using mutants of PMR1, which encodes for an intracellular calcium ATPase pump, was shown to affect

the transport of several heterologously-expressed cargo proteins through the yeast secretory pathway (Smith et al., 1985). A number of mammalian proteins such as bovine prochymosin, bovine growth hormone, and a nonglycosylated variant of human urinary plasminogen activator, when expressed in yeast, were recognized as foreign protein by the yeast quality control system and accumulated in the ER. However, *pmr1* mutants secreted 5 to 50 fold higher levels of these cargo proteins into the culture medium compared to wild-type cells, suggesting that lowered levels of calcium in the ER compromised the ability of chaperones to retain the foreign protein (Rudolph, et al., 1989). Similarly, in strains expressing TCR-α variant forms that are usually retained in the ER, lowering luminal calcium levels resulted in the dissociation of the mutant protein from BiP and subsequent transport out to the cell surface (Suzuki, et al., 1991). Thus, maintenance of calcium levels in the ER is crucial for the normal activities of protein folding and quality control.

Recycling of misfolded protein from the cis-Golgi back to the ER

The great majority of research investigating the quality control processes have focused on events in the ER, primarily because of the discovery that ER molecular chaperones bind to unassembled and misfolded protein. There are several examples of misfolded protein that elude long-term interactions with ER chaperones and escape to the cis-Golgi. However, some of these proteins appear to encounter a quality control mechanism in the cis-Golgi that recycles misfolded protein back to the ER, while allowing the rest of the cargo traffic to continue along the secretory pathway. This retrieval process provides the cell with additional opportunities to re-expose misfolded protein to ER folding enzymes, in effect giving the recycled substrate a second chance to acquire a correct conformation.

A recycling pathway that transports immature protein from the cis-Golgi back to the ER has been described for two mutant membrane proteins in mammalian cells and

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one mutant soluble protein in yeast that are strongly retained in the ER. Under certain growth conditions, incorrectly-assembled MHC class I molecules (Hsu et al., 1991) and the ts045 folding mutant of the VSV G protein (Hammond and Helenius, 1994) were localized to the Golgi by immunofluorescence studies. Both the MHC and ts045 proteins were subsequently shown to migrate from a Golgi to an ER fraction in subcellular fractionation experiments, implying that the mutant proteins were indeed being recycled. Most likely, retrieval was initiated from an early cis-Golgi compartment in which enzymes that modify N-linked carbohydrate chains were absent, since the MHC and ts045 proteins did not acquire endoH resistance, a hallmark of transport through the Golgi compartments.

In yeast, an ER-retained active site mutant of the soluble vacuolar protease CPY, CPY*, was also reported to undergo recycling from the cis-Golgi back to the ER (Knop et al., 1996). CPY* became modified with Golgi-derived $\alpha1,6$ mannose residues in a *der1Δ* mutant strain background, which rendered CPY* resistant to ER degradation. Expression of CPY* in a *der1Δ erd1Δ* double mutant resulted in increased levels of CPY* protein acquiring Golgi modifications compared to the single *der1Δ* mutant. Since *ERD1* has been proposed to function in the retrieval of soluble HDEL-tagged ER resident proteins (Hardwick et al., 1990), the enhanced level of $\alpha1,6$ mannose modification in the double mutant suggested that CPY* was being recycled back to the ER by the same retrieval machinery that recycles -HDEL-tagged proteins.

In chapter 3, recycling from the cis-Golgi to the ER of a fusion protein comprised of invertase and a thermally-unstable folding mutant of the N-terminal domain of λ repressor, Inv-(N)cI$_{L57A}$, is described. Our findings that the efficiency of recycling is lowered in a *kar2-ΔHDEL* mutant and that Kar2p forms a complex with the fusion protein under native conditions supports the hypothesis that HDEL-tagged and misfolded proteins utilize some of the same components of the recycling machinery.

II) Degradation processes in the cell

ER degradation

Cells have developed mechanisms to degrade misfolded proteins that are retained intracellularly. These degradation processes are necessary to prevent the continuous build-up of non-functional protein that would otherwise take up limited space in the cell interior and interfere with on-going cell processes. Furthermore, turnover of misfolded protein would allow the cell to salvage for amino acids that could be incorporated during the synthesis of other proteins. Since the majority of misfolded proteins that have been studied are retained in the ER lumen, a proteolytic system was predicted to be localized in the ER to mediate degradative function. ER degradation has been observed for many mutant or unassembled proteins, including the α and β subunits of the T cell receptor (Lippincott-Schwartz et al., 1988; Bonifacino et al., 1989), the δ subunit of the CD3 complex (Wileman, et al., 1991), the ΔF508 form of CFTR (Cheng et al., 1990), the H2a subunit of the human asialoglycoprotein receptor (Amara et al., 1989), and the heavy chain of secreted immunoglobulins (Bole et al., 1986) in mammalian cells, and an active site CPY* mutant (Finger et al., 1993) and heterologous expression of human alpha-1-proteinase inhibitor PiZ variant (A1PiZ) (McCracken and Kruse, 1993) in yeast.

Several lines of evidence have been used to categorize that these proteins were degraded in the ER. Degradation in semi-permeabilized cells of the α subunit of TCR, the δ subunit of CD3, and the initial proteolytic step in the H2a subunit of the human asialoglycoprotein receptor was shown to proceed even in the absence of ATP and postnuclear supernatant, factors that are required for the transport of cargo between the ER and Golgi (Stafford and Bonifacino, 1991; Wikstrom and Lodish, 1992). The mammalian proteins were not transported to the lysosome for proteolysis,

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since degradation was insensitive to reagents such as a weak base NH_4Cl or chloroquine, which inhibit the activity of lysosomal enzymes by raising the intraluminal pH. In addition, the α subunit of TcR in fibroblasts and the ΔF508 variant of CFTR did not undergo any Golgi-associated carbohydrate processing, nor did α-TcR acquire resistance to the deglycosylation enzyme endo H before degradation, implying that these subunits were not transported to Golgi compartments (Lippincott-Schwartz, et al., 1988).

ER degradation in yeast was characterized by demonstrating that the active site CPY* mutant did not bear any detectable α-1,6 or α-1,3 mannose linkages, which are only acquired in the Golgi compartments. In addition, proteolysis of the CPY mutant occurred independent of a *sec18* mutation, which blocks fusion of cargo-carrying secretory vesicles with the cis-Golgi compartment (Finger, et al., 1993). The A1PiZ variant was assumed to be degraded in the ER since only the core-glycosylated form of A1PiZ was detected in kinetic analysis before protein turnover, and proteolysis was not dependent on vacuolar function.

Interestingly, intracellular levels of A1PiZ accumulated in the ER upon institution of a *sec18* block, suggesting that in addition to ER degradation, A1PiZ may also be subjected to rapid post-ER degradation (McCracken and Kruse, 1993). In chapter 5, analysis of the secretory fates of a library of invertase fusions to random peptides 50, 75, and 100 amino acids in length will be discussed. The majority of the random peptides underwent varying degrees of proteolysis. For some peptides, degradation took place after transport out of the ER and before transport to the cell surface, but did not appear to be dependent on vacuolar proteases. This finding leads to the speculation that the Golgi compartments may house a degradative system that destroys unfolded protein.

Similar to the recognition and binding of immature proteins by chaperones, proteins to be degraded probably display a sequence or structural determinant that

signals for their destruction. Since some retained proteins are degraded more rapidly than others, the length of time that a protein remains in the ER is not indicative of susceptibility to proteolysis. Studies have been performed to identify the determinants on the α-chain of TcR that directs its degradation in the ER. From mutational analysis and the behavior of chimeric proteins, a 9 amino acid sequence containing two critical charged residues from the transmembrane domain of the α subunit was proposed to contain information that marked the subunit for ER degradation (Bonifacino et al., 1990a; Bonifacino et al., 1990b). Furthermore, introduction of single potentially charged residues, arginine and aspartic acid most effectively, into the transmembrane domain of the Tac antigen (interleukin-2 receptor α chain) conferred retention and rapid degradation in the ER (Bonifacino et al., 1991). These observations suggest that exposure of a charged residue in the transmembrane domain is sufficient to act as a signal for ER degradation, since the general folding and transmembrane topology of the Tac mutants did not appear to be affected. Most likely, as subunits assemble to form multimeric complexes, potential ER degradation signals on each subunit become masked by interaction with other subunits. Rapid degradation of the TCR α and β chains were inhibited by assembly with the CD3 ε and γ chains in fibroblasts upon co-expression (Wileman et al., 1990). Similarly, the intracellular degradation of IgM heavy chains was retarded by assembly with light chain even when transport out of the ER was blocked (Sitia et al., 1987).

The ubiquitin/proteasome proteolytic system

Extensive efforts to identify components of a degradative system in the ER lumen have proven to be fruitless. However, a series of recent studies have begun to characterize an unexpected pathway by which soluble and membrane misfolded protein in the ER are delivered to the cytosol, conjugated with several molecules of ubiquitin, a 76 amino acid highly conserved polypeptide, and then targeted for

destruction by the cytosolic 26S proteasome. Although this pathway has not yet been established as the only process by which ER-retained proteins are degraded, it is both logical and advantageous for the cell to maintain compartmental separation of protein folding and maturation that takes place in the ER from degradation of misfolded substrate that takes place in the cytoplasm. Thus nascent chains and proteins in the process of folding would not have to be exposed to a proteolytic system in the same luminal space.

Several observations led to the proposal that mutant ER membrane proteins were being subjected to ubiquitination and rapid degradation by the proteasome. Efficient proteolysis of a mutant form of Sec61p, a component of the yeast translocation complex, was shown to require the activities of both the ER membrane-bound Ubc6p ubiquitin transferase and the proteasome (Sommer and Jentsch, 1993). Treatment with two proteasome inhibitors, ALLN and lactacystin, inhibited degradation of the ER-retained ΔF508 CFTR variant and induced the accumulation of detergent-insoluble polyubiquinated forms of CFTR (Jensen et al., 1995; Ward et al., 1995). Degradation of ΔF508 CFTR was also compromised in a mutant cell line expressing a thermolabile form of the ubiquitin-activating enzyme E1, implying that ubiquitination must precede proteasome-mediated destruction. For mutant membrane proteins, it remains unclear whether the proteasome targets exposed polypeptide loops or termini that face out on the cytoplasmic side of the ER membrane or requires the removal of the entire protein from the membrane into the cytoplasm.

Ubiquitination also appears to signal for the endocytosis of membrane proteins at the cell surface for delivery to the vacuole. In endocytosis mutants, both Ste2p, the α-factor receptor, and Ste6p, which is required for the transport of a-factor, were shown to accumulate as polyubiquitinated forms at the plasma membrane (Kolling and Hollenberg, 1994; Hicke and Riezman, 1996). In these cases, ubiquitination marks

membrane proteins for internalization and targeting to the vacuole rather than for proteasome-mediated degradation.

Degradation of misfolded soluble proteins by the proteasome is subject to a logistical problem since they are completely contained within the ER lumen. The first indication that a factor in the cytoplasm was responsible for the proteolysis of misfolded luminal protein came from in vitro studies investigating the degradation of unglycosylated pro-alpha factor (pαF) in isolated yeast microsomes (McCracken and Brodsky, 1996). Addition of a cytosol fraction to the microsomes induced the selective and rapid degradation of pαF, whereas incubation with either heat-inactivated or trypsin-treated cytosol stabilized pαF, indicating that a protein component in the cytosol was required for pαF degradation. From a genetic screen to identify components of an ER proteolytic system, several mutants were isolated which blocked the degradation of the soluble CPY* active site mutant in the ER (Knop, et al., 1996). One of these mutants was discovered to be *UBC7*, which catalyzes the conjugation of ubiquitin to target substrate (Hiller et al., 1996). Degradation of CPY* was further linked to proteasome activity by kinetic studies in which the rate of degradation was decelerated in strains expressing defective subunits of the proteolytic core of the proteasome (*pre1*, *cim3*, and *cim5*).

Recent analysis of the strategy by which human cytomegalovirus (HCMV) eludes targeted destruction by cytolytic T cells has shed some light on the mechanism that may be used to transfer ER-retained proteins from the lumen out to the cytoplasm for destruction by the proteasome. As part of the immune response to viral invasions, the host cell would normally express a complex between MHC class I molecules and a virus-derived peptide at the cell surface to signal for recognition by cytolytic T cells. To circumvent this host defense, an HCMV-encoded protein US2 was demonstrated to escort newly-synthesized class I molecules into the cytoplasm, where the heavy chain was deglycosylated by an N-glycanase and degraded by the proteasome (Wiertz et

al., 1996). Interestingly, the deglycosylated heavy chain was found to be associated with Sec61p, a component of the translocation complex, suggesting that the translocation pore may be able to conduct both entry of nascent chains into the ER lumen as well as exit of protein out of the ER for exposure to the proteasome. Immature protein retained in the ER may utilize the Sec61 complex to enter the cytoplasm as well since an association between unassembled class I heavy chain and Sec61p was also detected in uninfected cells. Thus, the Sec61 complex is proposed to provide a channel whereby both soluble cargo and membrane-bound protein can be extruded into the cytoplasm for destruction by the proteasome.

Degradation by the lysosomal/vacuolar pathway

The mammalian lysosome and the yeast vacuole are analogous organelles that perform the same function of macromolecular degradation. Both house a wide assortment of proteases and other degradative enzymes, which mediates the turnover of many sources of substrate, including plasma membrane proteins imported from the cell surface by endocytosis, cellular organelles or cytosolic proteins absorbed into the lysosome/vacuole through autophagocytosis, and mutant membrane proteins being transported through the secretory pathway.

Degradation of mutant membrane proteins in the lysosome has been characterized by employing the same types of analysis used in the study of ER-based degradation. Mature-glycosylated erythropoietin receptor (EPO-R) was thought to be targeted to the lysosome for degradation, since proteolysis was inhibited by the addition of NH_4Cl and chloroquine, reagents that impair lysosomal function (Neumann et al., 1993). In support of EPO-R sorting to the lysosome, intact EPO-R and fragments of EPO-R were localized to the lysosome by immunofluorescence studies in cells treated with NH_4Cl. Production of excess amounts of the partial complex α-β or the free δ chains of TcR also resulted in their lysosomal degradation, since treatment with

NH$_4$Cl prevented any proteolytic activity (Minami et al., 1987). Finally, mutant forms of the E1 protein of the coronavirus mouse hepatitis virus A59, in which the first and second transmembrane domains were deleted, were visualized in lysosomal structures in cells treated with chloroquine (Armstrong et al., 1990).

There are analogous examples of mutant membrane proteins being targeted to the yeast vacuole for proteolysis as well. Mutants of the plasma membrane ATPase *PMA1* were sorted to the vacuole instead of being transported to the cell surface (Chang and Fink, 1995). Expression of Pma1p mutants in a *pep4* mutant, which blocks maturation of most vacuolar proteases, resulted in substantial stabilization of the mutant protein. In addition, a hybrid protein composed of the secretory marker invertase fused to mutant forms of the transmembrane and C-terminal domain of the ER membrane protein Wbp1p were also targeted to the vacuole (Gaynor et al., 1994). The C-terminus of wild-type Wbp1p bears the ER retrieval signal for membrane proteins, -KKXX, which is thought to be recognized by subunits of COPI-coated vesicles in the cis-Golgi and signal for recycling of escaped proteins back to the ER (Letourneur et al., 1994). Mutation of the two lysines in the -KKXX signal to glutamine in the invertase-Wbp1p fusion proteins crippled the cis-Golgi to ER recycling pathway, and resulted in re-direction of the fusions to the vacuole for proteolysis.

In chapter 2, the delivery of Inv-(N)cI$_{L57A}$ to the vacuole, where the unstable λ repressor domain undergoes degradation, will be described. We further demonstrate that Inv-(N)cI$_{L57A}$ is a soluble protein, implying that a receptor acts in the trans-Golgi to segregate misfolded protein away from correctly-folded protein being targeted to the plasma membrane.

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Chapter 2

A pathway for targeting soluble misfolded proteins to the yeast vacuole

Abstract

We have evaluated the fate of misfolded protein domains in the *S. cerevisiae* secretory pathway by fusing mutant forms of the N-terminal domain of λ repressor protein to the secreted protein invertase. The hybrid protein carrying the wild-type repressor domain is mostly secreted to the cell surface, whereas hybrid proteins with amino acid substitutions that cause the repressor domain to be thermodynamically unstable are retained intracellularly. Surprisingly, the retained hybrids are found in the vacuole, where the repressor moiety is degraded by vacuolar proteases. The following observations indicate that receptor-mediated recognition of the mutant repressor domain in the Golgi lumen targets these hybrid fusions to the vacuole. (i) The invertase-repressor fusions, like wild-type invertase, behave as soluble proteins in the ER lumen. (ii) Targeting to the vacuole is saturable since overexpression of the hybrids carrying mutant repressor increases the fraction of fusion protein that appears at the cell surface. (iii) Finally, deletion of the *VPS10* gene, which encodes the transmembrane Golgi receptor responsible for targeting carboxypeptidase Y to the vacuole, causes the mutant hybrids to be diverted to the cell surface. Together these findings suggest that yeast have a salvage pathway for degradation of non-native luminal proteins by receptor-mediated transport to the vacuole.

Introduction

Eukaryotic cells have the ability to discriminate between correctly-folded and misfolded proteins within the secretory pathway. Experimentally, secretory proteins can be prevented from achieving their native folded conformations by mutation (Doms et al., 1988; Cheng et al., 1990), expression of single subunits of multisubunit complexes (Lippincott-Schwartz et al., 1988; Wikstrom and Lodish, 1992), or by inhibition of glycosylation or disulfide bond formation (Olden et al., 1979; Braakman et al., 1992). In all of these cases, failure either to fold or to oligomerize properly causes the protein to be retained intracellularly, most often in the endoplasmic reticulum (ER), and then to be degraded. The capacity of the cell to retain and to degrade unfolded or unassembled secretory proteins constitutes a quality control process that prevents secretion of defective gene products to the cell surface and allows for the salvage of amino acids from nonfunctional proteins (de Silva et al., 1990; McCracken and Brodsky, 1996).

To gain access to the mechanisms that underlie quality control of secretory proteins, we have developed a method to examine systematically the fate of unfolded polypeptides in the S. cerevisiae secretory pathway. The idea is to direct a test polypeptide domain into the lumen of the ER as a fusion protein and to compare the fate of a folded, wild-type domain to that of mutant domains that are thermodynamically unstable. We chose as a test protein the 92 amino acid N-terminal DNA-binding domain of the phage λ repressor. Crystallographic and biochemical analyses have shown that the N-terminal domain of λ repressor is a compact globular structure without solvent-exposed hydrophobic regions or flexible strands that might be recognized as unfolded substrate (Pabo and Lewis, 1982). Moreover, folding of the repressor domain in the ER lumen should not be impeded by inappropriate disulfide-bond formation or carbohydrate addition since the amino acid sequence does not contain cysteine residues or potential sites for N-linked glycosylation. The effect of

unfolding of the N-terminal domain of λ repressor can be tested using mutants that reduce the thermal stability of the protein. For most of our work, we use a Leu[57] to Ala mutation that reduces the hydrophobicity of the core of the folded protein, thereby lowering the temperature of 50% thermal denaturation (T_m) from 54°C to 20°C for the purified N-terminal domain (Parsell and Sauer, 1989).

Wild type and mutant λ repressor domains were introduced into the secretory pathway by fusion to the C-terminus of the secretory glycoprotein invertase. Invertase confers several useful properties on the fusion proteins. First, transport of invertase from the ER to the Golgi can be detected by the extension of N-linked carbohydrate chains that occurs in the Golgi (Esmon et al., 1981). Second, addition of polypeptide sequences to the C-terminus of invertase does not interfere with folding of the active enzyme, allowing enzymatic assays to be performed to quantitate the amount of active fusion protein. Finally, once it has folded, dimeric invertase is remarkably stable (Gascon et al., 1968), allowing the invertase moiety to be used as a marker for the fate of the fusion protein, even under circumstances where the λ repressor sequences are degraded.

We find that invertase fused to the wild-type repressor domain is mostly secreted to the cell surface as an intact fusion protein, whereas fusions to thermally unstable repressor domains are mostly retained in the cell. This finding indicated a retention process within the secretory pathway that was able to discriminate between wild-type and mutant versions of the repressor domain. Previous work had shown that either additional peptide sequences or an uncleaved signal sequence at the N-terminus of invertase causes invertase to be retained in the ER (Schauer et al., 1985; Kaiser et al., 1987; Bohni et al., 1988). We expected that misfolded peptide sequences at the C-terminus of invertase would similarly cause retention in the ER. Instead we find that the fusion proteins bearing mutant repressor sequences are transported from the ER and then diverted to the vacuole by a receptor-mediated

targeting process.

Materials and Methods

Strains, reagents, and molecular biological techniques

S. cerevisiae strains are listed in Table I. Standard genetic manipulations, preparation of yeast rich medium (YEP) and minimal medium (SD) (Difco, Detroit, MI), and yeast transformations were performed as described (Kaiser et al., 1994). In strain constructions, the suc2-Δ9 allele was scored by invertase assay. Invertase anti-serum was elicited in rabbits to purified cytoplasmic invertase (gift of D. Botstein). Recombinant lyticase was prepared by expression of β-glucanase from Oerskovia xanthineolytica in E. coli and collection of the enzyme from a periplasmic osmotic shock extract. DNA manipulations and subcloning were carried out using standard techniques (Sambrook et al., 1989). DNA sequencing was performed using the Sequenase kit (United States Biochemical Corp., Cleveland, OH). Restriction enzymes used during plasmid constructions were obtained from New England Biolabs, Beverly, MA.

Construction of invertase and invertase fusion plasmids

The SUC2 gene on a 4.3 kb EcoRI fragment from pRB58 (Carlson and Botstein, 1982) was inserted into the centromeric vector pRS316 to create pEHB2. pEHB29 is pRS316 containing a mutant form of invertase (SUC2-s11) in which the signal sequence cleavage site has been altered from alanine to isoleucine at position 18 (Bohni et al., 1987).

A vector suitable for fusions to the C-terminus of invertase (pEHB9) was constructed by introduction of BglII and NheI restriction sites at the C-terminus of the invertase coding sequence in pEHB2 by site-directed mutagenesis (Kunkel et al., 1987). The mutagenic oligomer consisted of 17 nt of non-complementary sequence containing the BglII (bold) and NheI (underlined) sites, flanked by 16 nt and 18 nt

complementary to the *SUC2* sequence on the 5' and 3' ends respectively: (5'-TAA GTT TTA TAA CCT CTA <u>GCT AGC</u> TGC TG**A GAT CTT** TAC TTC CCT TAC TTG-3').
The mutagenesis was confirmed by DNA sequencing.

Four different versions of the 92 amino acid N-terminal domain of λ repressor (1) wildtype, (2) (N)cl$_{L57A}$, (3) (N)cl$_{L57G}$, and (4) (N)cl$_{LAGA}$, were cloned into pRB104 (Parsell and Sauer, 1989). Additional peptide sequences were appended to the C-terminal end of the repressor domains to facilitate later identification and purification. These are the M2 epitope, DYKDDDDK, for which a monoclonal antibody is available, and a 6x His tag (Davidson and Sauer, 1994). The λ repressor constructs were amplified by PCR using the following primers: (5'-CTC AAG ACC CGT TTA GAG GCC CCA AGG GG-3') complementary to the 3' end and (5'-GGT **GAG ATC** TCA AGC ACA AAA AAG AAA CCA TTA ACA C-3') complementary to the 5' end and which introduces a BglII site (bold). The BglII fragment from the amplified product containing the λ repressor, epitope, and tag was inserted into pEHB9 at the end of the *SUC2* coding sequence to produce: pEHB43 [Inv-(N)cl$_{wt}$], pEHB44 [Inv-(N)cl$_{L57A}$], pEHB51 [Inv-(N)cl$_{L57G}$], and pEHB53 [Inv-(N)cl$_{LAGA}$]. The 4.3 kb SalI-SacI fragment containing Inv-(N)cl$_{L57A}$ from pEHB44 was cloned into the high-copy vector pRS306-2μ to produce pEHB55.

Detection of invertase fusion proteins

Yeast strains were grown in SD medium containing 2% glucose and the appropriate supplements to the exponential growth phase. To induce invertase synthesis, cells were transferred to YEP medium containing 0.1% glucose at a density of 2 x 10^7 cells/ml. Invertase induction was carried out for 2 hours at 30°C (or at 37°C for the restrictive condition of temperature-sensitive strains) followed by a one hour incubation in 2% glucose to repress further synthesis of invertase and to allow the fusion proteins to reach their final cellular location. Cells from 3 ml of culture were

collected by centrifugation, washed with 50mM Tris-Cl pH 7.5, 10mM NaN$_3$, and then suspended in 20 µl of sample buffer (80mM Tris-Cl pH 6.8, 2% SDS, 1.5% DTT, 10% glycerol, 0.1% bromophenol blue). Protein extracts were prepared by heating samples to 100°C for 2 minutes (to rapidly inactivate proteases), breaking cells by vigorous agitation with acid-washed glass beads, and diluting to 0.1 ml with sample buffer.

To spheroplast cells, induced cultures of 6 x 10^7 cells were washed in distilled water, incubated in 0.3 ml of 100mM Tris-SO$_4$ pH 9.4, 50mM ß-merceptoethanol for 10 minutes, washed in 1.2M sorbitol, resuspended in 60 µl of spheroplasting buffer (1.2M sorbitol, 10mM Tris-Cl pH 7.5) containing 60U of recombinant lyticase, and incubated for 30 minutes at 30°C. The endpoint for complete spheroplasting was determined by >85% cell lysis upon dilution into 1% Triton X-100. Centrifugation at 500 x g for 5 minutes yielded a supernatant fraction containing extracellular enzyme and a spheroplast pellet. Pellets were washed in spheroplasting buffer and lysed by vigorous agitation with acid-washed glass beads. Both supernatant and pellet fractions were boiled in sample buffer. In spheroplasting experiments where protease inhibitors were used, 1mM PMSF and 0.5 µg/ml leupeptin (both Boehringer Mannheim Biochemicals, Indianapolis, IN) were added to the YEP containing 0.1% glucose induction medium, spheroplasting buffer, and lyticase.

Samples of 10-20 µl of extracts from whole cells, spheroplasts or spheroplast supernatants were resolved by SDS-PAGE (Laemmli, 1970) on 8% polyacrylamide gels and then electro-blotted to nitrocellulose filters. For Western blot detection, the following antibodies were used: rabbit anti-invertase at 1:1000 dilution, mouse anti-M2 (Eastman Kodak Co., Rochester, NY) at 1:10,000 dilution, HRP-coupled Protein A (Organon Teknika-Cappel, Westchester, PA), at 1:10,000 dilution, and HRP-coupled sheep anti-mouse Ig (Amersham Corp., Arlington Heights, IL) at 1:10,000. Blots were developed using the ECL detection system (Amersham Corp.).

For Endo H digestions, 5 µl of boiled protein extract were diluted with 2-3 volumes of 50mM sodium citrate pH 5.1 containing 100-250U of EndoH$_f$ (New England Biolabs) and incubated at 37°C for 2-16 hours. Samples were boiled prior to gel electrophoresis.

Invertase assays

To quantitate the invertase activity secreted to the cell surface and retained in the cell, spheroplasts and spheroplast supernatants were diluted to 1 ml with 50mM Tris-HCl pH 7.5, 10mM sodium azide. Invertase activity was assayed (Goldstein and Lampen, 1975) from two independent cultures in duplicate for each strain tested. Appropriate dilutions of the spheroplast and supernatant samples for CKY406 (wild-type) cells overexpressing Inv-(N)cl$_{L57A}$ were made to ensure that the assay was always conducted in its linear range.

Indirect immunofluorescence

Indirect immunofluorescence was performed essentially as described (Pringle et al., 1991). CKY414 (*pep4Δ*) cells expressing pEH44 [Inv-(N)cl$_{L57A}$] were fixed in a final concentration of 3.7% formaldehyde for 1 hour at 25°C, collected by centrifugation, and spheroplasted in 0.1M potassium phosphate pH 7.5, 2 µl/ml β-mercaptoethanol, 800U lyticase at 30°C for 30 minutes. Incubations in primary and secondary antibody were performed for one hour each on a coverslip in a dark, humid chamber at 25°C. Invertase was detected using a 1:100 dilution of affinity-purified anti-invertase and a 1:300 dilution of goat anti-rabbit IgG-FITC antibody (Boehringer Mannheim Biochemicals). Invertase antiserum was affinity-purified by adsorption to nitrocellulose strips bearing concentrated amounts of deglycosylated invertase, followed by elution of bound antibodies with 0.2M glycine pH 2.3, 1mM EGTA. Cells were mounted in medium containing 1% p-phenylenediamine and 4'6-diamidino-2-

phenylindole (DAPI) and photographed on a Zeiss axioscope (Carl Zeiss, Inc., Thornwood, NY) with T-Max 400 film (Eastman Kodak Co.) and developed according to the manufacturer's specifications.

Cell fractionation

To evaluate the solubility of invertase fusion proteins, CKY407 (*sec12-4*) cells expressing the invertase fusions at 37°C were spheroplasted as described above. 6 x 10^7 cell equivalents of spheroplast pellets were resuspended in 20 µl cell lysis buffer (200mM sorbitol, 50mM Tris-Cl pH 7.4, 1mM EDTA, 1mM PMSF), lysed with acid-washed glass beads by vigorous agitation 8 x 30 sec with cooling on ice, and the lysate was centrifuged at 500 x g for 5 minutes to remove large cell debris. Membranes were then pelleted at 100,000 x g at 4°C for 10 minutes in a TLA 100.3 ultracentrifuge rotor (Beckman Instruments, Palo Alto, CA). The pellet and supernatant samples representing equal amounts of cell extract were solubilized in sample buffer and invertase was detected by Western blotting.

Results

A fusion between invertase and the N-terminal domain of λ repressor is secreted to the cell surface

The C-terminal end of the *SUC2* gene was modified by the addition of restriction sites, and the N-terminal 92 residues of λ repressor followed by a peptide epitope (M2) and six histidine residues were inserted at this position. This gene fusion expressed a hybrid protein, designated Inv-(N)cl$_{wt}$, which consists of invertase with a total of 111 amino acid residues appended to the C-terminus (Fig. 1).

To examine whether this fusion protein was secreted to the cell surface, wildtype cells (CKY406) expressing Inv-(N)cl$_{wt}$ were converted to spheroplasts, and cell bodies harboring intracellular fusion protein were separated from extracellular fusion protein by centrifugation. Assays of invertase activity showed that the gene fusion expressed a quantity of active enzyme similar to that of the wild-type *SUC2* gene, demonstrating that the additional C-terminal sequences did not interfere with the folding and oligomerization of invertase enzyme in the ER (Table II). Most of the invertase activity from Inv-(N)cl$_{wt}$ was extracellular, indicating successful secretion of this protein to the cell surface. To estimate the fraction of Inv-(N)cl$_{wt}$ that is secreted, it was necessary to subtract the contribution to intracellular activity made by the constitutive, cytoplasmic form of invertase expressed from the *SUC2* promoter (Carlson et al., 1983). This quantity should be equal to the intracellular invertase activity expressed from the wild-type *SUC2* gene, since in steady state most of the intracellular activity is due to the cytoplasmic form of the enzyme. When this quantity of the cytoplasmic form of the fusion protein was subtracted from the total intracellular pool of activity for Inv-(N)cl$_{wt}$, an overall efficiency of secretion to the cell surface of 67% was obtained (Table II).

Examination of Inv-(N)cI$_{wt}$ by Western blotting using anti-invertase antibody demonstrated that both the extracellular and intracellular protein migrated as high molecular weight, heterodispersed forms (Fig. 2a, lanes 5 and 6). These fusion proteins had acquired outer chains on the N-linked carbohydrates in the Golgi since the fusion proteins co-migrated with mature extracellular invertase expressed from the wild-type *SUC2* gene (Fig. 2a, lane 1), and migrated much more slowly than core-glycosylated invertase expressed from *SUC2-s11*, which contains a mutation in the signal peptidase cleavage site that causes invertase to be retained in the ER (Bohni et al., 1987) (Fig. 2a, lane 4). The size of the deglycosylated hybrid protein was examined after removal of the N-linked carbohydrate by digestion with Endo H. The deglycosylated extracellular Inv-(N)cI$_{wt}$ migrated as a discrete band with the predicted molecular weight of the full-length fusion protein (Fig. 2b, lane 5). Antibody to the M2 epitope at the extreme C-terminus of the fusion protein also recognized this band, indicating that extracellular Inv-(N)cI$_{wt}$ was secreted as an intact fusion protein (data not shown). Deglycosylation of the residual intracellular Inv-(N)cI$_{wt}$ yielded a protein of about the size of deglycosylated wild-type invertase (Fig. 2b, compare lanes 1 and 6). This protein was not recognized by antibody to the M2 epitope, suggesting that the C-terminal portion of the fusion protein had been proteolytically removed. In summary, fusion of the N-terminal domain of λ repressor to invertase produced an enzymatically active hybrid protein that was transported to the cell surface at a somewhat lower efficiency than wild-type invertase; for the fusion protein that remained intracellular, the repressor moiety was degraded.

Fusions to thermally unstable λ repressor mutants are retained intracellularly

The behavior of the invertase fusion to the wild type repressor domain was compared to that of thermally unstable repressor mutants. For this analysis, we

examined a set of related mutant repressors: Leu[57] to Ala (L57A); Leu[57] to Gly (L57G); and the triple mutant Leu[57] to Ala, Gly[46] to Ala, Gly[48] to Ala (LAGA). All of these changes reduce the hydrophobicity of residue 57, which is buried in the hydrophobic core of the repressor domain, and thereby destabilize the folded protein. The temperature of 50% thermal denaturation (T_m) of the wild-type repressor and each of the mutants has been determined for purified N-terminal domains and is given in Figure 1 (Parsell and Sauer, 1989). Each mutant repressor was fused to invertase, and the resulting hybrid proteins were tested for transport to the cell surface in wildtype cells (CKY406) by following invertase activity. All of the fusion proteins express a total invertase activity similar to wild-type, demonstrating that the mutant repressor sequences did not interfere with folding and assembly of the enzyme (Table II). The efficiency of transport to the cell surface was determined by the fraction of invertase activity that was extracellular. In contrast to Inv-(N)cl$_{wt}$, which gave mostly secreted fusion protein, the three hybrids bearing unstable repressor domains expressed less than 25% of the invertase activity at the cell surface (Table II), indicating that the mutant domains caused retention of the fusion proteins in the cell.

Examination of the mutant fusion proteins by Western blotting revealed that the retained, intracellular protein bears outer-chain glycosylation, and thus resides in a post-ER compartment (Fig. 2a, lanes 8, 10, and 12). This behavior can be contrasted to that of the SUC2-s11 mutant, which showed a similarly low level of transport of invertase to the cell surface but, as was found previously (Bohni et al., 1987), most of the intracellular protein remained core-glycosylated, indicative of retention in the ER (Fig. 2a, lane 4). The size of the deglycosylated mutant fusions after Endo H digestion was smaller than that expected for the full-length fusion protein and was approximately the size of wild-type invertase (Fig. 2b, lanes 8, 10, and 12). The truncated intracellular protein was not recognized by antibody to the M2 epitope, indicating that the C-terminal sequences were degraded, removing the M2 epitope sequence and, most

likely, the repressor domain. These experiments demonstrate that, whereas the fusion bearing the wild-type repressor domain was secreted almost as well as native invertase, the mutant repressor domains cause retention of the fusion protein and degradation of the repressor sequences.

The denaturation of the repressor domain was further correlated to retention and degradation in the yeast secretory pathway by the behavior of the LAGA triple mutant at reduced temperatures. This mutant carries two Gly to Ala substitutions that stabilize folding of the N-terminal domain (Hecht et al., 1986). These changes partially offset the destabilizing effect of the Leu[57] to Ala substitution, bringing the measured T_m of the triple mutant from 20°C to between 26°C and 28°C (Reidhaar-Olsen et al., 1990). Decreasing the temperature of induction for Inv-(N)cl$_{LAGA}$ from 30°C to 25°C led to the secretion of some full-length Inv-(N)cl$_{LAGA}$ to the cell surface (Fig. 2b, lane 13 and Table II). Presumably, lowering the temperature of induction to 25°C partially stabilized the (N)cl$_{LAGA}$ domain, allowing a larger fraction of the molecules to escape retention and degradation. Reducing the temperature of induction to 25°C did not increase the fraction of Inv-(N)cl$_{L57A}$ secreted to the cell surface (data not shown). Given the lower T_m of the single mutant, most likely a 5°C reduction in temperature was not sufficient to significantly increase the fraction of Inv-(N)$_{L57A}$ that was properly folded.

Degradation of the repressor domain of Inv-(N)cl$_{L57A}$ occurs in the vacuole

We used the Inv-(N)cl$_{L57A}$ fusion to determine where degradation of the mutant hybrids was taking place in the cell. When Inv-(N)cl$_{L57A}$ was expressed in a *sec12* mutant (CKY407), which blocks transport of secretory proteins from the ER at the restrictive temperature, the fusion protein remained at its full length (Fig. 3, lane 2). Mutations in a set of other *sec* mutants that block transport from the ER (*sec13, sec16,*

sec17, *sec18*, *sec22*, *sec23*) similarly prevented degradation of the repressor moiety

of Inv-(N)cl$_{L57A}$ (data not shown), demonstrating that the repressor domain was stable

to proteolysis as long as the fusion protein resided in the ER. In contrast, when Inv-

(N)cl$_{L57A}$ was expressed in a *sec1* mutant (CKY413), which fails to fuse post-Golgi

vesicles with the plasma membrane at the restrictive temperature, proteolysis still

occurred (Fig. 3, lane 3). Together these results showed that transport to the Golgi, but

not to the plasma membrane, was required for degradation of Inv-(N)cl$_{L57A}$.

The possibility that degradation of Inv-(N)cl$_{L57A}$ was occurring in the vacuole

was examined by inactivating vacuolar proteases. The *PEP4* gene encodes the

principal proenzyme-activating protease in the vacuole, and a *pep4Δ* mutant exhibits

greatly reduced activity for most of the vacuolar proteases (Jones, 1991). Disruption of

the *PEP4* gene (in CKY414) largely blocked proteolysis of Inv-(N)cl$_{L57A}$, implicating

vacuolar proteases in the degradation of the repressor moiety (Fig. 3, lane 4).

Similarly, the *pep4Δ* mutation blocked degradation of Inv-(N)cl$_{L57G}$, Inv-(N)cl$_{LAGA}$, and

the intracellular form of Inv-(N)cl$_{wt}$ (data not shown). Targeting of Inv-(N)cl$_{L57A}$ to the

vacuole was further supported by cytological examination of the intracellular location

of the fusion protein. Immunofluorescent staining of Inv-(N)cl$_{L57A}$ with anti-invertase

antibody located the protein to large intracellular bodies, distinct from the DAPI-stained

nuclei, that appeared to be vacuoles (Fig. 4).

Inv-(N)cl$_{wt}$ and Inv-(N)cl$_{L57A}$ are soluble luminal proteins

This case of vacuolar targeting being specified by a mutated domain of a

luminal protein implies that misfolded proteins can be sorted away from properly

folded proteins within the lumen of the Golgi. While there is no precedent for such a

quality control system for sorting soluble proteins at this step of the secretory pathway,

there are several examples where non-native membrane proteins are segregated to

the vacuole (Gaynor et al., 1994; Chang and Fink, 1995). Although the sequence of

the N-terminal domain of λ repressor is not overly hydrophobic, the unfolded repressor

domain may have assumed some of the properties of an integral membrane protein.

To address this possibility, we examined the affinity that Inv-(N)cl$_{L57A}$ and Inv-(N)cl$_{wt}$

have for membranes. The comparison of membrane association was made in a $sec12$

mutant (CKY407) at the restrictive temperature to prevent vacuolar degradation of the

repressor moiety of Inv-(N)cl$_{L57A}$ and to make the comparison for binding to the same

membrane, namely that of the ER. Spheroplast pellets were vigorously lysed to

disrupt the ER, and lysates were centrifuged at 100,000 x g to separate membranes

from the soluble proteins. Wild-type invertase, used as a control, was present mostly

in the supernatant, as expected for a soluble secretory protein (Fig. 5, lanes 1-3).

Similarly, most of Inv-(N)cl$_{wt}$ and Inv-(N)cl$_{L57A}$ fractionated in the supernatant,

confirming that both hybrid proteins also behave as soluble proteins (Fig. 5, lanes 4-6

and 7-9). Thus in cell extracts, the mutant repressor domain of Inv-(N)cl$_{L57A}$ does not

increase the propensity of the protein to associate with membranes, and therefore

sorting to the vacuole most likely takes place in the lumen and not the membrane of

the Golgi.

Inv-(N)cl$_{L57A}$ is secreted to the cell surface upon overexpression

Segregation within the Golgi of misfolded luminal proteins from properly folded

proteins suggested the existence of a receptor that recognizes and binds to the mutant

repressor domain. If delivery to the vacuole is mediated by such a receptor, then

saturation of that receptor by overexpression of Inv-(N)cl$_{L57A}$ should result in secretion

of some of the excess fusion protein to the cell surface. Such a saturation

phenomenon has been observed for the soluble vacuolar proteins carboxypeptidase

Y (CPY) and proteinase A (PrA): in wild type cells, 95% of both proteins reach the

vacuole, but upon overexpression, greater than 50% of CPY and PrA are missorted to

the cell surface (Rothman et al., 1986; Stevens et al., 1986).

To determine whether the targeting process that sorts the mutant repressor fusions to the vacuole is saturable, Inv-(N)cl$_{L57A}$ was overexpressed from the high-copy plasmid pRS306-2μ in a wild-type strain (CKY406). Upon overproduction, 33% of Inv-(N)cl$_{L57A}$ reached the cell surface, compared to 20% in a strain expressing the fusion protein in low-copy (Table III). Analysis of the fusion protein on Western blots revealed that full-length Inv-(N)cl$_{L57A}$ expressed at high level was present in the spheroplast supernatant fraction (Fig. 6, lane 5), whereas no full-length fusion protein expressed in low-copy was secreted to the cell surface (Fig. 6, lane 1). This observation demonstrates that targeting to the vacuole is saturable.

Complicating this analysis is the finding that the extracellular invertase from overexpressed Inv-(N)cl$_{L57A}$ was present in two forms: a form that co-migrated with the undegraded intracellular fusion in a pep4Δ strain (Fig. 6, lane 4), and a partially-truncated form that migrates more slowly than the truncated protein in the vacuole (Fig. 6, lane 5). This partially-truncated form was sometimes detected to a much lesser extent in the extracellular fraction of both wild-type and pep4Δ strains expressing Inv-(N)cl$_{L57A}$ in low copy (Fig. 7, lane 3). The small amount of partially-truncated Inv-(N)cl$_{L57A}$ in the spheroplast pellet of CKY414 (Fig. 7, lane 4) was probably due to incomplete spheroplasting and this band was not visible in similar experiments. Since the partially-truncated form was found in spheroplast supernatants, we suspected that Inv-(N)cl$_{L57A}$ was being exposed to periplasmic proteases once the fusion protein has reached the cell surface. Presumably, this partially-truncated species was not generated when hybrid protein containing wild-type repressor was expressed at the cell surface because of the relative stability of the wild-type repressor domain. As expected for a degradation process that occurred only on the cell surface, none of the partially-truncated form of the hybrid protein was detected when transport to the cell surface of overexpressed Inv-(N)cl$_{L57A}$ was blocked in a sec1 mutant at the restrictive temperature (Figure 6, lanes 7-8). The only forms of Inv-(N)cl$_{L57A}$ detected in the sec1

mutant were the full-length fusion protein and the truncated fusion protein apparently produced by vacuolar proteolysis that comigrates with wild-type invertase (Figure 6, lane 8). We originally suspected that the partial truncation of the fusion protein on the cell surface resulted from the action of proteases in the lytic enzyme preparation used for spheroplasting. However, this did not appear to be the case since the partially-truncated fusion protein was detected in cell lysates that were prepared by mechanical disruption without the use of lytic enzymes. Attempts to eliminate the extracellular proteolysis of Inv-(N)cl$_{L57A}$ by adding the protease inhibitors PMSF and leupeptin to cultures during invertase induction and spheroplasting, also failed to prevent formation of the partially-truncated fusion protein. We conclude that both the full-length and partially-truncated proteins represent Inv-(N)cl$_{L57A}$ secreted to the cell surface during overexpression, most likely resulting from saturation of a receptor-mediated targeting system that sorts Inv-(N)cl$_{L57A}$ to the vacuole.

Inv-(N)cl$_{L57A}$ is missorted to the cell surface in *vps10Δ*

Recent studies have identified Vps10p as a transmembrane receptor in the late-Golgi that is required for targeting the soluble vacuolar resident proteins CPY and PrA (Marcusson et al., 1994; Cooper and Stevens, 1996). In a *vps10Δ* strain, ~90% of CPY and ~50% of PrA are diverted to the cell surface. Because Vps10p is the only known receptor for sorting luminal protein to the vacuole, we examined the possibility that Vps10p also participated in the delivery of the mutant repressor fusions to the vacuole. Indeed, in a *vps10Δ* strain (EMY3), the mutant repressor fusions were diverted to the cell surface rather than to the vacuole. Approximately 67% of the enzymatic activity in *vps10Δ* strains expressing Inv-(N)cl$_{L57A}$, Inv-(N)cl$_{L57G}$, and Inv-(N)cl$_{LAGA}$ partitioned with the spheroplast supernatant, compared to 20% in a wild-type strain (CKY406) expressing Inv-(N)cl$_{L57A}$ (Table III). On Western blots of *vps10Δ* strains, almost all of the mutant repressor fusions were present in the extracellular fraction (Fig. 7, lanes 5,

7, 9) with very little fusion protein retained intracellularly (Fig. 7, lanes 6, 8, 10). Similar to the case when Inv-(N)cl$_{L57A}$ was overexpressed, two forms of the extracellular fusion protein were seen: a full-length fusion that co-migrates with undegraded intracellular fusion in a *pep4Δ* strain (Fig. 7, lane 4) and the partially-truncated form derived from proteolysis of the full-length fusion in the periplasmic space (Fig. 7, lanes 5, 7, and 9). Thus in a *vps10Δ* strain, about as much fusion protein bearing mutant repressor domains reached the cell surface as fusions bearing the wild-type repressor in a wild-type strain. However, the majority of the hybrid protein bearing mutant repressor domains that reach the cell surface in *vps10Δ* are then partially degraded by an unknown extracellular protease to yield partially-truncated forms of the hybrid protein. These results demonstrate that Vps10p is required to target the mutant repressor fusions to the vacuole.

Discussion

A large body of both published experimental data and unpublished lore indicates that incorrectly-folded proteins or incompletely-assembled protein complexes are usually degraded within the secretory pathway before they can reach the cell surface. In an effort to understand how cells discriminate between correctly and incorrectly-folded proteins, we designed a system to determine the fate of the N-terminal domain of λ repressor protein within the secretory pathway of *S. cerevisiae*. The repressor protein domain was chosen because of its compact globular structure and the availability of mutations that cause thermal denaturation of this domain. Invertase fused to the N-terminal wild-type repressor domain passes through the ER and reaches the cell surface as a full-length fusion protein, demonstrating that the wild-type repressor domain has little effect on the transport of invertase through the secretory pathway. In contrast, mutations that reduce the T_m of the N-terminal repressor domain cause the invertase fusions bearing these mutant repressor domains to be targeted to the vacuole, where the repressor sequences are degraded by proteases. Thus, the mutant repressor does not elicit retention of the fusion protein in the ER, but instead has the capacity to act as a vacuolar targeting signal.

The yeast vacuole can be considered to be analogous to the mammalian lysosome as an organelle devoted principally to macromolecular degradation. The vacuole is characterized by its content of proteases and other degradative enzymes, which under conditions of nutrient deprivation, mediates much of the protein turnover in a yeast cell (Teichert et al., 1989). Several different pathways have been identified by which proteins can be targeted to the vacuole for degradation. A number of membrane proteins including pheromone receptors and permeases, whose abundance in the plasma membrane is regulated, are removed from the plasma membrane and transported to the vacuole for degradation via the endocytic pathway (Davis et al., 1993; Berkower et al., 1994; Schandel and Jenness, 1994; Volland et al.,

1994; Hicke and Riezman, 1996). A second path for protein import into the vacuole is autophagocytosis where, in response to nutrient deprivation, portions of the cytosol and organelles are engulfed and degraded by the vacuole (Takeshige et al., 1992). Autophagocytosis or direct translocation across the vacuolar membrane have been proposed as mechanisms for vacuolar degradation of the cytosolic enzymes fructose 1,6-bisphosphatase and the β subunit of fatty acid synthase (Chiang and Schekman, 1991; Egner et al., 1993; Schork et al., 1994). Finally, in a number of cases, mutant membrane proteins have been shown to be targeted to the vacuole. These include mutants of the plasma membrane ATPase that are directed to the vacuole rather than the plasma membrane (Chang and Fink, 1995) and a hybrid protein composed of the transmembrane domain of the ER membrane protein Wbp1p fused to invertase (Gaynor et al., 1994). An apparently analogous process for the degradation of incorrectly-assembled membrane protein complexes has been observed in mammalian cells, where the Golgi coronavirus E1 protein and excess α-β and δ subunits of the T-cell receptor complex were shown to be substantially stabilized by treatment of cells with agents that inhibit lysosomal proteases (Minami et al., 1987; Armstrong et al., 1990).

Here we describe a new mechanism for delivery of proteins to the vacuole, namely, targeting of a luminal protein to the vacuole as specified by an incorrectly-folded protein moiety. We considered the possibility that targeting of invertase fusions to the vacuole may occur by a mechanism similar to that described for mutant membrane proteins. Although the repressor sequence is quite hydrophilic, it seemed possible that unfolding of the repressor domain might expose hydrophobic regions of the protein that are normally buried and convert the hybrid protein into a membrane protein. We experimentally addressed this possibility by testing the solubility of fusions to mutant and wild-type repressor domains and found both to be as soluble as wild-type invertase. The most convincing evidence that the fusions to the mutant

repressor are sorted by a process that occurs in the Golgi lumen as opposed to the Golgi membrane is the dependence of vacuolar targeting on Vps10p, a membrane protein shown to sort luminal proteins.

Vps10p is a 178 kD type I integral membrane protein that acts as a receptor in the late-Golgi for targeting of the precursor forms of CPY and PrA to the vacuole (Marcusson et al., 1994; Cooper and Stevens 1996). The interaction between Vps10p and the ligand sequence in the pro-region of CPY has been extensively studied. Four contiguous amino acid residues Q^{24}-R-P-L^{27} in the pro-region of CPY define the sequence required for interaction with Vps10p as identified by deletion mapping and mutational studies (Johnson et al., 1987; Valls et al., 1990). The critical role that the QRPL sequence plays in vacuolar sorting was further illustrated by the demonstration that mutation of Q^{24} to K disrupts the ability to chemically cross-link proCPY to the Vps10p receptor (Marcusson et al., 1994).

Vps10p is also capable of acting as a vacuolar targeting receptor for proteins that do not have the QRPL determinant. Mutational analysis of the QRPL sequence in the pro-region of CPY revealed a tolerance for many different amino acid substitutions, especially at positions R^{25} and P^{26}, that show only a slight defect in the sorting of CPY to the vacuole (van Voorst et al., 1996). Vps10p has also been shown to be partly responsible for targeting proPrA to the vacuole (Westphal et al., 1996; Cooper and Stevens, 1996). Vps10p apparently recognizes the pro-region of proPrA since a hybrid protein composed of the pro-region fused to invertase is targeted to the vacuole in a Vps10p-dependent manner (Klionsky et al., 1988; Westphal et al., 1996), despite the fact that the pro-region of PrA does not contain QRPL or related sequences. Thus, recognition of luminal vacuolar proteins by Vps10p can apparently involve a wide variety of sequence determinants, some of which may have the qualities of unfolded polypeptides instead of a specific peptide motif. The degeneracy of vacuolar sorting signals in general, and Vps10p ligands in particular, suggests that one aspect of

vacuolar sortting signals may be an extended flexible polypeptide that can be encompassed by a large number of sequences with little common primary sequence. Indeed, spectroscopic evaluation of the conformation of the pro-region of CPY expressed in *E. coli* revealed that the pro-region assumes a flexible conformation with little regular secondary structure (Sorensen et al., 1993). It is therefore possible that accessibility to an extended polypeptide chain in an unfolded state may be part of the mechanism by which Vps10p binds to its ligands.

Although it is possible that the repressor missense mutations alter the primary structure so as to fortuitously create short peptide sequences that are recognized as vacuolar targeting signals, we greatly favor the interpretation that the mutations generate a more general type of vacuolar targeting signal by causing the repressor domain to be unfolded. The repressor folding defect caused by the Leu[57] to Ala mutation is partially suppressed by second-site mutations at two Gly residues that have been shown to increase the T_m of the N-terminal domain of λ repressor by 6-8°C (Hecht et al., 1986; Reidhaar-Olsen et al., 1990). Introduction of these mutations reduces the ability of the corresponding invertase fusion to be targeted to the vacuole, giving direct evidence that retention of the fusion proteins is related to the effect that the sequence alterations have on protein folding and stability. As a control to show that vacuolar targeting was not simply a consequence of changing Leu[57], we tested another destabilizing repressor mutation that contains an amino acid change at a different position, Leu[69] to Gly, which similarly lowers the T_m of the N-terminal domain measured *in vitro* (R. T. Sauer, unpublished observations). The corresponding invertase fusion of this mutant was also targeted to the vacuole, emphasizing that unfolded determinants are key in re-directing the mutant hybrids. Finally, the ability of unfolded polypeptides to specify vacuolar targeting appears to be quite general since invertase molecules with randomly generated amino acid sequences appended to the C-terminus also specify targeting of the fusion protein to the vacuole (E. Hong, A.

Davidson, M. Cordes, and C. Kaiser, unpublished observations). These results suggest that there is a general mechanism by which cells can recognize misfolded proteins for targeting to the vacuole.

Given that capture of ligands by Vps10p may partly involve recognition of an extended polypeptide chain, it is possible that the repressor domains containing destabilizing mutations mimic vacuolar targeting sequences. We tested the idea that Inv-(N)cl$_{L57A}$ binds to the same site on Vps10p as proCPY by determining whether overexpression of Inv-(N)cl$_{L57A}$ can cause secretion of proCPY into the extracellular medium. No effect on CPY secretion was seen when Inv-(N)cl$_{L57A}$ was expressed from a 2µ plasmid. Similarly, in the converse experiment, overexpression of CPY did not result in an increased level of Inv-(N)cl$_{L57A}$ at the cell surface. This apparent absence of competition leads us to conclude that the repressor sequences and proCPY bind to different sites on the luminal domain of Vps10p.

Alterations of the N-terminus of invertase that prevent cleavage of the signal peptide cause invertase to be retained within the ER (Schauer et al., 1985; Kaiser et al., 1987). Initially, we expected that additional sequences which assume an unfolded conformation appended to the C-terminus of invertase would also cause a similar retention in the ER. It appears that neither the wild-type repressor domain nor the mutant repressors possess the sequence or structural characteristics that elicit ER retention. Possibly the repressor sequences are not as hydrophobic as signal sequences, and hydrophobicity may be an important determinant for ER retention, whether by association with chaperone cofactors or binding to the ER membrane. Thus there appear to be at least two different stages at which proteins that are not correctly-folded are segregated from normally-folded proteins en route to the cell surface. Retention of misfolded proteins in the ER would allow additional opportunities for folding in the presence of chaperones such as BiP and PDI to take place. Eventually, if folding cannot be completed in the ER, it would be advantageous to rid

the cell of these polypeptides and to recover the amino acids. Clearly one way to accomplish this would be to have a mechanism whereby misfolded proteins are allowed to leave the ER but are then targeted to the vacuole for degradation. The mutant repressor sequences do not appear to have the characteristics needed for ER retention but do contain the necessary determinants for targeting to the vacuole.

Acknowledgements

Figure Legends

Figure 1: Structure of the invertase-λ repressor fusions. The coding sequence of invertase was fused to the N-terminal 92 amino acid DNA-binding domain of λ repressor (light stippled box), the 8 amino acid M2 epitope (dark stippled box) and a 6x histidine tag (black box). The amino acid sequence for residues 45-60 of the wild-type λ repressor and three mutants are shown. T_m measurements for the N-terminal λ repressor domains were obtained previously (Parsell and Sauer, 1989; Reidhaar-Olsen et al., 1996).

Figure 1

Hong et al., 1996

invertase

invertase

λ repressor N-terminal domain

. . . VDNLFYIDKFQVREVKKIS STKKKPLTQEQLEDARRLKAIYEKKKNQLGLSQESVADKMGMG

M2

QSGVGALFNGINALNAYNAALLAKILKVSVEEFSPSIAREIYEMTEAVSM LDYKDDDDKHHHHHH– (COOH)

residue 45   60

T_m (°C)
of λ (1-93)
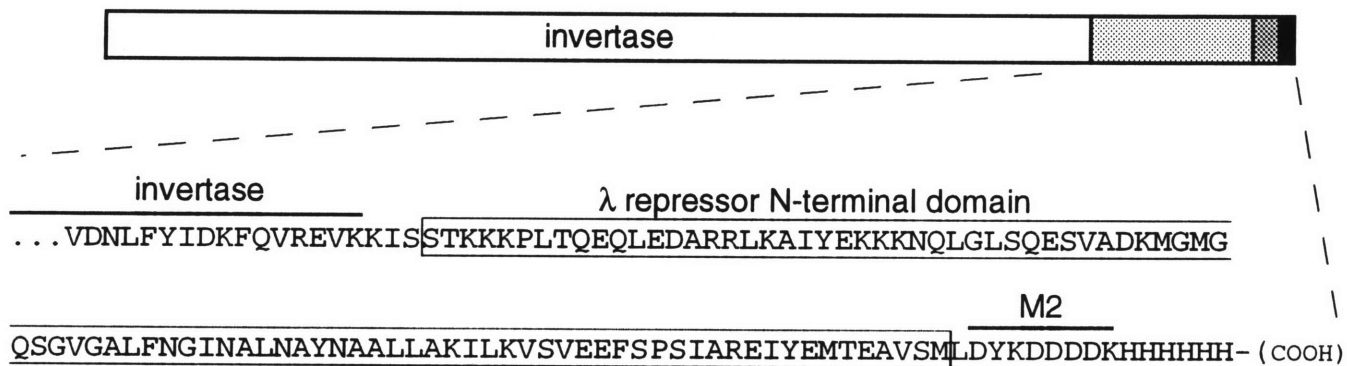
Sequence	Construct	T_m (°C)
S G V G A L F N G I N A L N A Y	Inv-(N)cl$_{WT}$	54
- - - - - - - - - - - - A - - -	Inv-(N)cl$_{L57A}$	20
- - - - - - - - - - - - G - - -	Inv-(N)cl$_{L57G}$	0-4
- A - A - - - - - - - A - - -	Inv-(N)cl$_{LAGA}$	26-28

Figure 2: Inv-(N)cl$_{wt}$ is secreted to the cell surface whereas fusions bearing mutant repressor domains are retained intracellularly, and the repressor domains are degraded. (a) CKY406 (wild-type) expressing different forms of invertase were converted to spheroplasts to separate extracellular protein in the supernatant (S) from the intracellular invertase in the pellet (P). The following forms of invertase were examined: wildtype invertase $SUC2^+$ (lanes 1-2), the $SUC2$-$s11$ signal sequence cleavage mutant (lanes 3-4), Inv-(N)cl$_{wt}$ (lanes 5-6), Inv-(N)cl$_{L57A}$ (lanes 7-8), Inv-(N)cl$_{L57G}$ (lanes 9-10), Inv-(N)cl$_{LAGA}$ induced at 30˚C (lanes 11-12), and Inv-(N)cl$_{LAGA}$ induced at 25°C (lanes 13-14). Protein was solubilized, resolved by SDS-PAGE on 8% polyacrylamide gels and invertase was detected by Western blotting with invertase anti-serum. Secreted, mature-glycosylated invertase migrates heterogeneously at ~140 kD. Core-glycosylated invertase migrates at ~90 kD for wildtype invertase and 110 kD for the repressor fusions. (b) Extracts were digested with Endo H to remove N-linked carbohydrate chains. Deglycosylated wildtype invertase migrates at 59 kD and fusion invertase at 71 kD.

Figure 2
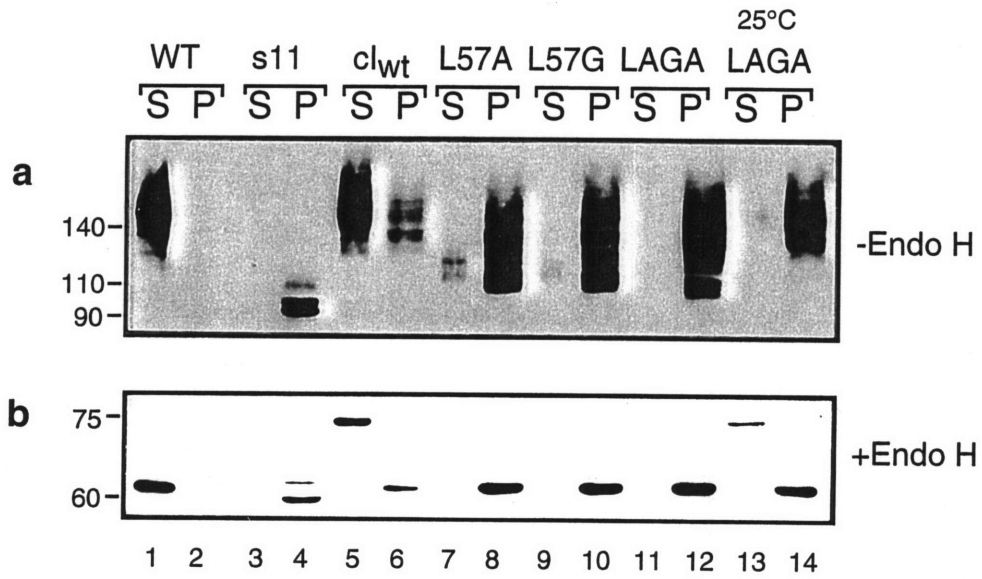
Figure 3: The repressor domain of Inv-(N)cl$_{L57A}$ is degraded in the vacuole. Inv-(N)cl$_{L57A}$ expression was induced in CKY406 (wild-type), CKY407 (*sec12-4*), CKY413 (*sec1-1*), and CKY414 (*pep4Δ*) at the restrictive temperature of 37°C. Protein extracts were treated with Endo H, resolved on an 8% SDS-polyacrylamide gel, and invertase visualized by Western blotting with invertase anti-serum.

Hong et al., 1996
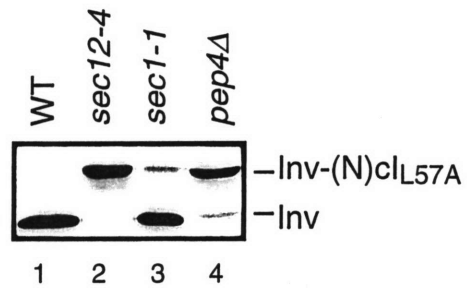
Figure 3

Figure 4: Inv-(N)cl$_{L57A}$ is localized in large intracellular bodies. CKY414 (*pep4Δ*) expressing Inv-(N)cl$_{L57A}$ was examined by indirect immunofluorescence using affinity-purified rabbit anti-invertase antibody and a fluorescein-conjugated anti-rabbit secondary antibody (FITC). Also shown are stained nuclear DNA (DAPI) and cell bodies visualized by light microscopy (DIC).

Figure 4

FITC   DAPI   DIC
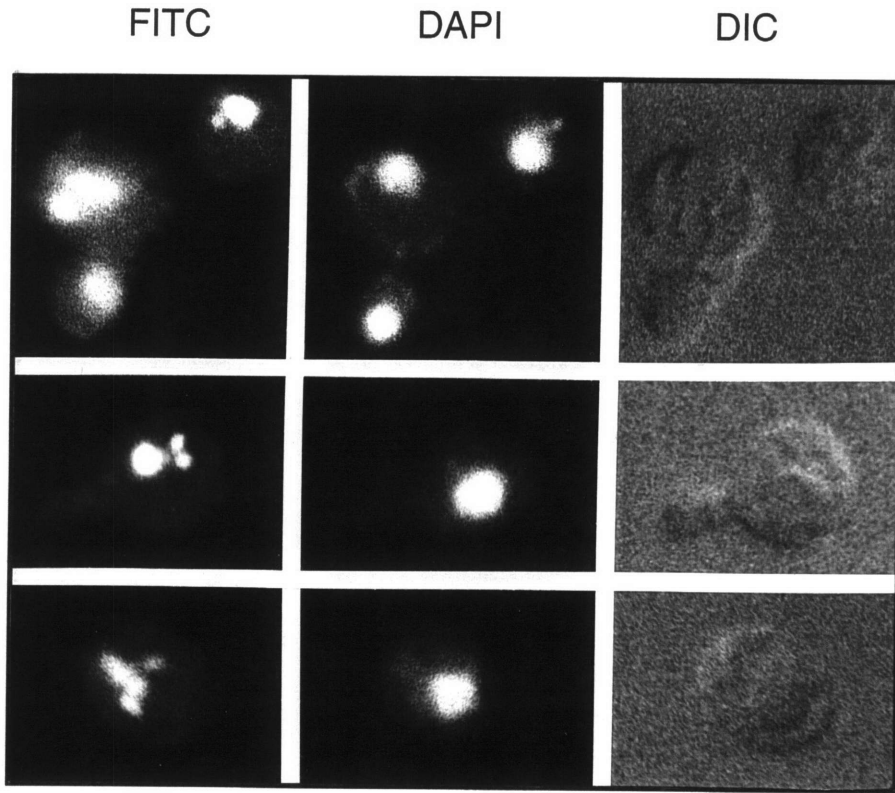
Figure 5: Inv-(N)cl$_{wt}$ and Inv-(N)cl$_{L57A}$ behave as soluble proteins in the ER lumen. CKY407 (*sec12-4*) was induced for expression of wildtype invertase (lanes 1-3), Inv-(N)cl$_{wt}$ (lanes 4-6), and Inv-(N)cl$_{L57A}$ (lanes 7-9) at the restrictive temperature of 37°C to keep invertase in the ER. Cells were converted to spheroplasts which were lysed by vigorous agitation with glass beads. Lysates were centrifuged at 100,000 x g to give high speed supernatant (HSS) and high speed pellet (HSP) fractions. Unfractionated lysate represents a control for total protein (T). Protein samples were analyzed on an 8% SDS-polyacrylamide gel and by Western blotting using invertase anti-serum.

Figure 5
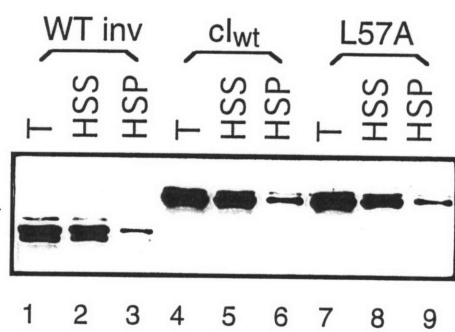
Figure 6: Overexpressed Inv-(N)cl$_{L57A}$ is secreted to the cell surface. CKY406 (wildtype) (lanes 1-2) and CKY414 (*pep4Δ*) (lanes 3-4) expressing pEHB46 [Inv-(N)cl$_{L57A}$-CEN] and CKY406 (wildtype) (lanes 5-6) and CKY413 (*sec1-1*) (lanes 7-8) expressing pEHB55 [Inv-(N)cl$_{L57A}$-2μ] were induced for invertase expression at 30°C (37°C for *sec1-1*) and spheroplasted to separate the supernatant (S) and pellet (P) fractions. Extracts were digested with Endo H, resolved on an 8% SDS-PAGE gel, and invertase visualized by Western blotting using invertase anti-serum. The protein amount in the fractions derived from expression of Inv-(N)cl$_{L57A}$-2μ, lanes 5-8, represent one-fifth of the protein loaded in lanes 1-4.

Figure 6
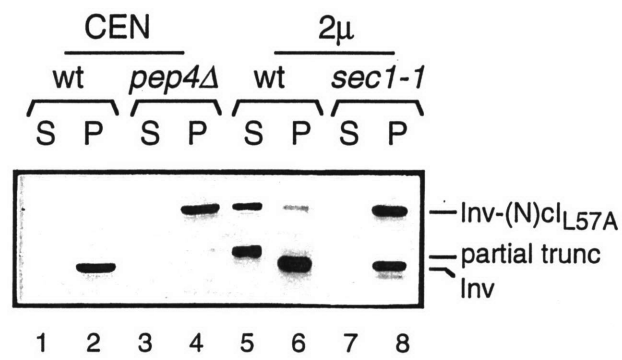
Figure 7: Vps10p is required to target Inv-(N)cl$_{L57A}$ to the vacuole. CKY406

(wildtype) (lanes 1-2), CKY414 ($\Delta pep4$) (lanes 3-4), EMY3 ($\Delta vps10$) (lanes 5-6)

expressing pEHB46 [Inv-(N)cl$_{L57A}$], EMY3 ($\Delta vps10$) expressing pEHB51 [Inv-(N)cl$_{L57G}$]

(lanes 7-8), and pEHB53 [Inv-(N)cl$_{LAGA}$] (lanes 9-10) were treated as described in the

legend of Figure 6. A wildtype strain (SEY6210), isogenic to EMY3, expressing

pEHB46 exhibited the same pattern of cleaved Inv-(N)cl$_{L57A}$ in the pellet fraction as

seen in CKY406 (lanes 1-2).

Figure 7
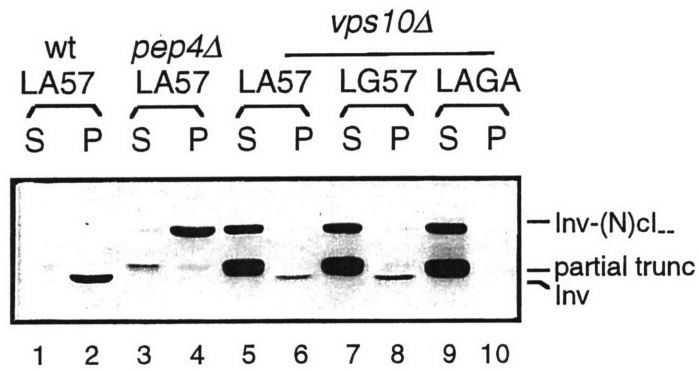
Figure 8: Model for the secretory fates of Inv-(N)cI$_{wt}$ and Inv(N)cI$_{L57A}$ in the cell.

(a) The majority (67%) of Inv-(N)cI$_{wt}$, invertase fused to the wild-type N-terminal domain of λ repressor, is successfully secreted to the cell surface. In contrast, the majority (77%) of Inv-(N)cI$_{L57A}$, invertase fused to a destabilizing mutant form of the N-terminal domain of λ repressor, is targeted to the vacuole, where the repressor moiety undergoes proteolysis. (b) In the trans-Golgi, the Vps10p receptor sorts proCPY and Inv-(N)cI$_{L57A}$ to the vacuole by recognition of the N-terminal pro-region of CPY and the C-terminal mutant repressor domain of Inv-(N)cI$_{L57A}$. Inv-(N)cI$_{wt}$ bears a folded repressor domain and does not elicit Vps10p sorting. This diagram emphasizes our hypothesis that Vps10p binding to ligand may require access to an unfolded polypeptide chain.

Figure 8
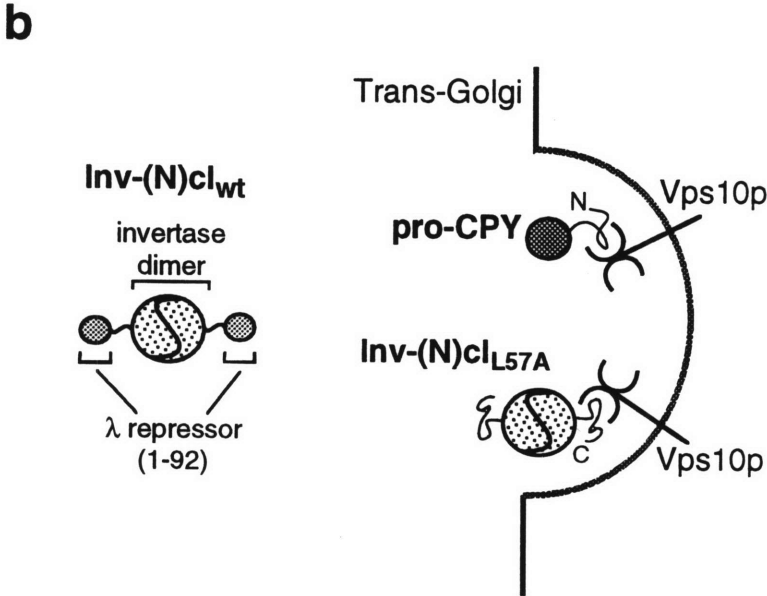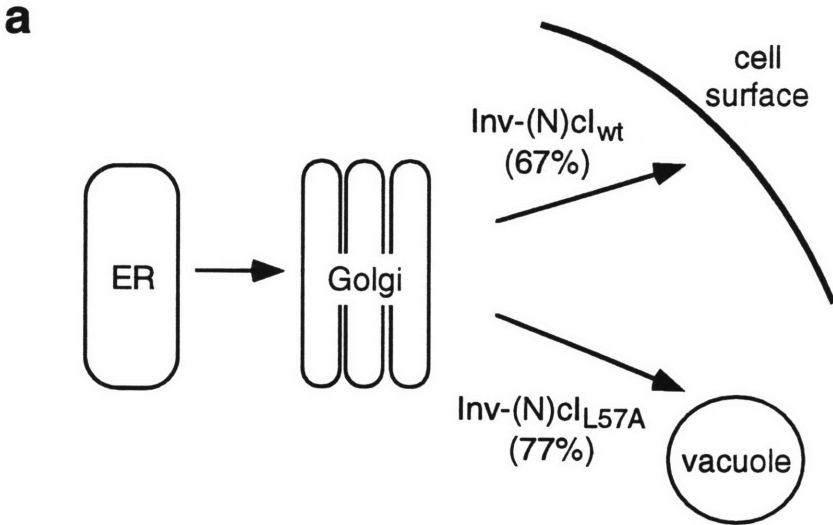
a

b

Table I. *Saccharomyces cerevisiae* Strains

Strain	Genotype	Source or Reference
CKY406	*MATa ura3-52 leu2-3,112 suc2-Δ9*	This study
CKY407	*MATa sec12-4 ura3-52 leu2-3,112 suc2-Δ9*	This study
CKY408	*MATα sec13-1 ura3-52 leu2-3,112 suc2-Δ9*	This study
CKY409	*MATα sec16-2 ura3-52 his4-619 suc2-Δ9*	This study
CKY410	*MATa sec17-1 ura3-52 suc2-Δ9*	This study
CKY420	*MATa sec18-2 ura3-52 leu2-3,112 suc2-Δ9*	This study
CKY411	*MATa sec22-3 ura3-52 leu2-3,112 suc2-Δ9*	This study
CKY412	*MATa sec23-1 ura3-52 his4-619 suc2-Δ9*	This study
CKY413	*MATα sec1-1 ura3-52 ade2-101 suc2-Δ9*	This study
CKY414	*MATa pep4::LEU2 ura3-52 leu2-3,112 suc2-Δ9*	This study
SEY6210	*MATa leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*	Marcusson et al., 1994
EMY3	*MATa Δvps10::HIS3 leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*	Marcusson et al., 1994

Table II. Distribution of periplasmic and intracellular activity for invertase-repressor fusions.

Invertase construct	Invertase Activity (U/OD) *		% secreted to the cell surface [b]
	Periplasmic	Intracellular	
WT invertase	1.22 ±0.14	0.12 ±0.03	100
Inv-s11	0.12 ±0.01	0.47 ±0.07	26
Inv-(N)cl$_{wt}$	0.75 ±0.06	0.49 ±0.04	67
Inv-(N)cl$_{L57A}$	0.29 ±0.06	1.08 ±0.18	23
Inv-(N)cl$_{L57G}$	0.27 ±0.06	1.03 ±0.18	23
Inv-(N)cl$_{LAGA}$ (30°C)	0.26 ±0.02	1.01 ±0.13	23
Inv-(N)cl$_{LAGA}$ (25°C)	0.25 ±0.01	0.62 ±0.05	33

The mean and standard deviation from four assays (two each from two independent cultures) are given.

* One unit (U) of invertase releases 1 μmol of glucose from sucrose per minute at 37°C.

[b] % secreted $= \dfrac{\text{periplasmic activity}}{\text{total activity-cytoplasmic activity}_{\text{(for WT inv.)}}}$

Table III. Redistribution to the cell surface for Inv-(N)cl$_{L57A}$ overexpression and mutant repressor fusions in vps10Δ.

Strain/ Invertase fusion	Invertase Activity (U/OD) *		% secreted to the cell surface [b]
	Periplasmic	Intracellular	
wildtype/ Inv-(N)cl$_{L57A}$ (CEN)	0.29 ±0.07	1.31 ±0.08	20
wildtype/ Inv-(N)cl$_{L57A}$ (2μ)	2.21 ±0.24	5.25 ±0.44	33
vps10Δ/ Inv-(N)cl$_{L57A}$ (CEN)	0.69 ±0.06	0.48 ±0.03	66
vps10Δ/ Inv-(N)cl$_{L57G}$ (CEN)	0.56 ±0.06	0.41 ±0.02	66
vps10Δ/ Inv-(N)cl$_{LAGA}$ (CEN)	0.66 ±0.07	0.44 ±0.03	67

The mean and standard deviation from four assays (two each from two independent cultures) are given.

* One unit (U) of invertase releases 1 μmol of glucose from sucrose per minute at 37°C.

[b] % secreted = $\dfrac{\text{periplasmic activity}}{\text{total activity-cytoplasmic activity}_{\text{(for WT inv.)}}}$

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Chapter 3

Recycling of misfolded protein from the Golgi to ER is dependent on BiP and COPI function in yeast

Abstract

We have characterized in yeast a quality control mechanism in which misfolded proteins are recycled from the Golgi back to the ER. We had previously observed that fusion proteins composed of invertase and thermally-unstable mutant forms of the N-terminal domain of λ repressor were targeted to the vacuole, where the repressor domain underwent degradation. In this study, we demonstrate that one of these mutant hybrids, Inv-(N)cI$_{L57A}$, bearing Golgi-derived α1,6-mannose modifications can be isolated from a fraction enriched for the ER. From kinetic analysis, the rate of vacuolar degradation of the mutant repressor domain is increased up to 2.5 fold in mutants of four COPI subunits, γ-COP, β'-COP, and newly-identified δ-COP and β-COP alleles, and in two mutants impaired for retrieval of Kar2p, the yeast analog of BiP. Furthermore, we are able to detect a physical interaction between Kar2p and α1,6-modified Inv-(N)cI$_{L57A}$. A simple interpretation of these findings is that Kar2p binds to the mutant repressor domain in the cis-Golgi and recycles the hybrids back to the ER through its association with the Kar2p receptor, Erd2p, via COPI-coated retrograde vesicles. Consistent with this proposed model, mutations in the four different COPI subunits cause more Kar2p to be secreted into the extracellular medium.

Introduction

The secretory pathway in eukaryotic cells displays stringent requirements for the transport of cargo molecules to the cell surface. Secretory proteins that do not acquire the native folded conformation (for review, see Gething and Sambrook, 1992) or proper oligomeric state (Kreis and Lodish, 1986) are retained in the cell by a quality control process that monitors for mutant protein and degrades irreversibly misfolded substrate (de Silva et al., 1990). The observation that several secretory proteins exhibit different rates of transport out of the endoplasmic reticulum (ER) (Lodish et al., 1983) has long focused study of the quality control system on mechanisms located in the ER. Extensive studies in yeast and mammalian systems have demonstrated that many mutant secretory proteins, such as invertase bearing an uncleaved signal sequence (Schauer et al., 1985), the temperature-sensitive ts045 folding mutant of vesicular stomatitis virus (VSV) G protein (Doms et al., 1987), and the ΔF508 form of the cystic fibrosis transmembrane conductance regulator (Cheng et al., 1990), are blocked from exiting the ER. Mutant proteins were presumed to be degraded by an ER-localized degradative system to salvage constituent amino acids for the synthesis of new protein. Although components of this ER system remain unidentified, degradation of some misfolded ER proteins has been shown to be mediated by the cytoplasmic proteasome (Ward et al., 1995; Hiller et al., 1996) through reverse translocation of ER-retained protein via the Sec61 pore complex into the cytoplasm (Wiertz et al., 1996).

In a recent study to evaluate the efficiency of quality control in yeast, we obtained evidence that administration of quality control is, in fact, not limited to the confines of the ER. We identified a novel sorting mechanism that acts in the late-Golgi to target misfolded protein to the vacuole for degradation (Hong et al., 1996). The secretory fate of hybrid proteins composed of the secretory marker protein invertase

fused to either wild-type or mutant destabilized forms of the N-terminal domain of λ repressor was examined. While the majority of invertase fused to the wildtype λ repressor was successfully secreted to the cell surface, fusions to several mutant forms of λ repressor were diverted to the vacuole, where the repressor domain was degraded by vacuolar proteases. These mutant repressor fusions were shown to be soluble proteins and delivered to the vacuole by a receptor-mediated process dependent on Vps10p, the receptor responsible for the targeting of vacuolar hydrolases (Marcusson et al., 1994; Cooper and Stevens, 1996). This analysis provided strong evidence that a mechanism functions in the late-Golgi to segregate misfolded protein to the vacuole from traffic continuing on to the cell surface.

During kinetic analysis of the repressor hybrids, we observed that Inv-(N)cl$_{L57A}$, one of the mutant repressor hybrids, acquired outer-chain glycosylation at a slower rate than wildtype invertase, as if transport of Inv-(N)cl$_{L57A}$ was being retarded between the ER and the cis-Golgi. In addition, we were able to isolate Inv-(N)cl$_{L57A}$ bearing Golgi-derived α1,6-mannoses from a fraction enriched for the ER, suggesting that the mutant repressor hybrids reached the cis-Golgi but then underwent a recycling process back to the ER. Recycling between the cis-Golgi and ER has been reported mostly for ER resident soluble and membrane proteins that have escaped ER retention but are then retrieved through recognition of their C-terminal retrieval signals, H(K)DEL (Munro and Pelham, 1987) and -KKXX (Nilsson et al., 1989), respectively. However, two examples of misfolded proteins, unassembled MHC class I molecules and the ts045 folding mutant of the VSV G protein, have been described to escape ER retention and be recycled from the cis-Golgi back to the ER in mammalian cells (Hsu et al., 1991; Hammond and Helenius, 1994). In this study, we find evidence that Inv-(N)cl$_{L57A}$ also undergoes recycling from the cis-Golgi back to the ER in a retrieval process that is dependent on the function of COPI coat proteins and the recycling of Kar2p. Thus a quality control mechanism in the cis-Golgi functions to retrieve

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misfolded protein back to the ER, presumably to re-expose immature protein to the ER folding environment.

Materials and Methods

Strains, Reagents, and Molecular Biological Techniques

S. cerevisiae strains are listed in Table I. Standard genetic manipulations, preparation of yeast rich medium (YEP) and minimal medium (SD) (Difco, Detroit, MI), and yeast transformations were performed as described (Kaiser et al., 1994). In strain constructions, the suc2-Δ9 allele was scored by invertase assay. Production of invertase antiserum and construction of pEHB2 (wildtype invertase), pEHB29 (Inv-s11), pEHB43 [Inv-(N)cl$_{wt}$], pEHB44 [Inv-(N)cl$_{L57A}$], and pEHB51 [Inv-(N)cl$_{L57G}$] were described previously (Hong, et al., 1996). pEHB29 contains a mutant form of invertase (Inv-s11) in which the signal sequence cleavage site has been altered from ala to ile at position 18 (Bohni et al., 1987). DNA sequencing was performed using the Sequenase kit (United States Biochemical Corp., Cleveland, Ohio).

Radiolabeling and Immunoprecipitations

Radiolabeling and immunoprecipiations were carried out essentially as described (Gimeno et al., 1995) with the following modifications. Strains were grown in selective SD medium containing 2% glucose and auxotrophic supplements to ~1 x 10^7 cells/ml at 25°C and then induced in medium containing 0.1% glucose for one hour at 30°C at 2 x 10^7 cells/ml. Cells were radiolabeled for 7 min and 1 OD of cells were taken at the time points indicated after the initiation of chase. For the kinetic analysis of COPI mutants, *kar2-ΔHDEL* and *erd2-B25*, labeled cultures were brought up to 1% glucose at the initiation of chase to turn off invertase expression. Protein extracts were prepared in 20 μl of 60mM Tris-HCl pH6.8, 100mM DTT, 2% SDS by vigorous agitation with acid-washed glass beads. Solubilized samples were rotated overnight at 4°C in 1ml of IP buffer (50mM Tris-HCl pH7.4, 150mM NaCl, 1% Triton X-100) containing the primary antibody. 1μl of anti-invertase or anti-CPY antibody (gift of

R. Schekman) was used per OD_{600} unit of labeled cell extract. For endo H digestions, $5\mu l$ of boiled protein extract was diluted with $15\mu l$ of 50mM sodium citrate pH 5.1 containing 100-250U of $EndoH_f$ (New England Biolabs, Beverly, MA) and incubated at 37°C for 2-16 hours. Images were analyzed using a 445si Phosphorimager and ImageQuant Software (Molecular Dynamics, Sunnyvale, CA).

In co-immunoprecipitation experiments, 20 ODs of cells were induced for invertase expression, radiolabeled for 30 min, and spheroplasted as previously described (Hong, et al., 1996). Spheroplast pellets were resuspended in 1 ml of cell lysis buffer (20mM MES pH 6.8, 150mM NaCl, 5mM Mg_2Cl, 1mM PMSF) and apyrase at 20U/ml, dounced 10 times using a tissue grinder homogenizer (Curtin Matheson Scientific, Inc., Florence, KY), and the crude lysate centrifuged at 500xg to remove large cell debris. The clarified supernatant was adsorbed to IgSorb for 10 min and re-centrifuged for 5 min. The resulting supernatant was brought up to 0.1% Triton X-100, rotated with $0.2\mu l$ of anti-BiP antibody and then protein A sepharose for 1 hour each. Protein samples were divided so that one-fifth was saved in the freezer and the other four-fifths immunoprecipitated with $2\mu l$ of anti-invertase antibody. The resulting protein samples following invertase precipiation were again divided so that one-third was saved and two-thirds immunoprecipitated with $2\mu l$ of anti-α1,6 mannose antibody and processed.

Subcellular fractionation

To detect the presence of α1,6-modified invertase in the ER, 4×10^8 cells expressing pEHB2 (wildtype invertase) or pEH44 [Inv-(N)cl$_{L57A}$] were labeled for 10 min and spheroplasted. The spheroplast pellet was resuspended in 1ml of cell lysis buffer (200mM sorbitol, 50mM Tris-Cl pH 7.4, 1mM EDTA, 1mM PMSF), dounced 10 times, and the lysate centrifuged at 500xg. The clarified supernatant was spun at 13,000 x g at 4°C for 10 min using the TL100.3 ultracentrifuge rotor (Beckman

Instruments, Palo Alto, CA) to generate a P13 fraction, which was diluted in 1 ml of IP

buffer, immunoadsorbed with 2 µl of anti invertase antibody and processed as

described above. After boiling to dissociate protein from the secondary reagent, half of

the sample was re-immunoprecipitated with 2 µl of anti-invertase antibody and the

other half with 3 µl of anti-α1,6 mannose antibody (gift of R. Schekman) and

processed. To verify purification of the ER, the unadsorbed supernatants following the

first invertase antibody incubation were re-immunoprecipitated with 1 µl of anti-CPY

antibody, and processed samples were analyzed alongside the p1, p2, and mature

forms of CPY isolated from a wildtype strain (CKY406) that had been labeled for 20

min and processed.

Western blotting and Kar2p quantitation

Protein extract preparation, spheroplasting, and western blotting were

performed as described (Hong, et al., 1996). The following antibodies were used:

polyclonal rabbit anti-invertase antibody at 1:1000 dilution, rabbit anti-

carboxypeptidase Y (CPY) antibody at 1:7000 dilution, HRP-coupled ProteinA

(Organon Teknika-Cappel, Westchester, PA), at 1:10,000 dilution, and HRP-coupled

sheep anti-rabbit antibody (Amersham Corp., Arlington Heights, IL) both at 1:10,000.

Kar2p secretion levels were quantified essentially as described (Elrod-Erickson

and Kaiser, 1996). 10^8 cells were harvested, lysed with glass beads, and solubilized

in 500µl of sample buffer. Proteins from the culture medium were TCA precipitated,

solubilized in 100µl sample buffer, and resolved by SDS-PAGE. Kar2p was detected

on nitrocellulose filters with affinity-purified rabbit anti-Kar2p antibody (gift of M. Rose)

and HRP-coupled goat anti-rabbit antibody (Amersham Corp.), both at 1:10,000

dilution, and quantified on an LKB 2202 Ultrascan laser densitometer. Serial dilutions

of culture medium from CKY190 were used to define the linear range of BiP detection

on western blots and to generate a standard curve for comparison.

Identification, Cloning and Genetic Analysis of *RET2*, δ-COP

The Hartwell collection of approximately 1,800 temperature-sensitive yeast

mutants (gift of M. Winey) was screened by Western blot for conditional accumulation

of core-glycosylated forms of invertase and CPY at 37°C. The ts⁻ growth and CPY

secretion defects in the *ret2* mutant isolate were followed through three backcrosses to

CKY273, CKY10, and CKY8 and co-segregated 2:2 in all tetrads examined in the final

cross, indicating that the *ret2* strains (CKY400 and CKY401) host a single, nuclear

mutation associated with both the ts⁻ growth defect and secretion block. The *ret2*

mutant complemented all of the *sec* mutants in our collection: CKY39 (*sec12-4*),

CKY45 (*sec13-1*), CKY50 (*sec16-2*), CKY78 (*sec23-1*), CKY54 (*sec17-1*), CKY58

(*sec18-1*), CKY69 (*sec21-1*), CKY100 (*sec27-1*), CKY65 (*sec20-1*), CKY71 (*sec22-3*),

CKY85 (*bet1-1*), and CKY90 (*ypt1^ts*).

RET2 was cloned from *GAL1*-promoted yeast cDNA (Liu et al., 1992) and

genomic pCT3 (Thompson et al., 1993) libraries. Approximately 10,000 *GAL1*-cDNA

and 33,000 pCT3 transformants of CKY400 on SC plates lacking uracil were assayed

for growth at 38°C. For the cDNA library, plasmid DNA (pAF40) was isolated from a

clone which grew at 38°C for 3 days on YEP-2 % galactose but not on YEP-2%

glucose plates. CKY400 retransformed with pAF40 grew at 38°C only on YEP-2%

galactose, confirming that rescue of the growth defect was plasmid-dependent. For

the genomic library, five transformants were isolated at 38°C that all reverted

phenotypically to ts⁻ after growth on SC plates containing 0.1% 5-fluoroorotic acid

(Boeke et al., 1984), but remained Ts⁺ after growth on SC plates lacking uracil.

CKY400 retransformed with plasmid DNA isolated from two of the Ts⁺ clones (pAF41

and pAF42) were shown to be Ts⁺.

Insert DNAs from pAF40, pAF41, and pAF42 were sequenced using the T3 and

T7 primers, and primer complimentary to the *GAL1* promoter. A BLAST search

identified sequence from pAF40 insert DNA as YFR051C on chromosome VI (The

Saccharomyces Genomic Information Resource [http://genome-www.stanford.edu/]).

pAF41 and pAF42 are overlapping clones from the same region. The predicted N-

terminal sequence of Ret2p matched that of peptide sequence obtained from the N-

terminus of purified yeast δ-COP (Cosson et al., 1996). The 2.6kb SnaB1-BamH1

fragment of pAF41 was cloned into Sma1/BamHI-digested centromeric vector pRS316

and integrating vector pRS306 to create pAF44 and pAF45, respectively. pAF44

rescued the sec80-1 ts⁻ growth defect. To test for linkage of the RET2 cloned

sequence to the ret2-2 mutation, CKY10 was transformed with pAF45 linearized by

NruI digestion. Three independent transformants were mated to CKY400 and the

diploids sporulated for tetrad dissection. In all tetrads scored, the Ts⁺ and Ura⁺

phenotypes co-segregated, verifying that our RET2 clone was linked to ret2-2.

To test for genetic interactions between ret2-2 and other ER-to-Golgi sec

mutations, CKY400 or CKY401 was mated to all of the temperature-sensitive sec

mutants from our collection listed above. Following sporulation and tetrad dissection,

spores were scored for growth at 24, 30, 33, 36, and 38°C. Synthetic lethality was

observed between ret2-2 and the second sec mutation when the corresponding cross

yielded tetrads where spore viability segregated in a 2:0, 3:0, or 4:0 pattern at room

temperature, and amongst the viable spores, 0, 2, or 4 were ts⁻, respectively.

Identification, Cloning, and Genetic Analysis of *SEC26*, β-COP

The sec26-1 mutant was obtained from a group of temperature-sensitive

mutants that were originally identified as being defective in ribosome synthesis (gift of

J. Warner). sec26-1 exhibits a conditional accumulation of the core-glycosylated form

of CPY at 37°C. The ts- growth and CPY secretion defects in sec26-1 were followed

through two backcrosses to CKY10 and co-segregated 2:2 in all tetrads examined in

the final cross. Approximately 10,000 YCp50 transformants of sec26-1 on SC plates

lacking uracil were assayed for growth at 38°C. Plasmid DNA (pEC4) isolated from two different clones which grew at 38°C, rescued the ts- growth defect of *sec26-1*. Insert DNA from pEC4 was sequenced using T3 and T7 primers, and a BLAST search identified a region of sequence on chromosome IV adjacent of YDR238c containing *SEC26*. A 2.7kb KpnI fragment of pEC4 containing *SEC26* was cloned into KpnI-digested pRS306 to create pEC13. To test for linkage of the SEC26 cloned sequence to the *sec26-1* mutation, CKY-- was transformed with pEC13 linearized with SnaBI digestion. Three independent transformants were mated to CKY10, and in ten tetrads scored for each transformant, the ts- and Ura+ phenotypes co-segregated. A 4.1kb ClaI/EcoRI fragment containing SEC26 from pEC4 was cloned into pRS316 and found to rescue the ts- growth defect. Synthetic lethality tests were performed between CKY- - (*sec26-1*) and CKY69 (*sec21-1*), CKY100 (*sec27-1*), and CKY400 (*ret2-2*).

Results

Inv-(N)cl$_{L57A}$ is recycled from the Golgi back to the ER

We have previously described the receptor-mediated sorting to the vacuole of
Inv-(N)cl$_{L57A}$, a hybrid protein composed of invertase and a mutant form of the N-
terminal domain of λ repressor that contains the point mutation Leu^{57} to Ala (Figure
1A) (Hong, et al., 1996). This mutation reduces the hydrophobicity of residue 57,
which is buried in the hydrophobic core of the repressor, thus destabilizing protein
folding and reducing the temperature of 50% thermal denaturation (T_m) of the purified
N-terminal domain from 54°C to 20°C (Parsell and Sauer, 1989). In the vacuole, the
mutant repressor domain of Inv-(N)cl$_{L57A}$ was degraded by vacuolar proteases,
leaving a truncated protein approximately the size of wildtype invertase.

During evaluation of the kinetics of Inv-(N)cl$_{L57A}$ secretion to the vacuole, we
observed that transport of Inv-(N)cl$_{L57A}$ through the early stages of the secretory
pathway was slower than that of wildtype invertase. Wild-type cells (CKY406)
expressing different forms of invertase were pulse-labeled for 7 min and chased for 10
min, and the invertase from the 0 and 10 min time points were recovered by
immunoprecipitation with anti-invertase antibody, resolved on SDS-polyacrylamide
gels, and visualized using a phosphorimager. After 10 min of chase, wildtype
invertase had completely acquired N-linked outer-chain mannose carbohydrates,
signifying transport through the Golgi compartments (Fig. 1B, lanes 1-2). Inv-(N)cl$_{wt}$, a
fusion between invertase and the wildtype N-terminal repressor domain, which was
transported fairly efficiently to the cell surface as an intact fusion protein in our
previous study, was also modified with Golgi carbohydrates (Fig. 1B, lanes 3-4), albeit
at a slower rate. In contrast, Inv-(N)cl$_{L57A}$ persisted primarily as core-glycosylated
protein after 10 min of chase (Fig. 1B, lanes 5-6), indicating that transport of Inv-

(N)cl$_{L57A}$ was retarded at an earlier step in the secretory pathway between the ER and the Golgi, before targeting to the vacuole.

Although Inv-(N)cl$_{L57A}$ did not receive any extensive Golgi modifications, a small population of the hybrid protein after 10 min of chase did migrate slightly more slowly compared to the core-glycosylated protein seen at the beginning of the chase (Figure 1B, compare lanes 5 and 6). This partial acquisition of outer chain carbohydrate over the period of the chase had been previously observed for several proteins that are recycled from the cis-Golgi back to the ER: (1) Sec12p, which bears the retrieval signal for soluble proteins -HDEL (Nishikawa and Nakano, 1993), (2) α-factor fused to -HDEL (Dean and Pelham, 1990), and (3) invertase fused to the cytoplasmic tail of Wbp1, which bears the retrieval signal for ER membrane proteins -KKXX (Gaynor et al., 1994). The slower migration of these recycled proteins compared to core-glycosylated protein was attributed to the addition of α1,6-mannose residues in the cis-Golgi. However, since these proteins are subsequently retrieved back to the ER, they did not acquire the complete repertoire of outer-chain mannoses characteristic of passage through the Golgi compartments.

To determine whether Inv-(N)cl$_{L57A}$ also undergoes a recycling process, Inv-(N)cl$_{L57A}$ isolated from an ER fraction was examined for the presence of Golgi-derived α1,6-mannoses. Wildtype cells (CKY406) expressing Inv-(N)cl$_{L57A}$ were labeled for 30 min and converted to spheroplasts by digestion of the cell wall. The cell pellets were gently lysed with a Dounce homogenizer and the lysate centrifuged at 13,000 x g to yield a pellet fraction (P13) enriched for ER membranes. Separation of the ER away from the Golgi and vacuole was verified by the presence of ER core-glycosylated p1 form of carboxypeptidase Y (CPY) in the P13 fraction, but not the Golgi-localized p2 or the mature form (Fig. 2A, lanes 1-3) (Dean and Pelham, 1990). Inv-(N)cl$_{L57A}$ was immunoprecipitated from the P13 fraction with anti-invertase antibody, and the processed samples were divided in half, one half re-precipitated with anti-invertase

antibody and the other half with antibody to anti-α1,6 mannoses. Anti-α1,6 mannose antibody precipitated the slower migrating form of Inv-(N)cl$_{L57A}$ from the P13 fraction (Fig. 2B, compare lanes 1 and 2), indicating that Golgi α1,6-modified hybrid protein did accumulate in the P13 fraction. In contrast, the anti-α1,6 mannose antibody precipitated very little wildtype invertase isolated from a similarly-prepared P13 ER fraction (Fig. 2B, lanes 3-4), as expected since α1,6-modified wildtype invertase would not normally accumulate in the ER. Both the retardation of Inv-(N)cl$_{L57A}$ transport early in the secretory pathway and the presence of α1,6-modified carbohydrates on Inv-(N)cl$_{L57A}$ isolated from a fraction enriched for the ER provides strong evidence that a recycling process recognizes and retrieves Inv-(N)cl$_{L57A}$ from the cis-Golgi back to the ER.

Transport of Inv-(N)cl$_{L57A}$ to the vacuole is accelerated in γ-COP and β'-COP mutant strains

For Inv-(N)cl$_{L57A}$ to be recycled, the hybrid protein must be packaged into vesicles in the cis-Golgi that specifically target back to the ER rather than forward to the other Golgi compartments. Several types of vesicles that mediate traffic in the secretory pathway have been characterized and are defined by the different multi-subunit coat structures that assemble around the vesicle (e.g. COPI, COPII, and clathrin). Although it is not known whether one specific type of coated vesicle directs all retrograde transport, recycling of ER resident membrane proteins bearing the -KKXX retrieval signal from the Golgi to the ER has been shown to be dependent on the COPI vesicle coat complex (Letourneur et al., 1994). We tested whether COPI is also required for the recycling of Inv-(N)cl$_{L57A}$ by determining the rate of vacuolar degradation of the repressor domain in two known COPI mutants *sec21-1* (γ-COP) and *sec27-1* (β'-COP) (Hosobuchi et al., 1992; Duden et al., 1994). We applied the assumption that Inv-(N)cl$_{L57A}$ not packaged into retrograde vesicles in the cis-Golgi

would proceed forward through the remainder of the Golgi compartments and be transported to the vacuole. Thus, if COPI-coated vesicles do participate in Inv-(N)cl$_{L57A}$ retrieval, then expression of a mutant COPI complex might be expected to impair the ability of Inv-(N)cl$_{L57A}$ to be recycled and lead to an increased rate of transport to the vacuole and subsequent processing of the repressor domain.

Strains were pulse-labeled for 7 min and chased for up to 30 min at 30°C, an intermediate temperature for growth for *sec21-1* and *sec27-1*. Processed time point samples were digested with endoH to remove N-linked carbohydrates and analyzed by gel electrophoresis using the phosphorimager. Quantitation of the deglycosylated forms of both the full-length and vacuole-degraded Inv-(N)cl$_{L57A}$ revealed that the rate of vacuolar processing was increased 2.5 and 2.3 fold in the *sec21* and *sec27* mutants respectively, over that of a wildtype strain (CKY406) (Fig. 3A, compare lanes 1-4 with 5-8 and 9-12, and Fig. 3C). The half-life of vacuolar processing was calculated to be 8.1 min and 8.9 min for the *sec21* and *sec27* mutants respectively, compared to 20 min for a wildtype strain. This acceleration of vacuolar processing of Inv-(N)cl$_{L57A}$ suggested that functional COPI complex normally acts to retard forward transport of the hybrid protein in the secretory pathway.

COPI-coated vesicles have also been proposed to function in forward transport between the ER and Golgi and between Golgi compartments (Hosobuchi, et al., 1992; Rothman and Orci, 1992; Pepperkok et al., 1993). Although transport of Inv-(N)cl$_{L57A}$ to the vacuole was accelerated in the COPI mutants, not impaired as would be expected if mutant COPI complex compromised forward transport, we analyzed the secretion of both CPY and wildtype invertase in the *sec21* and *sec27* strains to confirm that forward transport functioned normally under the experimental conditions of the pulse/chase studies. CPY was immunoprecipitated from the unadsorbed supernatants of the chase samples following incubation with anti-invertase antibody and analyzed by gel electrophoresis and the phosphorimager. The COPI mutant strains exhibited a

similar rate of CPY transport to the vacuole as a wildtype strain, converting p1 and p2 forms of CPY into the mature vacuolar form within 10 min (Fig. 3B, compare lanes 1-4 with 5-8 and 9-12).

The two COPI mutant strains expressing wildtype invertase were induced at 30°C, spheroplasted to separate protein secreted to the cell surface from protein retained in the cell body, and the two fractions analyzed by Western blot using anti-invertase antibody. Under steady-state conditions, mature-glycosylated invertase was present predominantly in the extracellular fraction of the *sec21* and *sec27* mutants (Fig. 4, lanes 5-6 and 7-8), as seen in a wildtype strain (CKY406) (Fig. 4, lanes 1-2), indicating that forward transport of wildtype invertase was also not compromised. As a control for ER-retained invertase, core-glycosylated Suc2-s11 invertase contains a mutation in the signal peptidase cleavage site, which renders the protein transport-incompetent (Bohni, et al., 1987) (Fig. 4, lanes 3-4).

Taken together, these results are consistent with functional COPI complex being required for efficient recycling of Inv-(N)cl$_{L57A}$ back to the ER and suggest that COPI-coated vesicles participate in the recycling of mutant proteins that have escaped the ER, in addition to membrane proteins bearing the -KKXX tag.

Isolation of alleles of *RET2* and *SEC26*, the δ-COP and β-COP subunits of the COPI complex

In our examination of COPI mutants, we included newly-identified alleles of both the δ subunit of the COPI complex, *RET2* (Cosson, et al., 1996), which we designated *ret2-2,* and the β-COP subunit, *SEC26* (Duden, et al., 1994), designated *sec26-1.* These two alleles failed to grow at temperatures above 36°C, accumulated ER core-glycosylated invertase and the p1 form of CPY at the restrictive temperature, and complemented all of the *sec* gene mutations in our collection. *RET2* and *SEC26* were isolated by complementation of the temperature-sensitive growth defect of the mutants,

and the cloned sequences were shown to be genetically linked to *ret2-2* and *sec26-1*, respectively.

In analysis of synthetic lethal interactions between *ret2-2* and other *sec* mutations, double mutant combinations of *ret2-2* with either *sec21-1* (γ-COP) or *sec27-1* (β'-COP) were inviable at 24°C, a temperature permissive for growth for each of the single mutants. Similarly, *sec26-1* demonstrated synthetic lethality with three mutants of COPI subunits, *sec21-1*, *sec27-1*, and *ret-2-2*. No genetic interactions were seen in combinations of *ret2-2* with mutations in COPII genes (*SEC13, SEC16* and *SEC23*) (Barlowe et al., 1994; Espenshade et al., 1995) or with genes involved in vesicle fusion (*SEC17* and *SEC18*) (Kaiser and Schekman, 1990). These observations strongly suggest that Ret2p, Sec26p, Sec21p, and Sec27p function together and support assignment of Ret2p and Sec26p as components of yeast coatomer.

The rate of degradation of the Inv-(N)cl$_{L57A}$ repressor domain was examined in both the *ret2-2* and *sec26-1* mutants at 30°C to determine whether defective δ-COP and β-COP subunits also accelerated transport of hybrid protein to the vacuole. Degradation of the repressor domain was shown to proceed at an increased rate of 1.7 fold in *ret2-2* and 2.1 fold in *sec26-1*, compared to the wildtype strain (CKY406) (Fig. 3A, compare lanes 1-4 with 13-16 and 17-20; Fig. 3C). Vacuolar degradation occurred with a half-life of 11.6 min and 9.6 min, respectively. Forward transport was not seriously compromised in the *ret2-2* and *sec26-1* mutants since almost all wildtype invertase was secreted out to the cell surface under steady-state conditions (Fig. 4, compare lanes 1-2 with 9-10 and 11-12). The maturation of CPY (Fig. 3B, compare lanes 1-4 with 13-16 and 17-20) in these two mutants did appear to be slightly slowed, since a small population of the p1 and p2 forms of CPY was still present after 10 min of chase. The finding that δ- and β-COP mutants exhibited a similar increase in the rate of vacuolar degradation of the repressor domain of Inv-(N)cl$_{L57A}$ as two other COPI

mutants, γ- and β'-COP further promotes a role for COPI-coated vesicles in mediating recycling of Inv-(N)cl$_{L57A}$ back to the ER.

Transport of Inv-(N)cl$_{L57A}$ to the vacuole is accelerated in mutant strains defective for retrieval of Kar2p back to the ER

Packaging of Inv-(N)cl$_{L57A}$ into COPI-coated retrograde vesicles in the cis-Golgi implies that components of the retrieval machinery must act to recognize and segregate the hybrid protein from the constant ongoing secretory traffic heading to the cell surface. In the case of recycling dilysine-tagged proteins, several COPI subunits have been shown to bind the cytoplasmic tail and -KKXX tag of the transmembrane protein Wbp1 *in vitro* (Cosson and Letourneur, 1994), suggesting that membrane proteins are held back in the cis-Golgi by direct interaction with the COPI coat. Inv-(N)cl$_{L57A}$, on the other hand, behaves as a soluble protein (Hong, et al., 1996) and would require a different mechanism to be retained in the cis-Golgi.

One possible candidate that may participate in binding Inv-(N)cl$_{L57A}$ in the cis-Golgi and initiating the recycling process is the ER chaperone Kar2p, the yeast analog of BiP. Extensive *in vivo* and *in vitro* studies in yeast and mammalian systems have implicated BiP in recognizing and binding to misfolded and non-native substrate (Bole et al., 1986; Hurtley et al., 1989; Machamer et al., 1990). In addition, the Kar2p that escapes to the cis-Golgi has been shown to be recycled back to the ER via recognition of the C-terminal -HDEL retrieval signal by the Kar2p receptor, Erd2p (Munro and Pelham, 1987; Dean and Pelham, 1990; Semanza et al., 1990). Thus Kar2p may also serve to carry Inv-(N)cl$_{L57A}$ back to the ER as it itself is being recycled.

We tested the involvement of Kar2p in the retrieval of Inv-(N)cl$_{L57A}$ by examining the rate of vacuolar processing of Inv-(N)cl$_{L57A}$ in two mutant strains that impair the ability of Kar2p to be recycled: (1) *kar2-ΔHDEL*, which fails to recycle Kar2p because the -HDEL retrieval signal has been deleted (Pelham et al., 1988) and (2) *erd2-B25*,

which expresses a form of Erd2p containing a Asp to Asn amino acid change at position 50 thought to comprise part of a hydrophilic pocket that accepts the -HDEL tag of Kar2p, thus rendering the receptor less efficient in recognizing the -HDEL signal (Semanza, et al., 1990; Townsley et al., 1993).

If retrieval of Kar2p is required for the transport of hybrid protein back to the ER, Inv-(N)cl$_{L57A}$ would be expected to be processed in the vacuole at a faster rate in these two mutants. From pulse/chase analysis, the rates of vacuolar processing in the *kar2-ΔHDEL* and *erd2-B25* mutants were increased 2.4 and 1.5 fold, respectively (Fig. 5A, compare lanes 1-4 with 5-8 and 9-12 and Fig. 5C), and the half-life for vacuolar processing was calculated to be 8.4 min and 13.7 min. Although, the *erd2-B25* mutant exhibited a small effect compared to the COPI and *kar2-ΔHDEL* mutants, the 1.5 fold increase in the rate of vacuolar processing was consistently observed. To demonstrate that differences in the strain background of the *erd2-B25* mutant wasn't responsible for producing this 1.5 fold effect, the *erd2-B25* mutant was backcrossed into the lab strain background and then re-examined for processing of Inv-(N)cl$_{L57A}$. The backcrossed *erd2-B25* also displayed an ~1.5 fold increase in the rate of mutant repressor processing, suggesting that defective Erd2p was responsible for allowing Inv-(N)cl$_{L57A}$ to be transported more rapidly to the vacuole. Since overexpression of Erd2-B25p was shown to be able to partially rescue the Kar2p hypersecretion phenotype in a *erd2-B25* strain, the lower fold effect in acceleration of Inv-(N)cl$_{L57A}$ transport to the vacuole compared to the *kar2-ΔHDEL* mutant may be explained by the mutant protein retaining some residual binding activity (Townsley et al., 1994). Forward transport was functioning normally in the *kar2-ΔHDEL* and *erd2-B25* mutants since CPY reached the vacuole within 10 min, similar to a wildtype strain (Fig. 5B, compare lanes 1-4 and 5-8 and 9-12). Thus, impairing both retrieval of Kar2p in the *kar2-ΔHDEL* mutant and the efficiency of Erd2p recognition of Kar2p in the *erd2-B25*

mutant removes the early kinetic block to Inv-(N)cl$_{L57A}$ transport, consistent with a role for Kar2p and Erd2p in recycling Inv-(N)cl$_{L57A}$ from the Golgi back to the ER.

Kar2p physically associates with α1,6 modifed Inv-(N)cl$_{L57A}$

From the preceding kinetic analysis, in which Inv-(N)cl$_{L57A}$ processing proceeded at an increased rate in COPI and Kar2p retrieval mutants, a simple model can be proposed in which Kar2p binds Inv-(N)cl$_{L57A}$ in the cis-Golgi and recycles the fusion protein back to the ER through interaction with the Erd2p receptor in COPI-coated vesicles. To obtain biochemical evidence for this model, co-immunoprecipitation experiments were performed to detect the existence of a complex between Kar2p and α1,6-modified Inv-(N)cl$_{L57A}$.

Wild-type cells (CKY406) expressing either wild-type invertase or Inv-(N)cl$_{L57A}$ were labeled for 30 min, spheroplasted, and the cell pellets lysed by homogenization in non-denaturing buffer. The lysates were then subjected to immunoprecipitation using anti-Kar2p antibody under native conditions to preserve any complexes in which Kar2p is a component. Processed samples were boiled, and the precipitated proteins were re-immunoprecipitated with anti-invertase antibody. Finally, processed samples using the invertase antibody were re-immunoprecipitated with anti-α1,6 mannose antibody. Protein extracts were resolved by gel electrophoresis and analyzed with the phosphorimager. Immunoadsorption using Kar2p antibody co-precipitated Inv-(N)cl$_{L57A}$ (Fig. 6, lanes 4-5), demonstrating that a complex between Kar2p and Inv-(N)cl$_{L57A}$ can be isolated under native conditions. Moreover, re-precipitation of Inv-(N)cl$_{L57A}$ with anti-α1,6 mannose antibody revealed that the fusion protein had acquired Golgi-derived α1,6 mannose modifications (Fig. 6, lane 6), eliminating the possibility that the observed interaction was solely the result of Kar2p in the ER binding to Inv-(N)cl$_{L57A}$ that had not yet been transported to the cis-Golgi.

In contrast, wild-type invertase did not appear to be associated with Kar2p (Fig. 6, lanes 1-2), since folded invertase mostly escaped interaction with a chaperone specific for binding misfolded substrate. Co-immunoprecipitation experiments with either the Suc2-s11 signal sequence cleavage mutant or invertase fused to the wild-type domain of λ repressor, Inv-(N)cl$_{wt}$, also did not reveal any interactions with Kar2p, presumably because the former is thought to be retained in the ER by association with the ER membrane (Schauer, et al., 1985) while the latter predominantly assumes the conformation of a folded protein (Hong, et al., 1996) (data not shown). The slight decrease in the rate of Inv-(N)cl$_{wt}$ transport to the Golgi compared with wild-type invertase seen in Figure 1B may be mediated by quality control factors other than interaction with Kar2p. The physical association between Kar2p and α1,6-modified Inv-(N)cl$_{L57A}$, combined with the kinetic evidence that Inv-(N)cl$_{L57A}$ retrieval is dependent on Kar2p itself being recycled, poses strong arguments that Kar2p plays a direct role in mediating recycling of misfolded protein from the cis-Golgi back to the ER.

Mutations in the γ-, β'-, δ-, and β-COP subunits cause Kar2p to be secreted

Although COPI-coated vesicles have been implicated in retrieval of ER membrane proteins bearing the -KKXX tag, the type of coated vesicle that recycles soluble proteins like Kar2p back to the ER has not been firmly established. Recent evidence demonstrating that Erd2p does not cycle back to the ER in a *sec21* mutant at the restrictive temperature does support a requirement for coatomer function in the recycling of Erd2p and by association, Kar2p as well (Lewis and Pelham, 1996). To test our hypothesis that Kar2p in the cis-Golgi is packaged into COPI-coated vesicles, we took advantage of the strong intracellular retention of Kar2p. In wild-type cells, barely any Kar2p could be detected in the extracellular medium, whereas ~12% of Kar2p was secreted into the medium in both *kar2-ΔHDEL* and *erd2* mutants (Hardwick

et al., 1990; Semanza, et al., 1990). If Kar2p is indeed recycled back to the ER in COPI-coated vesicles, then strains expressing defective COPI coats would be impaired in retrieval and would consequently secrete higher levels of Kar2p to the cell surface compared to a wild-type strain.

We examined the extracellular mediums from growing cultures of *sec21*, *sec27*, *ret2*, and *sec26* mutants by precipitating secreted protein from culture mediums with trichloroacetic acid and detecting the amount of Kar2p leaked to the cell surface compared to the amount remaining in the cell pellet by quantitative Western blot using anti-Kar2p antibody. In a wildtype strain, barely any Kar2p (~0.3%) was present in the culture medium, confirming what was observed in earlier work (Semanza, et al., 1990) (Fig. 7). In contrast, the *sec21*, *sec27*, *ret2*, and *sec26* mutants all exhibited an increase in the percentage of Kar2p secreted, 4.6%, 3.2%, 4.5%, and 1.8% respectively. The Kar2p secretion defect in *sec21*, *ret2*, and *sec26* can be partially rescued by introduction of a plasmid carrying a copy of the wild-type gene (data not shown). This demonstration that functional COPI proteins are required to prevent Kar2p from being secreted to the cell surface is consistent with our proposed model that COPI-coated vesicles recycle Kar2p from the Golgi back to the ER.

Discussion

The ER has long been viewed as the sole site in the cell where secretory proteins undergo scrutiny by a quality control process that permits correctly-folded proteins to be transported to the cell surface, but retains misfolded proteins for degradation. Thus it was to our surprise that analysis of the secretory fate of Inv-(N)cl$_{L57A}$, a hybrid protein composed of invertase and a destabilized mutant of the N-terminal domain of λ repressor, identified two novel quality control processes that function outside of the ER, in the Golgi compartments. The sorting of Inv-(N)cl$_{L57A}$ to the vacuole by a Vps10p-dependent receptor-mediated targeting process in the trans-Golgi and the subsequent vacuolar degradation of the mutant repressor domain was described in earlier work (Hong, et al., 1996). In this report, we propose that Inv-(N)cl$_{L57A}$ also undergoes recycling from the cis-Golgi back to the ER in a process dependent on functional COPI complex and Kar2p retrieval (Figure 8). Recycling would give mutant proteins additional opportunities to fold correctly into their native conformations in the presence of ER chaperones and folding enzymes and be subjected to degradation via an ER-based or proteasome-mediated system (Hiller, et al., 1996; McCracken and Brodsky, 1996).

Recycling from the cis-Golgi back to the ER was originally proposed for the retrieval of ER resident proteins that had escaped to the Golgi. The first retrieval signals identified were the tetrapeptide tags, -HDEL in yeast and -KDEL in mammalian cells, at the C-termini of soluble ER resident proteins. An -HDEL-tagged form of the secreted protein pro-α-factor in yeast (Dean and Pelham, 1990) and a -KDEL-tagged form of the lysosomal protein cathepsin D in COS cells (Pelham, 1988) both accumulated in the ER, but were shown to be modified by the Golgi enzymes, α1,6-mannosyltransferase and N-acetylglucosamine-1 phosphotransferase, respectively, indicating that the fusions were transported to the Golgi but then retrieved back to the

ER. An additional C-terminal retrieval signal, -KKXX, was subsequently identified as the retrieval signal for type I membrane-bound ER resident proteins from the Golgi (Jackson et al., 1993). We employed a similar strategy by isolating Inv-(N)cl$_{L57A}$ modified with Golgi-specific α1,6-mannose residues from an ER fraction to demonstrate that the thermally unstable N-terminal repressor domain was capable of eliciting the recycling process in the absence of the -H(K)DEL or -KKXX tags.

Although recycling has been most closely examined for resident ER proteins, two mutant membrane proteins in mammalian cells and one mutant soluble protein in yeast that are strongly retained in the ER have been suggested to also undergo retrieval from the cis-Golgi back to the ER. In mutant cell lines where MHC class I heavy and light chains fail to assemble correctly, the MHC molecules were localized by immunofluorescence to the Golgi upon incubation of the cells at 16°C, a growth condition that inhibits retrograde transport as assayed by the loss in brefeldin A-induced redistribution of Golgi proteins into the ER (Hsu, et al., 1991). Similarly, the second mutant membrane protein, the thermoreversible ts045 folding mutant of the vesicular stomatitis virus G protein, co-localized with Golgi and the intermediate compartment marker proteins at the restrictive temperature (Hammond and Helenius, 1994). Both the MHC and ts045 proteins were shown to migrate from a Golgi to an ER fraction in subcellular fractionation studies upon shifting to 25°C for the MHC protein or incubating in DTT to block forward transport for the ts045 G protein, demonstrating that the mutant proteins were indeed being recycled. Most likely, the two mutant proteins were retrieved from a very early cis-Golgi compartment in which enzymes that modify N-linked carbohydrate chains are absent, since they did not exhibit the expected mammalian Golgi-localized characteristics of endo H resistance.

In yeast, an ER-retained active site mutant of the soluble vacuolar protease CPY, CPY*, has also been reported to undergo recycling from the cis-Golgi back to the ER (Knop et al., 1996). CPY* became modified with Golgi-derived α1,6 mannose

residues in a $der1\Delta$ mutant strain background, which is impaired for the ER-degradation of CPY*. To examine whether generation of $\alpha1,6$-modified of CPY* can be further perturbed by a defective HDEL-mediated retrieval system, CPY* was expressed in a $der1\Delta$ $erd1\Delta$ double mutant. *ERD1* has been proposed to function in the retrieval of soluble HDEL-tagged ER resident proteins (Hardwick, et al., 1990). In the double mutant, more CPY* protein acquired Golgi modifications compared to the single $der1\Delta$ mutant, suggesting that CPY* was being recycled back to the ER by the same retrieval machinery that acts on -HDEL-tagged proteins.

Since studies had already revealed that the COPI coat complex (Letourneur, et al., 1994) and the Erd2p receptor (Semanza, et al., 1990) played key roles in the recycling of membrane and soluble ER resident proteins, respectively, we decided to investigate whether these components also functioned in the retrieval of Inv-(N)cl$_{L57A}$, despite it not bearing a C-terminal retrieval signal. In four COPI mutants tested, *sec21* (γ-COP),*sec27* (β'-COP), and newly-identified alleles of *ret2* (δ-COP) and *sec26* (β-COP), the rate of Inv-(N)cl$_{L57A}$ transport to the vacuole was increased up to 2.5 fold, indicating that the COPI proteins are most likely involved in an early kinetic block that retards forward transport of Inv-(N)cl$_{L57A}$. It is unclear if these four mutant COPI complexes are impaired for the exact same step in the recycling process, whether by an inability to target to the Golgi membrane, to form a retrograde vesicle, to load proteins to be recycled into the vesicle, or to pinch off a cargo-filled vesicle from the Golgi. In vitro studies demonstrating that *sec21* mutant coatomer retained the ability to bind dilysine motifs, whereas *sec27* and *ret1* (α-COP) coatomer lost binding capability (Cosson and Letourneur, 1994; Letourneur, et al., 1994), illustrates that different COPI subunits may perform different functions in the recycling process.

The role of the COPI complex in intracellular trafficking remains controversial since COPI-coated vesicles have also been proposed to function in forward transport from the ER to the Golgi and within the Golgi compartments (Rothman and Orci, 1992;

Pepperkok, et al., 1993). During initial characterization of COPI subunits in yeast, both *sec21* (Hosobuchi, et al., 1992) and *sec27* (Duden, et al., 1994) mutants were shown to accumulate the p1 ER form of CPY at the restrictive temperature of 37°C, implying that COPI was required for ER to Golgi transport. Indeed, we originally isolated the *ret2-2* and *sec26-1* alleles by screening for ts- mutants that exhibited an ER block in CPY and invertase secretion at the restrictive temperature. However, in our studies, wild-type invertase partitioned with the spheroplast supernatant in the four COPI mutants at either 30°C or 37°C, thus behaving as a protein secreted to the cell surface. Curiously, the secreted invertase migrated as an underglycosylated form intermediate in size between core-glycosylated and mature-glycosylated invertase at both temperatures, although the underglycosylation defect was more severe at 37°C. A recent study also reported that underglycosylated invertase was transported to the cell surface in all COPI mutants examined, including *ret2-1* (δ-COP), *ret-1* and *sec33-1* (α-COP), *sec27-1* (β-COP), *ret3-1* (ζ-COP), and several newly-generated ts alleles of *sec21* (γ-COP) (Gaynor and Emr, 1997). The authors further demonstrated that the underglycosylated forms of invertase can be precipitated with antibodies to both α1,6 and α1,3 mannose linkages, suggesting that the glycosylation defects may be due to impaired elongating α1,6 mannosyltransferase activity. In light of the strong genetic evidence that COPI-coated vesicles play an integral role in recycling, the observed defects of COPI mutants in forward transport for some cargo proteins have been interpreted to be a direct consequence of the inability to retrieve essential components which are required for forward transport to progress (Pelham, 1995).

A role for Kar2p in recycling mutant proteins from the cis-Golgi back to the ER introduces a new function for this yeast homolog of BiP, in addition to its already established roles in translocation into the ER lumen (Vogel et al., 1990) and mediating protein folding (Simons et al., 1995). Involvement of BiP in the retrieval of mutant protein had been suggested by immunofluorescence studies in which BiP co-localized

with the ts045 VSV G folding mutant in the intermediate compartment and cis-Golgi at the restrictive temperature, placing both BiP and mutant protein at sites where recycling was initiated (Hammond and Helenius, 1994). In our study, we were able to establish the existence of a physical interaction between Kar2p and Inv-(N)cl$_{L57A}$ bearing α1,6 mannoses. This biochemical evidence that Kar2p recognizes and binds to mutant proteins that had been modified in the cis-Golgi implied that binding most likely takes place in the cis-Golgi. It remains a formal possiblility that Golgi-modified Inv-(N)cl$_{L57A}$ is retrieved independent of Kar2p, and subsequently becomes associated with Kar2p in the ER lumen. However, the kinetic studies demonstrating that Inv-(N)cl$_{L57A}$ is transported to the vacuole at a faster rate in two mutants defective in Kar2p retrieval, *kar2ΔHDEL* and *erd2-B25*, strongly favors our model that Kar2p plays a role in retarding early Inv-(N)cl$_{L57A}$ transport by binding and retrieving the mutant protein from the cis-Golgi back to the ER through interaction with the Erd2p receptor.

Thus far, the mechanisms of recycling for two categories of retrieved protein have been studied separately for the most part: (1) -HDEL-tagged soluble protein bound by the Erd2 receptor, and (2) -KKXX-tagged membrane protein interacting with subunits of the COPI complex. Our analysis that increased levels of Kar2p are secreted into the extracellular medium in COPI mutants provides evidence that soluble and membrane protein cargo may be recycled via a single pathway in which COPI-coated vesicles are capable of ferrying -HDEL tagged protein back to the ER, in addition to -KKXX tagged protein. Consistent with our hypothesis, recycling of Erd2p has been shown to be impaired in a *sec21* mutant at the restrictive temperature (Lewis and Pelham, 1996). However, the fact that the levels of Kar2p secreted in the COPI mutants are lower compared to the amount released in a *kar2-ΔHDEL* mutant suggests that either the COPI mutant alleles retain some partial function for recycling

or that Kar2p may also be recycled back to the ER by an alternative COPI-independent pathway.

In addition to Inv-(N)cl$_{L57A}$, we have also examined whether two other invertase fusions to non-native domains undergo retrieval. The first hybrid, Inv-(N)cl$_{L57G}$, consists of invertase fused at the C-terminus to the N-terminal domain of λ repressor containing a leu to gly amino acid change at position 57, which reduces the temperature of 50% thermal denaturation (T_m) of the purified N-terminal domain from 54°C to 0-4°C (Parsell and Sauer, 1989). The second hybrid, Inv-3Ab, is a member of a collection of randomly generated peptides appended to the C-terminus of invertase that contains an additional ninety-three amino acids, of which seventy-five are randomized (E. Hong, A. Davidson, M. Cordes, and C. Kaiser, unpublished results). From kinetic analysis, the rates of vacuolar degradation of the non-native domains in Inv-(N)cl$_{L57G}$ and Inv-3Ab were shown to be accelerated in both a COPI mutant, sec21-1, and kar2-ΔHDEL, similar to what was seen for Inv-(N)cl$_{L57A}$. Applying our conclusions from study of Inv-(N)cl$_{L57A}$, we surmise that the two mutant domains appended to invertase also elicit recycling of the fusion protein from the cis-Golgi back to the ER. Evidence for recycling of invertase fused to three different mutant domains and the aforementioned examples of MHC class I, ts045 G protein and CPY* misfolded protein lead us to propose that recycling of mutant protein from the cis-Golgi back to the ER constitutes a general quality control mechanism that monitors for misfolded cargo early in the secretory pathway.

Acknowledgements

Alison Frand performed the BiP secretion assays, the cloning of *RET2* and genetic characterization of the *ret2-2* mutant. Elizabeth Chitouras initiated the mutant screen of the Hartwell collection to identify new secretory mutants and isolated the *ret2-2* and *sec26-1* mutants. Esther Chen cloned *SEC26* and studied its genetic interactions with the other COP I mutants.

Figure Legends

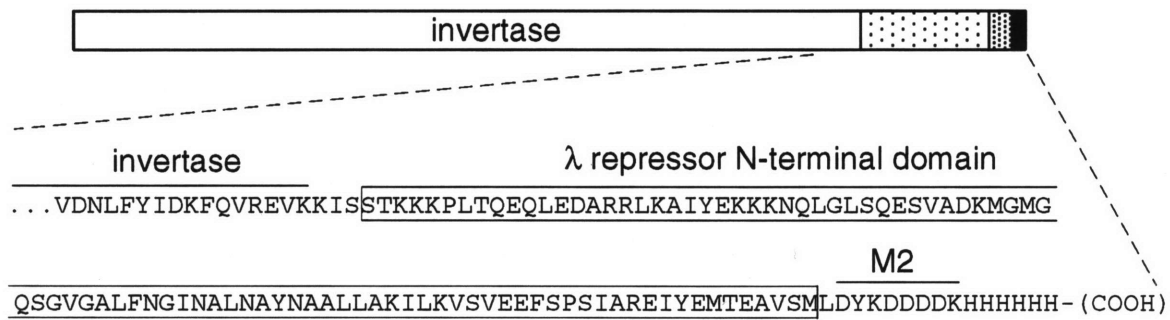**Figure 1:** An early secretory block in the transport of Inv-(N)cI$_{L57A}$.

(A) Structure of the invertase-λ repressor hybrids. The coding sequence of invertase was fused to the N-terminal 93 amino acid DNA-binding domain of λ repressor (light stippled box), the 8 amino acid M2 epitope (dark stippled box), and a 6x histidine tag (black box). The amino acid sequence for residues 55-60 of the wildtype λ repressor and two mutants are shown. T$_m$ measurements for the N-terminal λ repressor domains were obtained previously (Parsell and Sauer, 1989).

(B) CKY406 (wild-type) expressing pEHB2 (wild-type invertase), pEHB45 [Inv-(N)cI$_{wt}$], and pEHB46 [Inv-(N)cI$_{L57A}$] were induced for one hour at 30°C, labeled with [^{35}S]-methionine for 7 min and chased for 10 min. Cells from time points taken at 0 and 10 min after the start of the chase were lysed, immunoprecipitated with anti-invertase antibody, resolved on an 8% SDS polyacrylamide gel, and visualized using a phosphorimager. Secreted, mature-glycosylated invertase and Inv-(N)cI$_{L57A}$ migrates heterogeneously at about 140kD. Core-glycosylated invertase migrates at about 90kD for wildtype invertase and 110kD for the repressor hybrids.

Figure 1

A

invertase

invertase   λ repressor N-terminal domain

...VDNLFYIDKFQVREVKKIS STKKKPLTQEQLEDARRLKAIYEKKKNQLGLSQESVADKMGMG

M2

QSGVGALFNGINALNAYNAALLAKILKVSVEEFSPSIAREIYEMTEAVSMLDYKDDDDKHHHHHH-(COOH)

55   60
NALNAY   Inv-(N)cl_{wt}

--A---   Inv-(N)cl_{L57A}

T_m (°C) of λ (1-93)

	T_m (°C) of λ (1-93)
Inv-(N)cl$_{wt}$	54
Inv-(N)cl$_{L57A}$	20

B

	wt inv		Inv-(N)cl$_{wt}$		Inv-(N)cl$_{L57A}$	
chase (min)	0	10	0	10	0	10

mature glycosylated invertase

— core-glycosylated fusion

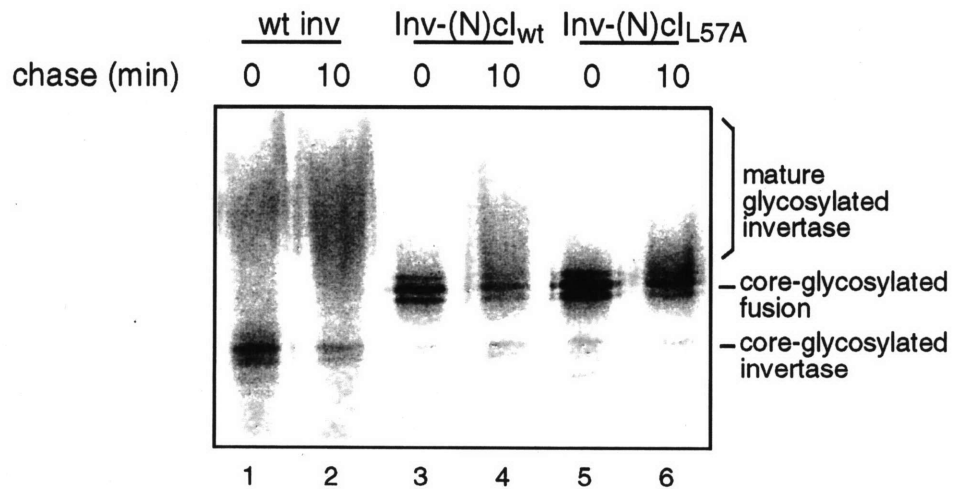— core-glycosylated invertase

1   2   3   4   5   6

Figure 2: Inv-(N)cl$_{L57A}$ bearing α-1,6 mannose modifications sediment in an ER-enriched fraction.

(A) CKY406 (wild-type) expressing pEHB2 (wild-type invertase) or pEHB46 [Inv-(N)cl$_{L57A}$] were induced for one hour at 30 C, labeled with [^{35}S]-methionine for 30 min, and spheroplasted. Spheroplast pellets were lysed and centrifuged at 13,000 x g, and the P13 pellet fractions of wildtype invertase (lane 1) and Inv-(N)cl$_{L57A}$ (lane 2) were immunoprecipitated with anti-CPY antibody. Samples were resolved on an 8% SDS-polyacrylamide gel and visualized using a phosphorimager. To visualize the p1 (ER), p2 (Golgi), and m (vacuole) forms of CPY, CKY406 expressing pEHB2 was labeled for 20 min and immunoprecipitated with anti-CPY (lane 3).

(B) The P13 pellet fractions of wild-type invertase (lanes 4 and 5) and Inv-(N)cl$_{L57A}$ (lanes 6 and 7) from (A) were also immunoprecipitated with anti-invertase antibody. Samples were divided in half, one half re-immunoprecipitated with anti-invertase antibody (lanes 4 and 6) and the other half with anti-α 1,6 mannose antibody (lanes 5 and 7), and processed.
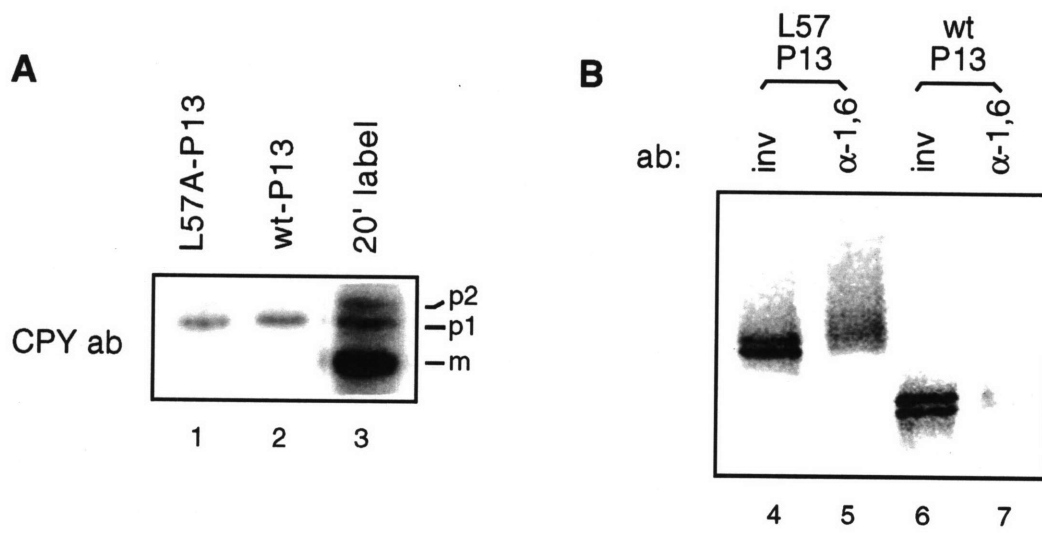
Hong et al., 1997

Figure 2

Figure 3: The repressor moiety of Inv-(N)cl$_{L57A}$ is degraded more rapidly in γ, β', δ, and β-COP mutant strains.

(A) Inv-(N)cl$_{L57A}$ was expressed in CKY406, wildtype (lanes 1-4); CKY415, *sec21-1* (lanes 5-8); CKY416, *sec27-1* (lanes 9-12); CKY417, *ret2-2* (lanes 13-16); and*sec26-1* (lanes 17-20). Strains were induced for one hour at 30°C, labeled with [^{35}S]-methionine for 7 min and chased for up to 30 min. Cells from time points taken at 0, 10, 20, and 30 min were lysed, immunoprecipitated with anti-invertase antibody, treated with Endo H, resolved on an 8% SDS polyacrylamide gel, and visualized using a phosphorimager.

(B) CPY was recovered by immunoprecipitation from the unadsorbed supernatants following the immunoprecipitation with anti-invertase antibody and resolved on 8% SDS polyacrylamide gels. The position of the p1, p2, and mature (m) forms of CPY are indicated.

(C) The fraction of Inv-(N)cl$_{L57A}$ converted to the vacuolar processed form in the wildtype strain and COPI mutants over the period of chase was quantified using the PhosphorImager software and plotted using Cricket graph.
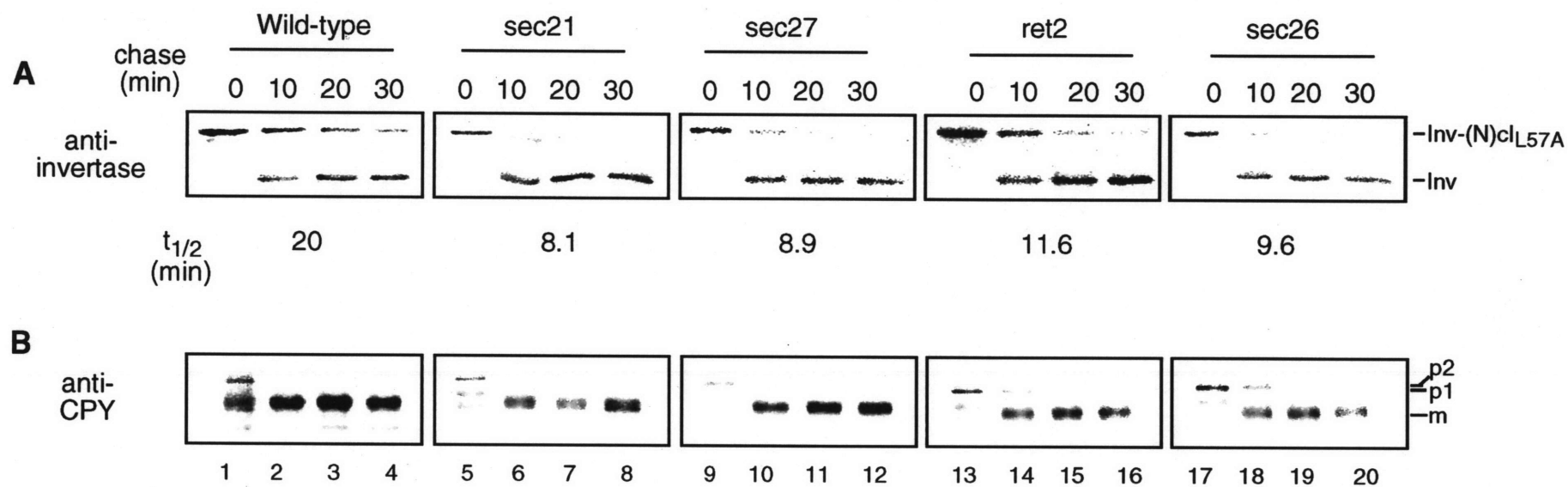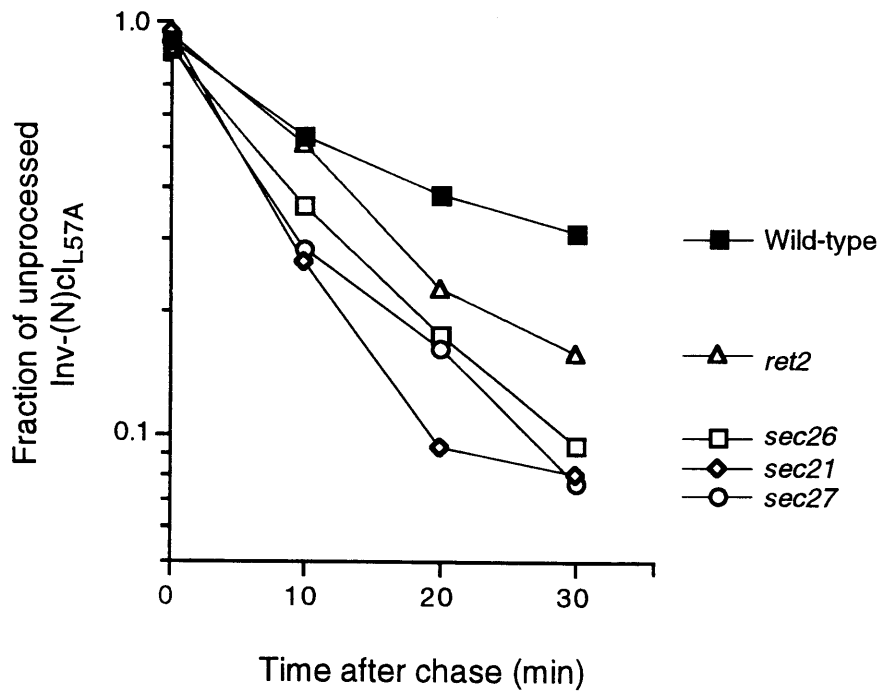
Figure 3

Figure 3b

Figure 4: Wildtype invertase is secreted to the cell surface in COPI mutants at 30°C. Strains expressing invertase at 30°C were converted to spheroplasts to separate extracellular protein in the supernatant (S) from the intracellular invertase in the pellet (P). pEHB2 (wild-type invertase) was expressed in CKY406, wild-type (lanes 1-2); CKY415, *sec21-1* (lanes 5-6); CKY416, *sec27-1* (lanes 7-8); CKY417, *ret2-2* (lanes 9-10); and *sec26-1* (lanes 11-12), and pEHB29 (s11-invertase) was expressed in CKY406 (lanes 3-4). Protein was solubilized, resolved on 8% SDS-polyacrylamide gels, and invertase was detected by Western blotting with invertase antiserum.
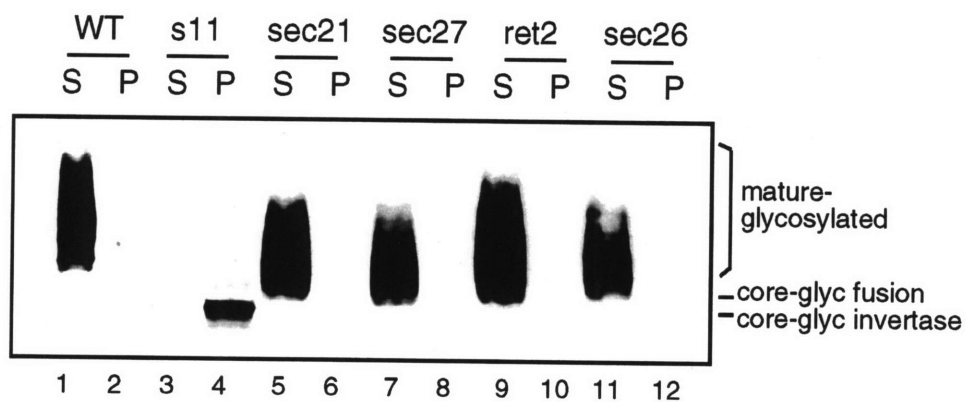
Figure 4

Figure 5: Inv-(N)cl$_{L57A}$ is transported to the vacuole more rapidly in strains defective for retrieval of BiP from the Golgi back to the ER.

(A) Inv-(N)cl$_{L57A}$ was expressed in CKY406, wildtype (lanes 1-4); CKY190, *kar2-ΔHDEL* (lanes 5-8); and CKY418, *erd2-B25* (lanes 9-12). Strains were induced for one hour at 30°C, labeled with [^{35}S]-methionine for 7 min and chased for up to 30 min. Time points taken at 0, 10, 20, and 30 min were immunoprecipitated with anti-invertase antibody, treated with Endo H, resolved on an 8% SDS polyacrylamide gel, and visualized using a phosphorimager.

(B) CPY was recovered by immunoprecipitation from the unadsorbed supernatants following the immunoprecipitation with anti-invertase antibody and resolved on 8% SDS polyacrylamide gels. The position of the p1, p2, and mature (m) forms of CPY are indicated.

(C) The fraction of Inv-(N)cl$_{L57A}$ converted to the vacuolar processed form in the wildtype, *kar2-ΔHDEL*, and *erd2-B25* strains over the period of chase was quantified using the PhosphorImager software and plotted using Cricket graph.
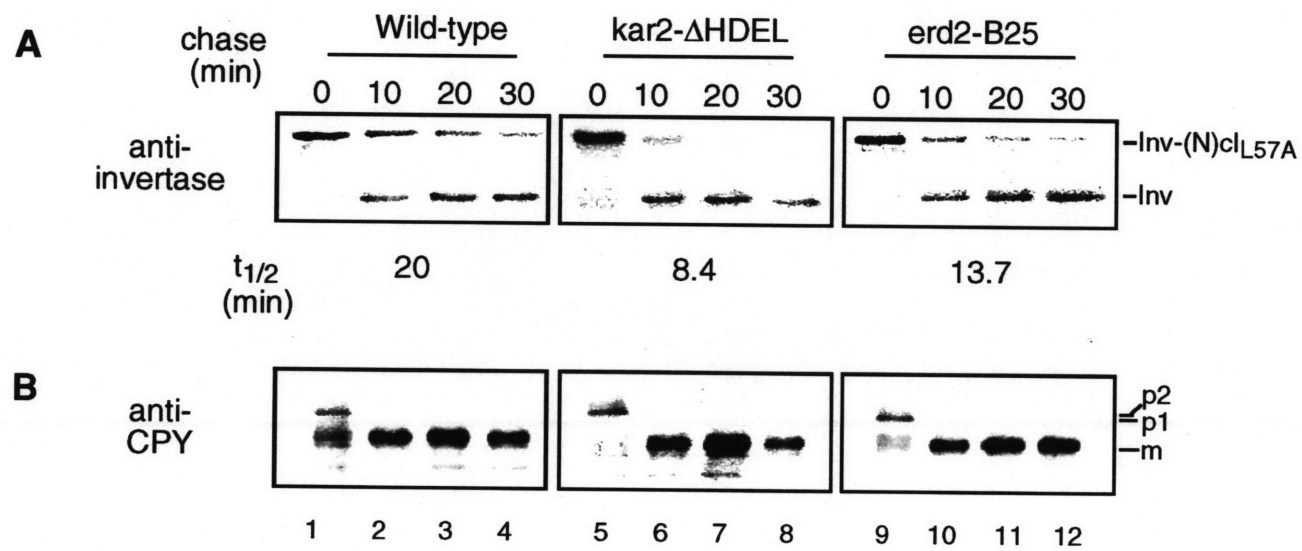
Figure 5

Figure 5b
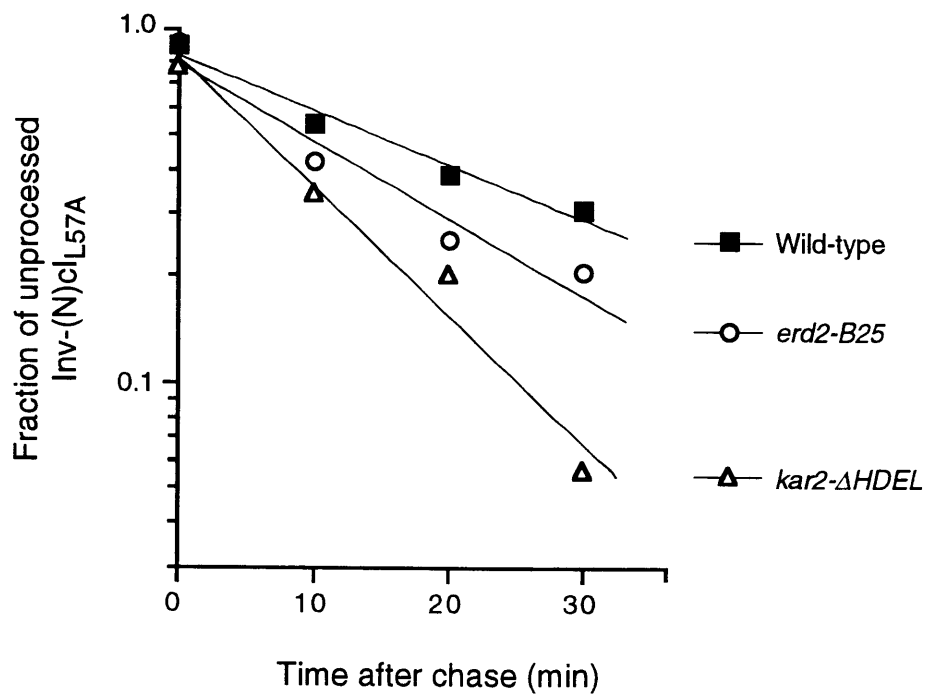
Figure 6: Kar2p physically interacts with $\alpha 1,6$-modified Inv-(N)cl$_{L57A}$.

Wild-type cells (CKY406) expressing either pEHB2 (wild-type invertase) or pEHB44 (Inv-(N)cl$_{L57A}$) were induced for 1 hour, labeled with [^{35}S]-methionine for 30 min, and spheroplasted. Spheroplast pellets were lysed by homogenization in a non-denaturing buffer and the lysates subjected to three sequential immunoprecipitations using anti-Kar2p, anti-invertase, and anti-$\alpha 1,6$ mannose antibodies. Protein extracts were resolved on 8% SDS-polyacrylamide gels and analyzed with the phosphorimager. The ratio of OD equivalents of protein extract loaded onto the gel is 1:3:4 for Kar2p, invertase, and $\alpha 1,6$ mannose antibodies, respectively.

Figure 6

	wt inv			Inv-(N)cl$_{L57A}$		
1st ab - Kar2	+	+	+	+	+	+
2nd ab - inv	−	+	+	−	+	+
3rd ab - α-1,6	−	−	+	−	−	+

− Inv-(N)cl$_{L57A}$

− Kar2p
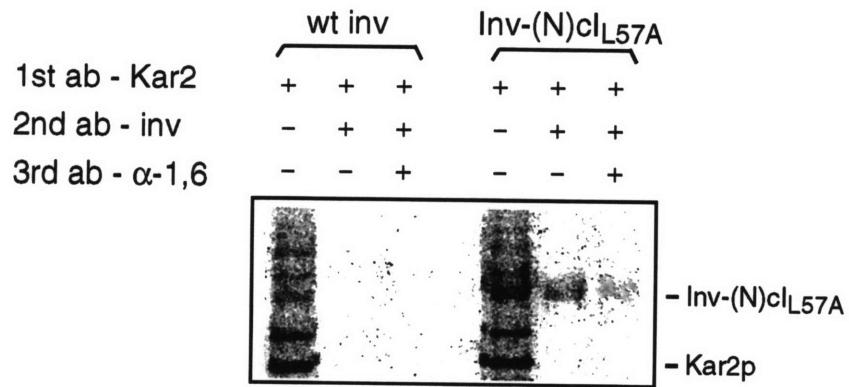
Figure 7: COPI mutants secrete more BiP than wildtype strain.

CKY406 (wild-type), CKY415 (*sec21-1*), CKY416 (*sec27-1*), CKY418 (*ret2-2*), and

CKY-- (*sec26-1*) expressing Inv-(N)cl$_{L57A}$ were grown till mid-exponential phase in

YPD medium and centrifuged to separate the cell pellet from the extracellular medium.

Cell pellets and TCA-precipitated mediums were solubilized, resolved on 8% SDS-

polyacrylamide gels, and Kar2p was detected by Western blotting with Kar2p

antiserum followed by densitometry analysis. The y-axis depicts the percentage of

Kar2p present in the extracellular medium compared to the total amount of Kar2p.

Figure 7

Figure 8: Model of misfolded protein being recycled from the cis-Golgi back to the ER.

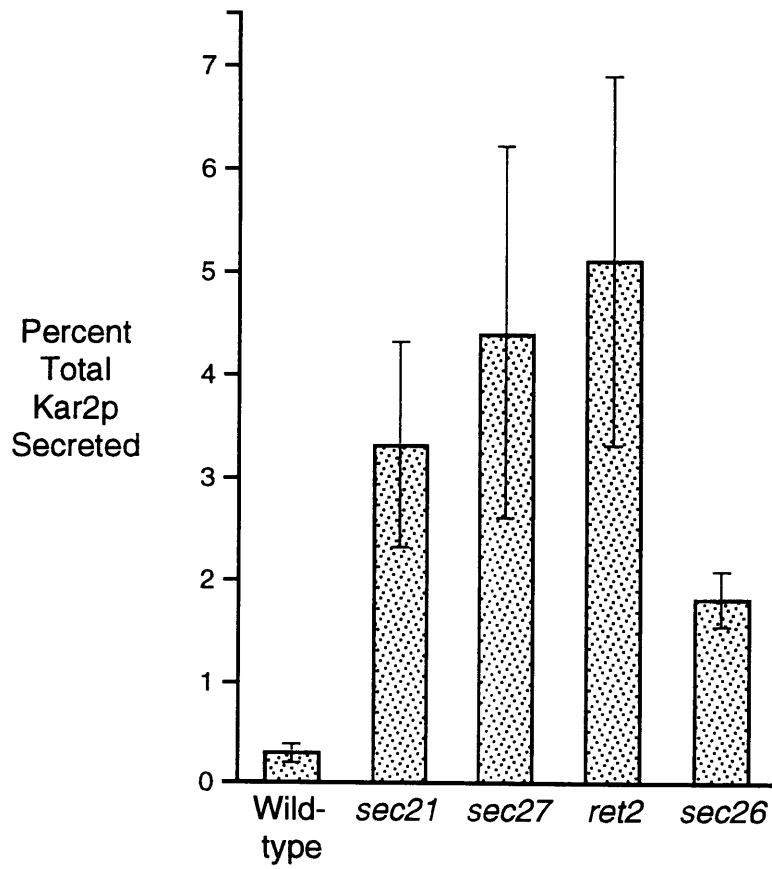Retrograde vesicles that mediate Golgi-to-ER transport are coated with the COPI complex and selectively carry misfolded protein via binding through Kar2p and the Kar2p receptor, Erd2p.
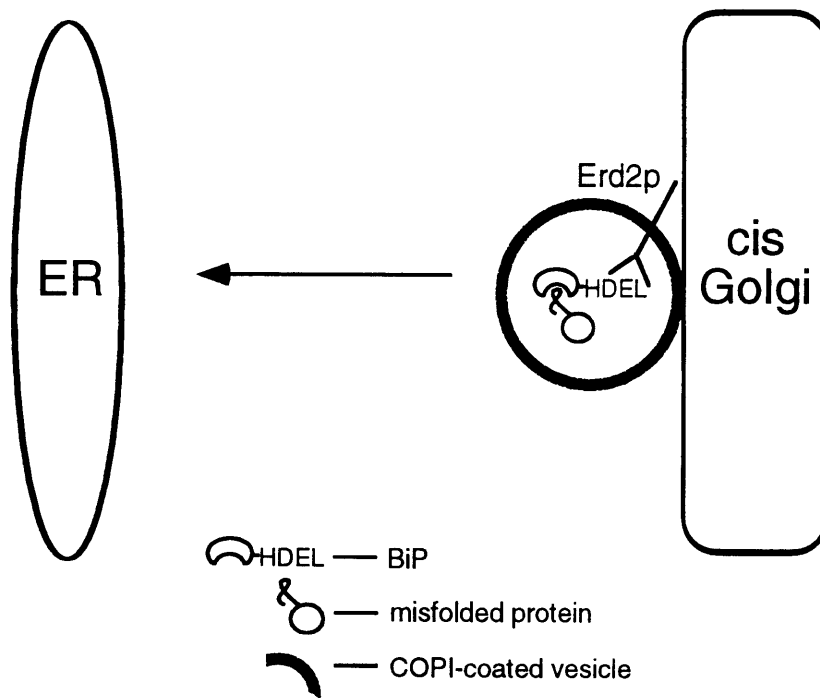
Figure 8

Erd2p

cis
Golgi

ER

HDEL — BiP

— misfolded protein

— COPI-coated vesicle

HDEL

Table I. *Saccharomyces cerevisiae* Strains

Strain	Genotype	Source or Reference
CKY406	*MATa ura3-52 leu2-3,112 suc2-Δ9*	This study
CKY415	*MATα sec21-1 ura3-52 suc2-Δ9*	This study
CKY416	*MATα sec27-1 ura3-52 suc2-Δ9*	This study
CKY417	*MAT sec80-1 ura3-52 suc2-Δ9*	This study
CKY400	*MATα sec80-1 ura3-52*	This study
CKY399	*MATa sec80-1*	This study
CKY190	*MATα kar2ΔHDEL ura3-52 leu2-3,112 his- suc2-Δ9*	M. Rose (Princeton University)
CKY418	*MATa ura3-52 leu2-3,112 his4-519 suc2-Δ9 erd2-B25*	M. Rose (Princeton University)
CKY8	*MATα ura3-52 leu2-3,112*	Kaiser Lab Collection
CKY10	*MATa ura3-52 leu2-3,112*	Kaiser Lab Collection
CKY273	*MATα can1r lys2-801 his4-619*	Kaiser Lab Collection
CKY39	*MATα sec12-1 ura3-52*	Kaiser Lab Collection
CKY45	*MATα sec13-1 ura3-52 his4-619*	Kaiser Lab Collection
CKY50	*MATα sec16-2 ura3-52 his4-619*	Kaiser Lab Collection
CKY54	*MATα sec17-1 ura3-52 his4-619*	Kaiser Lab Collection
CKY58	*MATα sec18-1 ura3-52 his4-619*	Kaiser Lab Collection
CKY65	*MATa sec20-1 ura3-52 his4-619*	Kaiser Lab Collection
CKY69	*MATa sec21-1 ura3-52 his4-619*	Kaiser Lab Collection
CKY71	*MATa sec22-3 ura3-52*	Kaiser Lab Collection
CKY78	*MATα sec23-1 ura3-52 his4-619*	Kaiser Lab Collection
CKY85	*MATa bet1-1 ura3-52*	Kaiser Lab Collection
CKY90	*MATa ypt1::LEU2 leu2 his3*	Kaiser Lab Collection
CKY100	*MATa sec27-1 ura3-52 leu2-3,112*	Kaiser Lab Collection

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Chapter 4

Invertase fusions bearing random 10-mer peptides enriched for hydrophobic residues are retained in the ER and associated with BiP

Abstract

We have constructed and expressed a library of 240 invertase fusion proteins bearing a random ten amino acid peptide at the C-terminus in yeast *Saccharomyces cerevisiae* to screen for short sequences that resemble a localized, misfolded determinant *in vivo* and elicit selective retention of the invertase fusion in the ER. Eight fusion proteins were isolated that accumulated core-glycosylated protein in the ER by steady-state and kinetic analysis. Seven out of the eight fusions were shown to be enzymatically active, indicating that retention was not caused by the peptide disrupting protein folding of invertase. A representative two fusions examined behaved as a soluble protein, implying that the peptides did not convert the fusion into a transmembrane domain. The amino acid sequences of these eight peptides were enriched up to 3.6 fold for bulky, hydrophobic residues arranged in alternating positions, a consensus sequence that had been identified as the optimal binding sequence for the ER Hsp70 chaperone BiP or Kar2p in vitro. We have subsequently detected a physical interaction between Kar2p and the two representative fusion proteins bearing hydrophobic peptides in cell lysates, providing in vivo evidence consistent with a model in which BiP recognizes and binds hydrophobic peptides exposed on mutant protein as a means of selectively retaining them in the ER.

Introduction

In eukaryotic cells, secretory proteins become exposed to an oxidizing environment and an extensive folding machinery in the ER lumen, where chaperones and modifying enzymes facilitate the folding of nascent protein into mature, disulfide-bonded forms to be transported to the Golgi compartments and then to the cell surface (Gething and Sambrook, 1992; Hwang et al., 1992). Cargo proteins which do not acquire a transport-competent form are recognized as misfolded substrate by quality control mechanisms and retained in the cell. Retention of misfolded protein in the ER has been investigated most extensively, including cases examining mutant forms of carboxypeptidase Y (CPY) (Knop et al., 1996), the cystic fibrosis transmembrane conductance regulator (CFTR) (Cheng et al., 1990), and unassembled subunits of immunoglobulins (Bole et al., 1986).

Historically, quality control in the ER has been studied by inducing the formation of grossly misfolded or incompletely-assembled protein with the glycosylation inhibitor tunicamycin (Marquardt and Helenius, 1992), addition of DTT to prevent disulfide bond formation (Braakman et al., 1992; Marquardt and Helenius, 1992), expression of single subunits of a multi-subunit complex (Bole et al., 1986; Lippincott-Schwartz et al., 1988; Wikstrom and Lodish, 1993), or protein sequence alterations (Doms et al., 1988; Cheng, et al., 1990; Knop, et al., 1996), and examining the identities of proteins that are specifically associated with the misfolded forms. The two most well-characterized chaperones, BiP and calnexin, were identified in this way. A member of the Hsp70 family, BiP was identified independently as an induced protein under conditions of glucose deprivation and as a protein associating with immunoglobulin heavy chain subunits not already bound to light chains in myeloma cell lines (Pouyssegur et al., 1977; Haas and Wabl, 1983). BiP was subsequently shown by in vitro studies to bind preferentially to short peptide sequences enriched for alternating, hydrophobic residues (Flynn et al., 1991; Blond-Elguindi et al., 1993). Similarly, calnexin was

found to be complexed with incompletely-assembled class I major histocompatibility complex (MHC) and membrane immunoglobulin subunits in lymphoma cell lines (Hochstenbach et al., 1992; David et al., 1993), although this chaperone recognizes a different type of target site, namely monoglucosylation of N-linked core oligosaccharides displayed on misfolded substrate (Hammond et al., 1994).

Since these chaperones were identified in association with nascent chains, grossly-misfolded protein, and incompletely-assembled membrane protein complexes, the sequence or structural determinants that mark a protein as being immature were unable to be characterized. To try and better define what comprises a misfolded determinant that causes ER retention under in vivo conditions, we have devised a method in which the yeast quality control system is exposed to short random sequences as target sites rather than to a grossly misfolded protein. We constructed a library of ten amino acid random peptides fused to the secretory marker protein invertase at its C-terminus. As discussed in Chapter 2, using invertase as a carrier protein for appended peptides offers several advantages, including the ability to follow transport from the ER to the Golgi based on glycosylation levels, the availability of an enzymatic assay to test whether the peptides interfere with invertase folding, and finally, a remarkable protein stability once the invertase folds and dimerizes properly. We hoped that upon translocation of the fusions into the ER lumen, the invertase moiety would fold into its native structure, but leave the random peptide projecting from the folded invertase to act as bait for interaction with components of the quality control system. Using the random peptide to mimic a small, localized, non-native region establishes a single target site for binding and avoids the severe cellular stress resulting from the massive accumulation of misfolded protein seen in previous studies.

In this study, we report that peptide sequences from invertase fusions retained in the ER were enriched for bulky hydrophobic residues in alternating positions, similar to the consensus sequence identified as the optimal target site for the ER chaperone

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BiP by in vitro studies. In addition, we were able to detect a physical complex between BiP and a representative two of the fusions bearing hydrophobic peptides. Our data is consistent with a model in which BiP recognizes and forms a complex with hydrophobic sequences on the surface of misfolded protein and blocks their transport from the ER.

Methods and Materials

Strains, reagents, and molecular biological techniques

Yeast strains used in this study were CKY16 (*MATa ura3-52 ade2-101 suc2-Δ9*), CKY406 (*MATa ura3-52 leu2-3,112 suc2-Δ9*), CKY407 (*MATa ura3-52 leu2-3,112 suc2-Δ9 sec12-4*), and CKY413 (*MATα ura3-52 ade2-101 suc2-Δ9 sec1-1*). The *kar2 suc2-Δ9* mutant strains were constructed from crosses between CKY16 and four *kar2* strains obtained from Mark Rose: MS174 (*MATa kar2-159 ura3-52 leu2-3,112*), MS194 (*MATa kar2-133 ura3-52 ade2-101 leu2-3,112*), MS1111 (*MATa kar2-1 ura3-52 leu2-3,112 ade2-101*), and MS1032 (*MATa kar2-203 leu2-3,112 ura3-52 ade2-101*) to produce EHY31 (*MATα kar2-159 ura3-52, suc2Δ9*), EHY35 (*MATa kar2-133 ura3-52 leu2-3,112 ade2-101, suc2-Δ9*), EHY40 (*MATa kar2-1 ura3-52 ade2-101 suc2-Δ9*), and EHY38 (*MATα kar2-203 ura3-52 ade2-101 suc2-Δ9*), respectively.

Standard genetic manipulations , preparation of yeast rich medium (YEP) and minimal medium (SD) (Difco Laboratories Inc., Detroit, MI), and yeast transformations were performed as described (Kaiser et al., 1994). DNA manipulations and subcloning were carried out using standard techniques (Sambrook et al., 1989). DNA sequencing was perfomred using the Sequenase kit (United States Biochemical Corp., Cleveland, OH).

Construction of invertase fusions

The plasmid pEHB9 carries the *SUC2* invertase coding seqence that had been modified by the addition of BglII and NheI sites at the C-terminus (Hong et al., 1996). The strategy to introduce random sequences into invertase was adapted from a protocol designed to display peptide libraries on the surface of phage (Cwirla et al., 1990). To construct the random peptide, three oligonucleotides were synthesized - (1) 5' GATCTCTGCATCA [NNK]$_{10}$ GCGGCCG 3'; (2) 5' TGATGCAGA 3'; and (3) 5'

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CTAGCGGCCGC $3'$ where N=G,A,T, or C and K=G or T, so that the 51-mer contains a random sequence of 30 nucleotides, while the 9-mer and 11-mer contain sequences that are complementary to the ends of the long oligo. Annealing of the three oligonucleotides yield a tri-oligo complex that recreates BglII and NheI sticky ends flanking the random sequence. The 9-mer, 11-mer, 51-mer, and gel-purified pEHB9 digested with BglII and NheI were incubated in a molar ratio of 100:100:5:1 at 75°C for 5 min, cooled slowly to room temperature, and incubated with 1U of ligase for 2 hours. The reaction mixture was then transformed into XL1-Blue *E. coli*, where the bacterial repair mechanisms filled in the complementary sequence in the gapped region spanning the random sequence of 30 nucleotides of the plasmid.

pEHB2 (wild-type invertase), pEHB29 (s11-invertase), and pEHB46 [Inv-(N)cI$_{L57A}$] have been described previously (Hong, et al., 1996). The 4.3 kb SalI-SacI fragments from pEHB2, pEH611 and pEH736 were cloned into high-copy vector pRS306-2μ to create pEHB2-2μ, pEH611-2μ and pEH736-2μ for the co-immunoprecipitation experiments.

Detection of invertase fusion proteins by western blotting

Invertase induction conditions, spheroplast formation, and western blotting techniques were performed essentially as described (Hong, et al., 1996). Cells were lysed immediately after the two hour induction in 0.1% glucose without an additional one hour incubation in 2% glucose.

To test whether the hydrophobic 10-mer peptides were anchoring the fusion protein in the membrane, 10^8 cell equivalents of spheroplast pellets were resuspended in 1 ml of cell lysis buffer (20mM Hepes pH 6.8, 0.1M NaCl, 5mM MgCl$_2$, 1mM PMSF), lysed with acid-washed glass beads by vigorous agitation 8x30 sec with cooling on ice, and the lysate then clarified at 500g for 5 min to remove large cell debris. 200μl aliquots of the resulting supernatant were incubated with an equal

volume of either cell lysis buffer, 200mM Na_2CO_3 pH 11, or 0.2% Triton X-100 for one hour on ice. Treated lysates were centrifuged at 100,000 g at 4°C for 10 min in an ultracentrifuge rotor (model TLA 100.3, Beckman Instruments, Fullerton, CA) to separate extracted proteins in the supernatant from the pellet fraction.

Invertase activity gels

10^8 equivalents of spheroplast pellets were lysed by vigorous agitation with glass beads in native sample buffer (0.1M Tris-PO4 pH 6.7, 0.1% Triton X-100, 10% glycerol, 0.1% bromophenol blue), diluted to 50μl with native sample buffer, and heated at 50°C for 15 min in the presence of 0.1% SDS. Samples were loaded in the following volume ratios: *sec1* and *sec12* - 1x; *Suc2-s11* - 2x; and pEH576, 611 and 736-CEN - 5x, onto a 5% non-denaturing polyacrylamide gel and run at 4°C for ~18 hours. To visualize the active invertase protein, the gel was washed in 0.1M Tris-PO4 pH 6.7 for 2 hours, incubated in 0.1M sucrose, 0.1M sodium acetate pH 5.1 for 20 min at 37°C, and then stained for activity by heating to 90°C in 50mM NaOH, 0.1% 2,3,5 triphenyl tetrazolium chloride until the invertase protein developed a red color. After quenching with 7.5M acetic acid, the gel was washed with distilled water and dried onto filter paper.

Radiolabeling and immunoprecipitations

Pulse/chase experiments and immunoprecipitations were performed essentially as described in Chapter 3, Materials and Methods. Labeled cells were brought up to 1% glucose at the initiation of chase to turn off invertase expression. For kinetic analysis of the *kar2* mutants, strains were labeled for 7 min at 25°C to permit Kar2p-dependent translocation and then chased at the restrictive temperature of 34°C.

To detect whether the ER-retained 10-mer fusions were modified with α1,6-mannoses, 4 x 10^8 wild-type cells (CKY406 or CKY16) expressing pEH44 [Inv-

(N)cl$_{L57A}$], pEH611, or pEH736 were labeled for 30 min and spheroplasted. The spheroplast pellet was resuspended in 1ml of cell lysis buffer (200mM sorbitol, 50mM Tris-Cl pH 7.4, 1mM EDTA, 1mM PMSF), dounced 10 times using a tissue grinder homogenizer (Curtin Matheson Scientific, Inc., Florence, KY), and the lysate centrifuged at 500xg. The clarified supernatant was spun at 13,000 x g at 4°C for 10 min using the TL100.3 ultracentrifuge rotor (Beckman Instruments, Palo Alto, CA) to generate a P13 fraction, which was diluted in 1 ml of IP buffer (50mM Tris-HCl pH7.4, 150mM NaCl, 1% Triton X-100), rotated with IgSorb, and then centrifuged for 5 min. The resulting supernatant was immunoadsorbed with 2 μl of anti-invertase antibody and processed. After boiling to dissociate protein from the secondary reagent, the sample was divided in half, one half was re-immunoprecipitated with 2 μl of anti-invertase antibody and the other half with 3 μl of anti-α1,6 mannose antibody (gift of R. Schekman) and processed.

For the co-immunoprecipitation experiments, 10^9 wild-type cells expressing high-copy 2μ versions of wild-type invertase, pEH611, and pEH736 were radiolabeled for 30 min, and spheroplasted. Spheroplast pellets were resuspended in 1 ml of cell lysis buffer (20mM MES pH 6.8, 100mM NaCl, 5mM Mg$_2$Cl, 1mM PMSF) and apyrase at 20U/ml, dounced 10 times, and the crude lysate centrifuged at 500xg. The clarified supernatant was adsorbed to IgSorb for 10 min and re-centrifuged for 5 min. The resulting supernatant was brought up to 0.1% Triton X-100, rotated with 0.2 μl of anti-BiP antibody and then protein A sepharose for 1 hour each. Processed protein samples were divided in half, one-half was saved and the other half immunoprecipitated with 2 μl of anti-invertase antibody and processed.

Results

A screen to identify invertase fusions to ten residue random peptides that are retained in the ER

A library of 240 invertase fusions to an eighteen residue peptide, ten of which are random amino acids, was constructed to screen for sequences that blocked transport of the invertase fusion out of the ER. To reduce the severity of the bias in the genetic code and maximize the equal distribution of amino acids, an (NNK) codon conformation (where N=G,A,T or C and K=G or T) was used in the synthesis of the random sequence. We hoped that a random peptide of ten amino acids would be a length long enough to elicit binding by a retention protein, but short enough to both allow for analysis of the sequence or structural patterns that emerged and not interfere with invertase folding. The constant region of eight amino acids, four flanking each side of the random sequence, contains the restriction sites required to insert the peptide, and was designed to be comprised of alanine or serine, both small, generally unreactive residues, in seven of the eight positions.

Each of the 240 invertase fusions was expressed in a wild-type yeast strain (CKY16) and then analyzed by Western blot using anti-invertase antibody. A majority of the invertase fusions, 219 total, co-migrated with wild-type invertase as high molecular weight, heterodispersed forms (Fig. 1A, lane 1), which is indicative of variable mannose addition to N-linked carbohydrate chains during transit through the Golgi compartments. Thus, the mature-glycosylated state of the 219 fusion proteins signified that these peptides appended to invertase did not severely block transport through the secretory pathway, and these fusions were presumed to be secreted out to the cell surface.

In contrast, the remaining twenty-one invertase fusions accumulated varying levels of core-glycosylated protein, implying that their peptides were somehow

causing retention of the fusions in the ER lumen and blocking transport to the Golgi (Fig. 1A, lanes 3-10). Bearing the additional eighteen amino acid peptide, core-glycosylated fusion protein migrated with a slightly higher molecular weight than Suc2-s11, an ER-retained mutant form of invertase with an uncleavable signal sequence (Fig. 1A, compare lane 2 and lanes 3-10) (Schauer et al., 1985). The majority of the twenty-one fusions also expressed mature-glycosylated fusion protein under steady state conditions, suggesting that the accumulation of the core-glycosylated forms was the result of a decreased rate in transport of the fusion proteins from the ER to the Golgi rather than a complete block in exit from the ER.

ER-retained fusion proteins bear peptides enriched for bulky, hydrophobic residues

Sequencing of the ER-retained fusion proteins revealed that in thirteen of the twenty-one peptides, the number of nucleotides comprising the random sequence were not in multiples of three, leading to a frameshift that lengthened the additional C-terminal peptide from eighteen to an average of thirty-two amino acids. Since analysis of longer sequences for ER retention consensus patterns would most likely prove to be more complicated, we did not characterize these extended fusions any further.

The remaining eight peptide sequences, which encoded for the expected ten random amino acids, exhibited a preponderance towards bulky, hydrophobic residues, such as tryptophan and phenylalanine (Fig. 1B). By taking into account the genetic code and the (NNK) configuration in the design of the peptides, we calculated the predicted number of times that a particular hydrophobic amino acid would be expected to be present in these eight random sequences (Fig. 1D). For example, tryptophan is represented one time for every 32 triplet configurations, and thus would be predicted to appear 2.5x in 80 random residues. In fact, nine tryptophan residues were present in the random sequences, a 3.6x increase over the expected number.

Similarly, the random sequences were enriched for other bulky, hydrophobic residues, such as phenylalanine, leucine, and methionine, exhibiting 2.8x, 2.0x, and 3.6x increases, respectively (Fig. 1D). To examine whether this enrichment for hydrophobic residues was specific for invertase fusions retained in the ER, we also sequenced representative random peptides from fusions that were secreted out to the cell surface (Fig 1C). The number of hydrophobic residues in these peptides more closely followed the expected distribution, with tryptophan and phenylalanine represented 0.8x, leucine 1.2x, and methionine 1.6x (Fig. 1D). Thus, increased levels of hydrophobic residues in peptide fusions correlated with the retention of these fusions in the cell.

Short peptide sequences (8 and 12-mers) enriched for bulky, hydrophobic residues have already been described as being a sequence motif for the binding of the ER chaperone BiP in phage display binding experiments (Blond-Elguindi, et al., 1993). Study of these peptide sequences had further elucidated a consensus pattern in which four hydrophobic residues were arranged in alternating positions. Upon inspection of the peptide sequences of retained fusions from our screen, we observed a similar alternating pattern of hydrophobic residue placement, most notably in the random sequences from pEH611 and pEH681, which contain four consecutive alternating hydrophobic residues (Fig. 1B). The ER retention of fusions bearing peptides that closely resemble target sequences for BiP in vitro suggested that this ER chaperone may be binding to the hydrophobic peptides and preventing the fusions from exiting the ER.

ER-retained fusion proteins are enzymatically active

In designing our invertase fusions, we had made the assumption that a ten residue random sequence would most likely not interfere with the normal folding of active invertase enzyme, so that retention of fusions in the ER could be ascribed to the

presence of the peptide as a retention signal rather than to the formation of grossly misfolded invertase. However, the possibility remained that the short hydrophobic peptides from the ER-retained fusions may be able to disrupt the invertase folding pathway. To determine whether the ER-retained fusions assumed a correctly-folded and enzymatically active conformation, we analyzed the fusion proteins on native activity gels.

A wild-type strain (CKY16) expressing different retained invertase fusions were converted to spheroplasts to separate cell pellets harboring intracellular fusion protein from extracellular protein. The cell pellet fractions were subjected to non-denaturing gel electrophoresis, and the gel was subsequently stained for invertase activity. For the fusion proteins EH576, EH611, and EH736 (Fig. 2, lanes 4-6), active core-glycosylated fusion protein was shown to co-migrate with a condensed lower molecular weight species from both wild-type invertase retained in the ER by a *sec12* block, which prevents the formation of budding vesicles from the ER (Fig. 2, lane 2), and ER-retained Suc2-s11 bearing a signal sequence (Fig. 2, lane 3). Similarly, EH609, EH681, EH718, and EH738 all exhibited active core-glycosylated invertase in the spheroplast pellet (data not shown), implying that these hydrophobic peptides did not completely disrupt proper folding of the entire invertase protein population. Only pEH639 fusion protein remained devoid of enzymatic activity, suggesting that its extremely hydrophobic (9 out of 10 residues) peptide did interfere with invertase maturation. The appearance of enzymatically active, core-glycosylated fusion protein in the spheroplast pellets provided strong evidence against the possibility that retention of these fusions was caused solely by the ER quality control system recognizing and binding to severly misfolded invertase fusion protein.

In addition to the core-glycosylated species, the ER-retained fusion proteins (except pEH639) were also resolved as mature-glycosylated forms that co-migrated with the active, heterodispersed, high molecular weight wild-type invertase retained in

the cell by a *sec1* mutation, which prevents the fusion of post-Golgi vesicles with the plasma membrane (Fig. 2, lane 1). The presence of active mature-glycosylated invertase fusions supports our hypothesis from western blot analysis that the transport of the retained fusion proteins out of the ER to the Golgi was being slowed down rather than completely blocked.

The amount of active invertase in the *sec1* and *sec12* spheroplast pellets appeared to be much higher than in strains expressing the 10mer fusions, partially because the two secretory mutants completely block secretion to the cell surface, while a population of the 10mer fusions was transported to the cell surface and released into supernatant during spheroplasting. However, invertase activity assays and quantitative western blots did reveal a decreased level of fusion protein in the cell compared to wild-type invertase (data not shown), although the reasons for this difference are unknown.

ER-retained fusion proteins exhibit a decreased rate of transport to the Golgi

The presence of mature-glycosylated fusion protein under steady state conditions implied that the ER-retained fusions were being transported out of the ER to the Golgi, but at a slower rate that resulted in the observed accumulation of fusion protein in the ER. To more closely analyze the kinetics of fusion protein transport, pulse/chase experiments were performed to determine the rate at which the ER-retained fusions acquired Golgi modifications.

A wild-type strain (CKY16) expressing wild-type invertase and various invertase fusions were radiolabeled with [^{35}S]-methionine for 7 min and chased for up 30 min. The invertase fusions from the 0, 10, 20, and 30 min time points were recovered by immunoprecipitation with anti-invertase antibody, resolved on SDS-polyacrylamide gels, and visualized using a phosphorimager. Wildtype invertase was completely

converted from core-glycosylated to mature-glycosylated protein very rapidly, within ten minutes of chase (Fig. 3, lanes 1-4). The half-life for transport of invertase from the ER to the Golgi has been measured to be ~1.5 min (Schauer, et al., 1985). In contrast, the four ER-retained fusions examined all exhibited a marked decreased rate of conversion to Golgi-modified fusion protein and still harbored core-glycosylated fusion through 30 min of chase (Fig. 3, lanes 5-20). The half-lives of EH576, EH611, and EH736 for ER to Golgi transport were calculated to be 14, 20, and 25 min, respectively. The variability in the half-lives between the four ER-retained fusions most likely reflected the different degrees with which the random peptides interacted with components of the ER quality control system. EH639, which had not displayed any enzymatic activity, persisted primarily as core-glycosylated protein through the chase period, suggesting that this fusion was being tightly retained in the ER.

ER-retained fusion proteins are not anchored in the ER membrane

Since wild-type invertase normally behaves as a soluble protein, we had presumed that the retained fusions were being confined in the ER lumen and blocked from becoming packaged into secretory vesicles. However, the enrichment of hydrophobic residues in the random sequences of retained fusions suggested the possibility that the peptide tag may have assumed the properties of a transmembrane domain. Upon translocation of the fusion protein into the ER lumen, the C-terminal peptide tag would become anchored in the ER membrane and convert the fusion protein into a type I transmembrane protein. Although the random sequence was considerably shorter than the 20 hydrophobic residues usually required to span the membrane lipid bilayer, we examined whether the ER-retained fusion proteins acted as an integral membrane protein. We chose to analyze EH611 and EH736 as representative of the ER-retained fusions for the remainder of the experiments described.

Spheroplast pellets from a wild-type strain (CKY16) expressing either pEH611 or pEH736 were lysed with glass beads, and the lysates were centrifuged at 100,000 g to separate soluble protein in the supernatant fraction from membrane-associated protein in the pellet fraction. The two fractions were analyzed by gel electrophoresis and western blotting using anti-invertase antibody. EH611 and EH736 invertase fusion protein were detected in both the supernatant (Fig. 4, lanes 2 and 9) and pellet fractions (Fig. 4, lanes 3 and 10), indicating that both soluble and membrane-associated forms of the retained fusions were present in the cell. To determine whether fusion protein in the pellet fraction were peripheral or integral membrane proteins, lysates were incubated with a high pH solution of sodium carbonate, which disrupts protein-protein interactions and extracts peripherally-associated proteins off of membranes, but leaves integral membrane proteins intact. After sodium carbonate treatment, all of the fusion protein partitioned with the high speed supernatant for both EH611 (Fig. 4, lanes 4-5) and EH736 (Fig. 4, lanes 11-12), implying that the membrane-bound fusion protein had been peripherally-associated with the ER membrane. Treatment of lysates with the detergent Trition X-100 also completely released membrane-bound fusions into the supernatant fraction (Fig. 4, lanes 6-7 and 13-14). Thus the hydrophobic peptides did not cause retention of the fusion proteins by behaving as transmembrane domains that anchored the fusions in the ER membrane. Rather, the hydrophobic peptides conferred on invertase the ability to partially associate with either proteins bound to the ER membrane or a large protein complex that can sediment following centrifugation at 100,000 g.

ER-retained fusions are not recycled from the cis-Golgi back to the ER

We had previously investigated the secretory fate of an invertase fusion to a thermally unstable mutant of the N-terminal DNA-binding domain of λ repressor [Inv-(N)cl$_{L57A}$] (see chapters 2 and 3). Transport of Inv-(N)cl$_{L57A}$ from the ER to the Golgi

was slowed when compared to that of wild-type invertase, indicating that the mutant repressor domain caused partial retention of the fusion protein at this early step of the secretory pathway. In addition, Inv-(N)cl$_{L57A}$ was observed to migrate at a slightly higher molecular weight than core-glycosylated fusion protein, suggesting that Inv-(N)cl$_{L57A}$ had acquired some modification. We were subsequently able to isolate α-1,6 mannose-modified Inv-(N)cl$_{L57A}$ from a fraction enriched for the ER (see chapter 3, Fig. 2B), implying that Inv-(N)cl$_{L57A}$ had been transported to the Golgi to receive the α-1,6 modifications but was then recycled back to the ER, as had been described for several other misfolded proteins (Hsu et al., 1991; Hammond and Helenius, 1994; Knop, et al., 1996). From the kinetic analysis of the 10-mer fusions, there wasn't any indication that the fusion protein had acquired modifications during the chase period, since conversion from core-glycosylated to mature-glycosylated fusion did not proceed through an observed intermediate molecular weight form. However, because our study of the behavior of Inv-(N)cl$_{L57A}$ had revealed that ER retention could also be mediated by recycling from the cis-Golgi back to the ER as well as prevention of transport from the ER, we examined whether 10mer fusions isolated from an ER fraction were modified by α-1,6 mannoses.

Wildtype cells (CKY406 and CKY16) expressing Inv-(N)cl$_{L57A}$, pEH611 or pEH736 were labeled for 30 min and converted to spheroplasts by digestion of the cell wall. The cell pellets were gently lysed with a Dounce homogenizer and the lysate centrifuged at 13,000 x g to yield a pellet fraction (P13) enriched for ER membranes. Invertase was immunoprecipitated from the P13 fraction with anti-invertase antibody, and the processed samples were divided in half, one half re-precipitated with anti-invertase antibody and the other half with antibody to anti-α1,6 mannoses. As demonstrated in Chapter 3, anti-α1,6 mannose antibody precipitated the slower migrating form of Inv-(N)cl$_{L57A}$ from the P13 fraction (Fig. 5, compare lanes 1 and 2), indicating that Golgi α1,6-modified Inv-(N)cl$_{L57A}$ did accumulate in the P13 fraction. In

contrast, the anti-α1,6 mannose antibody precipitated very little pEH611 and pEH736 isolated from a similarly-prepared P13 ER fraction (Fig. 5, lanes 3-4 and 5-6). We conclude that ER retention of these two invertase fusions do not involve recycling from the cis-Golgi back to the ER.

Kar2p physically associates with the ER-retained fusion proteins

Since in vitro studies had already established a consensus sequence of hydrophobic residues arranged in alternating positions for BiP binding, the finding that peptides from a group of invertase fusions retained in the ER were enriched for hydrophobic amino acids strongly suggested that BiP might be associated with these fusions in the ER lumen. To obtain biochemical evidence that Kar2p, the Bip homolog in the yeast ER, specifically interacted with the ER-retained fusions, co-immunoprecipiation experiments were performed to detect the existence of these complexes.

Wild-type cells (CKY16) expressing wild-type invertase, pEH611, or pEH736 from a high copy 2μ plasmid were labeled for 30 min, spheroplasted, and the cell pellets lysed by homogenization in non-denaturing buffer. The lysates were immunoprecipitated with anti-Kar2p antibody, followed by a second immunoprecipitation of one half of the resulting protein sample with anti-invertase antibody. Protein extracts were resolved by gel electrophoresis and analyzed with the phosphorimager. Wild-type invertase did not appear to be associated with Kar2p (Fig. 6, lanes 1-2), since folded invertase should mostly avoid long-term interactions with a chaperone specific for binding misfolded substrate. In contrast, immunoadsorption using Kar2p antibody co-precipitated both the EH611 and EH736 invertase fusions (Fig. 6, lanes 3-4 and 5-6), demonstrating that Kar2p specifically interacts with these two fusions. The ability to isolate these complexes under native conditions is

consistent with a model in which BiP recognizes and binds to the hydrophobic peptide of these invertase fusions and prevents their transport from the ER.

We also examined the secretory fate of EH611 and EH736 when expressed in four temperature-sensitive *kar2* mutants, which contain mutations in both of the major domains that comprise BiP: *kar2-1* and *kar2-133* contain mutations in the ~30kD C-terminal peptide binding domain, while *kar2-159* and *kar2-203* bear mutations in the ~44kD N-terminal ATPase domain but not specifically in the ATP binding site. Kinetic analysis of invertase fusion transport in these mutants was complicated by the fact that Kar2p is also required for efficient translocation of nascent chains through the ER membrane (Vogel et al., 1990). Thus, *kar2* strains expressing either pEH611 or pEH736 were induced and labeled at the permissive temperature of 25°C to allow translocation of invertase into the ER and then chased at the restrictive temperature of 34°C to impair the Kar2p chaperone function. Given that BiP was found to be complexed with at least two of the ER-retained fusions, we had expected that expression of these fusions in the peptide-binding mutants *kar2-1* and *kar2-133* would result in the loss of ER retention and an increased rate of transport of the fusions to the cell surface. Instead, pulse/chase experiments did not reveal a significant increase in the rate of fusion protein transport to the cell surface for these two *kar2* mutants compared to a wild-type strain (data not shown).

We expected the *kar2-159* and *kar2-203* mutants to exhibit any number of defects, including the rate of ATP hydrolysis, the conformation change induced upon ATP binding, or the coupling between ATP hydrolysis and peptide release. Although these two mutants should bind hydrophobic targets since their peptide-binding domains remain intact, they could demonstrate increased binding to substrate because peptide release mediated through ATP hydrolysis may be impaired. However, similar to the peptide-binding *kar2* mutants, we did not observe any significant change in the rate of transport of EH611 and EH736 through the secretory

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pathway. Possible explanations for these findings with the *kar2* mutants will be

presented in the Discussion.

Discussion

Retention of misfolded protein in the ER has generally been studied by forming grossly misfolded or unassembled substrate with such disruptive reagents as tunicamycin or DTT. Unfortunately, use of these disruptive reagents generates a randomly misfolded structure, for which it is impossible to determine what sequence or structural determinants were recognized by the ER quality control system. To the best of our knowledge, there haven't been any reports in which an in vivo system was used to systematically select for specific target sequences that comprise an unfolded determinant marking a protein for retention in the ER. We have addressed this line of experimentation by designing a screen to identify short random peptides that when appended to the secretory protein invertase, retains the fusion protein in the ER.

The eight fusion proteins identified from this screen exhibited varying degrees of an ER to Golgi transport defect and bore random sequences that were enriched for bulky hydrophobic amino acids compared to the peptides of fusions secreted to the cell surface. In general, misfolded protein are thought to be retained in the ER because their hydrophobic cores unfold and become exposed to the ER aqueous environment. Interestingly, the ER chaperone BiP was shown to preferentially bind to short peptides enriched for hydrophobic residues arranged in alternating positions in in vitro affinity panning studies of bacteriophage libraries that displayed random octapeptide and dodecapeptide sequences (Blond-Elguindi, et al., 1993). The authors proposed that the hydrophobic peptides bound to BiP in an extended conformation, with the bulky side chains of the alternating residues making contacts with binding pockets on the BiP protein. The 10-mer peptides from the ER-retained fusions also exhibit a similar pattern of alternating hydrophobic residues, suggesting that the fusion proteins are retained in the ER through interactions with BiP. In support of this hypothesis, we were able to detect a physical interaction between Kar2p, the BiP homolog in yeast, and the two fusion peptides tested, EH611 and EH681. Thus, our

finding that hydrophobic peptides identified by in vivo means were bound by BiP and caused retention of invertase in the ER very nicely complements the data gathered from the in vitro affinity panning studies.

Perhaps the most challenging aspect of this study was to demonstrate that the invertase moiety of the ER-retained fusions was folded properly in the ER and that retention was not caused by the formation of an abnormal or grossly misfolded fusion protein. We were able to detect enzymatically active, core-glycosylated invertase in strains expressing the ER-retained fusion proteins. In general, barely any core-glycosylated wild-type invertase protein can be observed under steady state conditions, as in a western blot or the enzymatic activity gels, since transport of invertase to the cell surface occurs extremely rapidly. Thus, accumulation of active, core-glycosylated fusion protein strongly indicates that the hydrophobic peptides are causing retention of a population of properly-folded fusion molecules in the ER.

An uncleavable signal sequence was reported to cause retention of invertase in the ER through association with ER membranes, even after treatment with the detergent saponin, which permeabilizes membranes and releases soluble protein (Schauer et al., 1985). Although EH611 and EH736 were shown to exist in both soluble and membrane-bound forms, treatment of cell lysates with high pH shifted all fusion protein from the pellet fraction into the supernatant fraction, implying that the membrane-bound population were peripherally-associated rather than converted into an integral membrane protein.

One puzzling feature in our investigation was the decreased protein levels of the ER-retained invertase fusions compared to wild-type invertase by both activity assays and quantitative western blot analysis. Inspection of endo H-digested time point samples from the pulse/chase experiments in Figure 3 revealed that more wild-type invertase was expressed upon induction in low glucose compared with the four fusion protein examined (data not shown). However, quantitation of the endo H-

digested fusion protein revealed fairly constant protein levels over the course of the chase period, suggesting that the fusion proteins weren't being degraded, at least for the thirty minutes of the chase. Possibly the cell modulates the transcriptional or translational efficiencies of the invertase fusions because it detects that the fusions are not quite the same as wild-type invertase or that the fusions are accumulating in the ER instead of being secreted to the cell surface.

We investigated whether secretion of EH611 and EH736 would be affected in several kar2 mutants: kar2-159 and kar2-203, which are defective in their ATPase domain, and kar2-1 and kar2-133, which are defective in their peptide-binding domain. These kar2 mutants had previously been studied in the context of whether Kar2p plays a role in the folding and maturation of carboxypeptidase Y (CPY) in the yeast ER lumen (Simons et al., 1995). In these experiments, kar2 mutant strains were grown at the permissive temperature to allow translocation of secretory protein into the ER and incubated in the reducing agent DTT to delay the folding and intracellular transport of CPY. Upon shift up to the restrictive temperature, the DTT was diluted from the medium to follow transport of CPY through the secretory pathway. Inactivation of Kar2p in the kar2-159 and kar2-203 mutants resulted in the sequestration of CPY in large, partially disulfide-linked, BiP-associated aggregates and increased binding between BiP and CPY, consistent with a model in which ATP hydrolysis or the conformational change following ATP binding mediates peptide release and overall protein folding.

Expression of EH611 and EH736 in the mutants kar2-159 and kar2-203 did not appear to lead to any change in their delayed transport through the secretory pathway by kinetic analysis. A possible explanation for this observation is that the mutant Kar2p proteins exhibit only a partial loss of function at the restrictive temperature (Simons, et al., 1995), which provides enough activity to still mediate substrate binding and release. The misfolding and aggregation effects observed in the kar2-159 and

kar2-203 mutants for CPY may have been more exaggerated than what would normally occur in these mutants since the DTT added to delay CPY folding may have precipitated more global effects on the ER environment, although the DTT was diluted out during the chase. For example, DTT may have modulated the folding activities of other ER chaperones that affect CPY maturation. Biochemical studies measuring the rate of ATP hydrolysis and peptide release need to be performed to determine whether the Kar2p mutant proteins expressed in *kar2-159* and *kar2-203* retain partial function.

The two peptide-binding mutants, *kar2-1* and *kar2-133*, also did not exhibit a change in the rate of EH611 and EH736 transport at the restrictive temperature. If these two mutants were completely defective in substrate binding, then the ER-retained fusions would be expected to sidestep any interaction with the non-functional Kar2p and be transported to the cell surface at a faster rate. However, the efficiency with which the mutant Kar2p proteins expressed in *kar2-1* and *kar2-133* binds to substrate have not been examined. Although *kar2-1* and *kar2-133* contain mutations that map to the peptide-binding domain, they may still be retain the ability to bind to the hydrophobic peptides of EH611 and EH736. In these two *kar2* mutants, CPY was shown to exhibit a partial folding and transport defect and accumulated in disulfide cross-linked aggregates. However, the same argument used for the *kar2-159* and *kar2-203* mutants can also be applied, namely that secondary effects caused by the addition of DTT precipitated CPY misfolding rather than the presence of mutant Kar2p protein.

A second explanation for why *kar2-1* and *kar2-133* did not exhibit an increased rate of transport to the cell surface for EH611 and EH736 is that other ER chaperones whose target sites overlap with those of BiP may have complexed with the fusion protein immediately after dissociation of the mutant Kar2p. Several ER chaperones have been identified whose binding specificities have not yet been established, including Grp94, ERp72 (Dorner et al., 1990), and Grp170 (Lin et al., 1993). Examples

of chaperone relays in mammalian cells have been reported in which immature protein is passed from one chaperone to another during the folding and maturation process (Melnick et al., 1994; Kim and Arvan, 1995). Possibly, one of these other chaperones in the ER recognizes the hydrophobic peptides on the ER-retained fusions and takes the place of Kar2p upon Kar2p inactivation at the restrictive temperature.

Figure legends

Figure 1: Invertase fused to ten amino acid random peptides that are enriched for bulky,hydrophobic residues accumulate in the ER.

(A) The following forms of invertase were expressed in a wild-type strain (CKY16): pEHB2 (lane 1), s11-invertase (lane2), pEH576 (lane 3), pEH609 (lane 4), pEH611 (lane 5), pEH639 (lane 6), pEH681 (lane 7), pEH718 (lane 8), pEH736 (lane 9), and pEH738 (lane 10). Protein was solubilized, resolved on 8% SDS-polyacrylamide gels and detected by Western blotting using anti-invertase antibody.

(B) The sequences for the random peptides of the eight ER-retained fusions are listed, with the bulky hydrophobic amino acids (tryptophan, phenylalanine, methionine, and leucine) underlined. The ISAS and AAAS residues flanking the random sequence contain the restriction sites used for plasmid construction.

(C) The sequences for random peptides of eight fusions that were secreted to the cell surface are listed, with bulky hydrophobic amino acids underlined.

(D) The number of hydrophobic amino acids in the peptides appended to the ER-retained fusions were compared to the number in peptides from fusions secreted to the cell surface and to the number expected by random distribution.

Figure 1
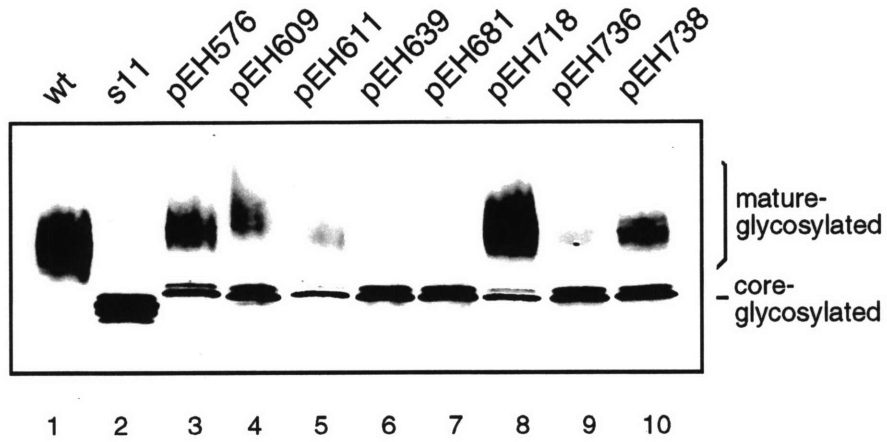
Designation	Sequence
WT invertase	...EVK*
pEH576	...EVKISAS MGLSITYLLL AAAS*
pEH609	...EVKISAS LTMTYTVMWI AAAS*
pEH611	...EVKISAS PLFRMVLSFG AAAS*
pEH639	...EVKISAS LLLRLWLFFM AAAS*
pEH681	...EVKISAS FLWAIIWSPW AAAS*
pEH718	...EVKISAS AGPWGWTVSF AAAS*
pEH736	...EVKISAS GWVRWMMVCT AAAS*
pEH738	...EVKISAS MLVMFIQPLH AAAS*

C

Designation	Sequence		
wt invertase	...LVK		
pEH514	...LVKISAS	SRSRDPCTV<u>L</u>	AAAS
pEH591	...LVKISAS	PKRY<u>L</u>CKPHK	AAAS
pEH618	...LVKISAS	<u>IL</u>STC<u>L</u>V<u>M</u>DG	AAAS
pEH644	...LVKISAS	<u>L</u>V<u>L</u>W<u>R</u>AD<u>W</u>T<u>L</u>	AAAS
pEH657	...LVKISAS	R<u>F</u>Y<u>F</u>CRG<u>IM</u>*	
pEH683	...LVKISAS	SV<u>I</u>TYGNQSS	AAAS
pEH709	...LVKISAS	<u>L</u>T<u>M</u>TSGGPN*	
pEH722	...LVKISAS	RA<u>MI</u>NKSTSS	AAAS

D

amino acid	# expected per 80 aa	retained fusions		secreted fusions	
		#observed	fold	#observed	fold
W	2.5	9	3.6x	2	0.8x
M	2.5	9	3.6x	4	1.6x
F	2.5	7	2.8x	2	0.8x
L	7.5	15	2.0x	9	1.2x
I	2.5	5	2.0x	4	1.6x

Figure 2: Core-glycosylated ER-retained fusion proteins exhibit enzymatic invertase activity.

CKY413 (*sec1-1*) (lane 1) and CKY407 (*sec12-4*) (lane 2) expressing pEHB2 (wild-type invertase) and CKY16 expressing pEHB29 (s11-invertase) (lane 3), pEH576 (lane 4), pEH611 (lane 5), and pEH736 (lane 6) were converted to spheroplasts. The spheroplast pellet was lysed in non-denaturing buffer, resolved on a 5% non-denaturing protein gel and active invertase was visualized on the gel by an enzymatic colorimetric assay. Samples were loaded in the following volume ratios: *sec1* and *sec12* - 1x; s11-invertase - 2x; and pEH576, 611 and 736-CEN - 5x.
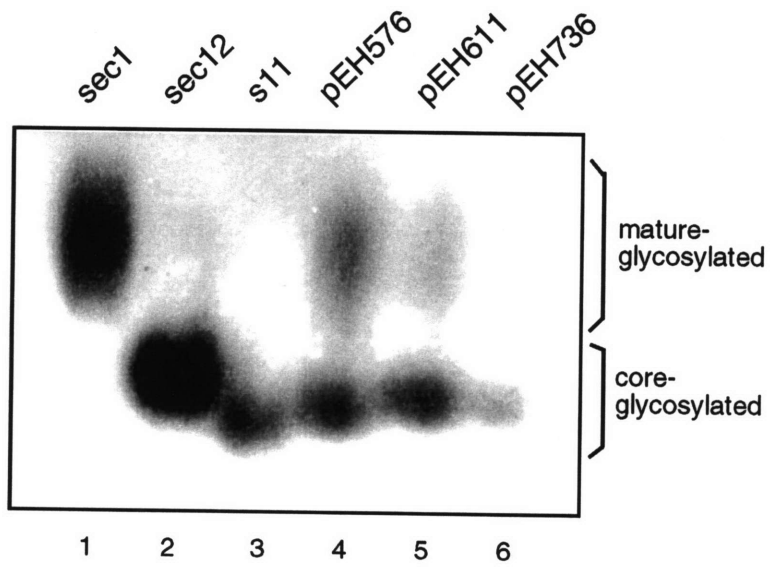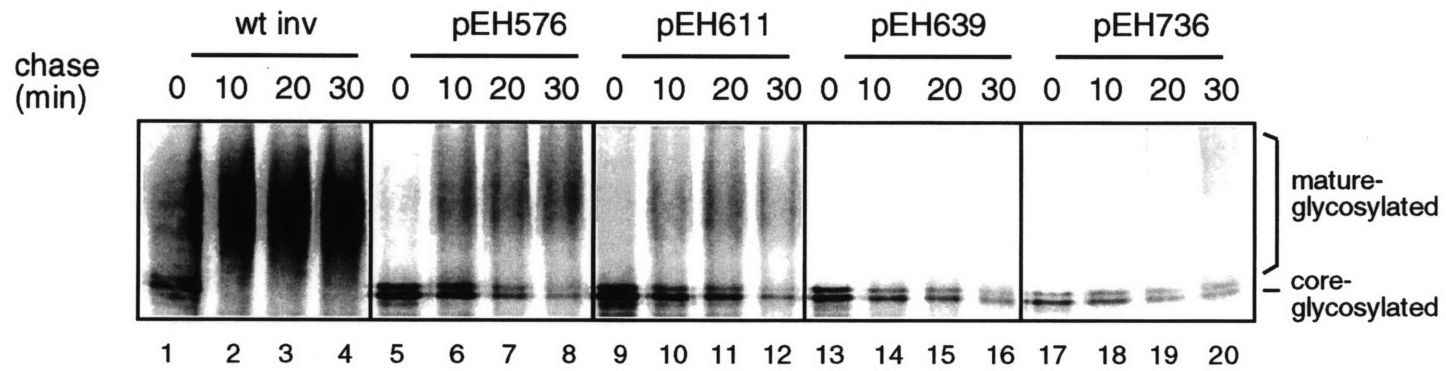
Figure 2

Figure 3: The ER-retained invertase fusions are transported to the Golgi at a slower rate than wild-type invertase.

(A) CKY16 (wild-type) expressing pEHB2 (wild-type invertase) (lanes 1-4); pEH576 (lanes 5-8); pEH611 (lanes 9-12); pEH639 (lanes 13-16); and pEH736 (lanes 17-20) were induced for one hour at 30°C, labeled with [^{35}S]-methionine for 7 min and chased for up to 30 min. Cells from time points taken at 0, 10, 20, and 30 min were lysed, immunoprecipitated with anti-invertase antibody, resolved on an 8% SDS polyacrylamide gel, and visualized using a phosphorimager.

(B) The fraction of core-glycosylated invertase or invertase fusion over the period of chase was quantified using the PhosphorImager software and plotted using Cricket graph.

Figure 3

A

B

fraction of core-glycosylated invertase (log)

time of chase (min)

□ wild-type

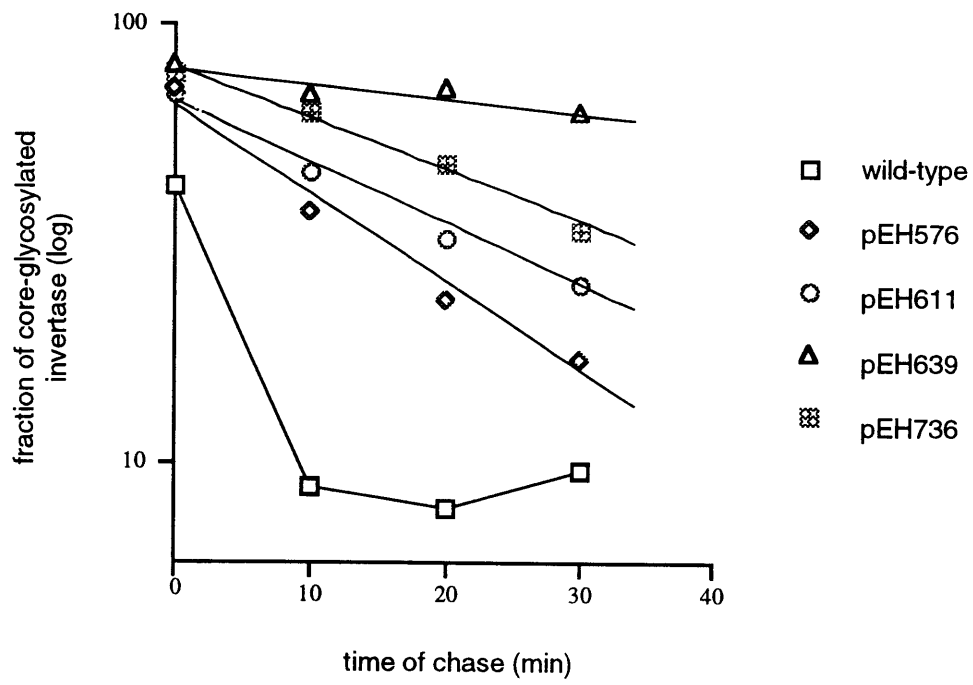◆ pEH576

○ pEH611

△ pEH639

▦ pEH736

Figure 4: The ER-retained fusions do not behave as transmembrane proteins.

CKY16 (wild-type) induced for expression of pEH611 (lanes 1-7) and pEH736 (lanes 8-14) were converted to spheroplasts and lysed by vigorous agitation with glass beads. Lysates were treated with buffer (lanes 2-3 and 9-10), 0.1M Na_2CO_3 pH 11 (lanes 4-5 and 11-12), or 1% Triton X-100 (lanes 6-7 and 13-14) and centrifuged at 100,000 x g to give supernatant (S) and pellet (P) fractions. Untreated/unfractionated lysate represents a control for total protein (T). Protein samples were analyzed on an 8% SDS-polyacrylamide gel by Western blotting using invertase anti-serum.
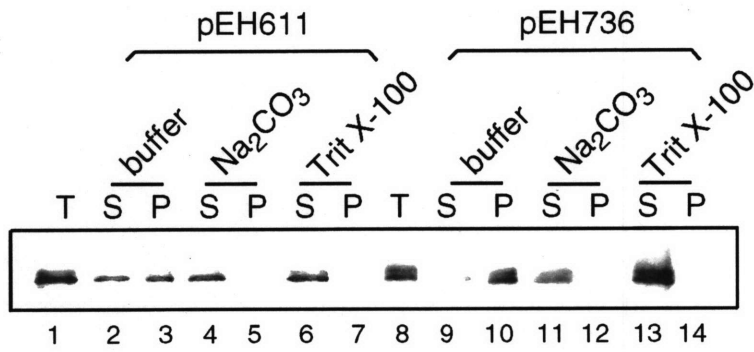
Figure 4

Figure 5: The ER-retained fusions are not recycled from the cis-Golgi back to the ER

CKY406 (wild-type) expressing pEHB46 [Inv-(N)cl$_{L57A}$] (lanes 1-2) and CKY16 (wild-type) expressing pEH611 (lanes 3-4) or pEH736 (lanes 5-6) were induced for one hour at 30°C, labeled with [^{35}S]-methionine for 30 min, and spheroplasted. Spheroplast pellets were lysed and centrifuged at 13,000 x g, and the P13 pellet fractions were immunoprecipitated with anti-invertase antibody and processed. Samples were divided in half, one half re-immunoprecipitated with anti-invertase antibody (lanes 1, 3 and 5) and the other half with anti-α 1,6 mannose antibody (lanes 2, 4, 6) and processed. Samples were resolved on an 8% SDS-polyacrylamide gel and visualized using a phosphorimager.
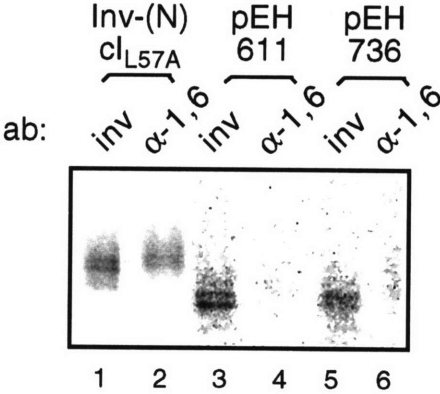
Figure 5

Figure 6: Kar2p physically interacts with ER-retained fusions.

Wild-type cells (CKY16) expressing high-copy 2μ versions of pEHB2 (wild-type invertase), pEH611, and pEH736 were induced for 1 hour, labeled with [^{35}S]-methionine for 30 min, and spheroplasted. Spheroplast pellets were lysed by homogenization in a non-denaturing buffer and the lysates subjected to immunoprecipitation using anti-Kar2p antibody. Processed samples were divided in half, and one half was immunoprecipitated with anti-invertase antibody. Protein extracts were resolved on 8% SDS-polyacrylamide gels and analyzed with the phosphorimager. The ratio of OD equivalents of protein extract loaded onto the gel is 1:1.

Figure 6
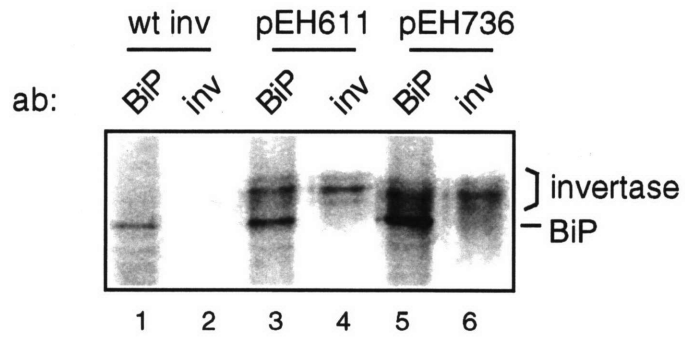
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Chapter 5

Analysis of the secretory fates of invertase fusions to random peptides of 50, 75, and 100 residues

Abstract

Invertase fusions to 50, 75, and 100 random amino acid peptides were constructed to provide a broad range of substrates that can be targeted by quality control mechanisms during transport through the yeast secretory pathway. Several general categories of secretory fates for these fusion proteins emerged from this analysis. About half of the fusions in these libraries were expressed as truncated proteins because stop codons were most likely introduced into the the random peptides. After subtracting out this category, approximately 40% of the remaining fusion proteins were retained in the ER with an intact random peptide. The remaining 60% were roughly evenly divided between two categories of proteolysis that degraded the random peptides: a *pep4*-dependent process and a *pep4*-independent process. Degradation of fusions by the *pep4*-independent process was shown to take place after transport from the ER and before secretion out to the cell surface, suggesting that a proteolytic system that targets unfolded protein may be present in the Golgi compartments.

Introduction

Quality control processes perform a crucial function in the secretory pathway by segregating misfolded protein away from correctly-folded cargo to prevent the cellular incorporation or secretion of defective protein. We have tested the efficiency of the yeast quality control system by examining the secretory fate of soluble fusion proteins between the secretory marker invertase and mutant forms of the N-terminal DNA-binding domain of λ repressor, most notably Inv-(N)cI$_{L57A}$, which bears a leucine to alanine change at position 57, rendering the domain thermally unstable. This investigation revealed that two types of quality control acted on Inv-(N)cI$_{L57A}$ to prevent secretion of the fusion protein out to the cell surface, as described in chapters 2 and 3. First, Inv-(N)cI$_{L57A}$ underwent recycling from the cis-Golgi back to the ER, presumably to re-expose the mutant λ domain to the folding environment of the ER lumen. Second, Inv-(N)cI$_{L57A}$ that eventually break out of the recycling loop was targeted from the trans-Golgi to the vacuole by a receptor-mediated process, where vacuolar proteases degrade the mutant repressor domain, leaving behind the stable, intact invertase moiety (Hong et al., 1996).

The finding that the invertase-mutant repressor fusions were delivered to the vacuole for proteolysis was unexpected since the vast majority of misfolded or unassembled protein were thought to be retained in the ER lumen, usually in association with molecular chaperones, before degradation (Bole et al., 1986; Cheng et al., 1990; Finger et al., 1993). One of the reasons that we originally chose to use invertase as the carrier for the repressor domains was because of the remarkable stability of invertase upon dimerization (Gascon et al., 1968), which would allow us to follow the fate of the fusion protein, even under circumstances where the λ repressor sequences are degraded. Most likely, we were only able to detect the targeting of the invertase-repressor fusions to the vacuole because the invertase moiety was not

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susceptible to vacuolar degradation and provided a marker for determining the final destination of the fusions. This line of thinking led us to suspect that other soluble misfolded proteins may, in fact, escape ER retention and be targeted to the vacuole for degradation. The reason why this process may easily have remained undetected was because complete vacuolar degradation of substrate would have eliminated any physical evidence that misfolded protein had been targeted to the vacuole.

Since expression of invertase fusions to the λ repressor domain had allowed us to discern the targeting of misfolded protein to the vacuole, we decided to expand on the scope of our investigation of the yeast quality control system by constructing a library of invertase fusions to random sequences of 50, 75, or 100 amino acids and determining what different categories of secretory fates these fusion proteins experience. We expected that the random peptides of these fusions would most likely undergo degradation, either by the fusions being retained and degraded in the ER or by targeting to the vacuole. Very few fusions should be secreted to the cell surface intact, since the chances that a random peptide can fold into a stable enough conformation to completely elude the quality control system are exceedingly small. In addition to observing the secretory fates that we had predicted, we also identified a group of invertase fusions whose random peptides were proteolyzed in a post-ER compartment, but in a *pep4*-independent manner. Possibly, these fusions may be degraded by proteases in the Golgi compartments, which would provide yet an additional level of quality control in the secretory pathway.

Materials and Methods

Strains, plasmids, and molecular biological techniques

The following strains were used in this study: CKY406 (*a ura3-52, leu2-3,112 suc2Δ9*); CKY407 (*a ura3-52 leu2-3,112 sec12-4 suc2-Δ9*); CKY413 (*α ura3-52 ade2-101 sec1-1 suc2-Δ9*); CKY420 (*a ura3-52 leu2-3,112 sec18-2 suc2-Δ9*); CKY414 (*a ura3-52 leu2-3,112 pep4::LEU2 suc2-Δ9*). The *gaa1* mutant was obtained from the Riezman lab. The *yap3Δ* and *mkc7Δ* mutant strains were generated by using knock-out plasmids obtained from R. Fuller. DNA cassettes containing coding information for multiples of 25 random amino acids (except for lysine, proline or cysteine) were inserted into pEHB9 at the C-terminal BglII site (Hong et al., 1996) to create the libraries expressing invertase fusions to random peptides. Standard genetic manipulations, preparation of yeast rich medium (YEP) and minimal medium (SD) and yeast transformations were performed as described (Kaiser et al., 1994).

Detection of invertase fusion proteins

Yeast strains were grown in SD medium containing 2% glucose and the appropriate supplements to the exponential growth phase. To induce invertase synthesis, cells were transferred to YEP medium containing 0.1% glucose at a density of 2×10^7 cells/ml. Invertase induction was carried out for 2 hours at 30°C (or at 37°C for the restrictive condition of temperature-sensitive strains) followed by a one hour incubation in 2% glucose to repress further synthesis of invertase and to allow the fusion proteins to reach their final cellular location. Cells from 3 ml of culture were collected by centrifugation, washed with 50mM Tris-Cl pH 7.5, 10mM NaN$_3$, and then suspended in 20 μl of sample buffer (80mM Tris-Cl pH 6.8, 2% SDS, 1.5% DTT, 10% glycerol, 0.1% bromophenol blue). Protein extracts were prepared by heating

samples to 100°C for 2 minutes, breaking cells by vigorous agitation with acid-washed glass beads, and diluting to 0.1 ml with sample buffer.

Samples of 10-20 μl of extracts from whole cells, spheroplasts or spheroplast supernatants were resolved by SDS-PAGE (Laemmli, 1970) on 8% polyacrylamide gels and then electro-blotted to nitrocellulose filters. For Western blot detection, the following antibodies were used: rabbit anti-invertase at 1:1000 dilution, mouse anti-M2 (Eastman Kodak Co., Rochester, NY) at 1:10,000 dilution, HRP-coupled Protein A (Organon Teknika-Cappel, Westchester, PA), at 1:10,000 dilution, and HRP-coupled sheep anti-mouse Ig (Amersham Corp., Arlington Heights, IL) at 1:10,000. Blots were developed using the ECL detection system (Amersham Corp.).

For Endo H digestions, 5 μl of boiled protein extract were diluted with 2-3 volumes of 50mM sodium citrate pH 5.1 containing 100-250U of EndoH$_f$ (New England Biolabs) and incubated at 37°C for 2-16 hours. Samples were boiled prior to gel electrophoresis.

Results and Discussion

Screen to identify intact invertase fusions to random peptides that are transported out to the cell surface

We constructed a library of plasmids expressing the secretory marker invertase fused at the C-terminus to random peptides consisting of 50, 75, and 100 amino acids in length. The very C-terminus of the random peptides contains a flag M2 epitope to easily identify the full-length fusion protein. The construction of the plasmids was designed in such a way that the cassette containing the random sequence can be inserted in either orientation. One orientation results in expression of a fusion protein, while the other one leads to a truncated protein immediately after the end of the invertase protein. Thus, approximately half of the constructs will consist of invertase fused to a random peptide. These fusions are designated by the following nomenclature: invertase bearing 50 residues are referred to starting with the number 1 (e.g. 1Af or 1-20), while fusions bearing 100 and 75 residues start with the number 2 and 3, respectively. A letter following the first number indicates that these peptide fusions were originally detected as an intact fusion at the cell surface (1Af), while a number indicates that it was not (1-20) (see below).

To investigate whether some of the invertase fusion proteins can be secreted to the cell surface as an intact hybrid, we screened ~5000 transformants (~2500 in the correct orientation), representing all three lengths of random peptide, by transferring colonies onto nitrocellulose filters, placing the filters on low glucose plates overnight, rinsing the filters of all cell material, and then probing the filters with anti-M2 antibody to detect full-length fusion protein that became immobilized on the filter. Forty colonies (~1.6%) exhibited a M2 signal, implying that very few random peptides are able to assume a folded enough conformation to evade complete degradation by the quality control system and be secreted to the cell surface. The sequences of these forty

random peptides are shown in Figure 1. In studies invesigating the folding of random peptides (QLR) consisting of 40% glutamine, 40% leucine, and 10% arginine, only 5% of expressed QLR peptides were detected in *E. coli* (Davidson and Sauer, 1994), demonstrating that the majority of random peptides in bacteria are also most likely recognized as unfolded structures and degraded.

The intact fusion proteins detected at the cell surface are predominantly retained in the ER

To more closely examine the secretory fate of the forty fusion peptides, whole cell extracts were made from strains expressing the fusion proteins and analyzed by SDS-polyacrylamide gels and Western blotting using both anti-M2 and anti-invertase antibodies. Surprisingly, with the M2 antibody, only three [2Cd (Fig. 2B, lane 6), 3Bp, and 3Eh] out of the forty fusions exhibited a strong level of heterodisperse mature-glycosylation, which signifies passage of secretory protein through the Golgi compartments to the cell surface. In contrast, both the M2 and invertase antibodies recognized a much more intense signal of core-glycosylated fusion protein for all forty fusion proteins, indicating that a large population of each of these fusion protein are retained in the ER (Fig. 2A and B, lanes 5-8). As controls, wild-type invertase (Fig. 2A, lane 1) and the invertase fusion to wildtype λ repressor, Inv-(N)cI$_{wt}$ (Fig. 2A and B, lane 3), appear predominantly in a mature-glycosylated form at steady state, while the invertase fusion to a thermally-unstable mutant of λ repressor, Inv-(N)cI$_{L57A}$ (Fig. 2A and B, lane 4), was visualized in both core and mature-glycosylated forms, representing its vacuolar targeting and recycling fates (Hong, et al., 1996). From these findings, we conclude that although these forty fusion peptides were identified by virtue of their secretion out to the cell surface as an intact protein, most likely, only a small population of each fusion protein made it out to the cell surface while the vast majority of the fusions were retained in the cell.

Endo H digestions were performed to examine the deglycosylated protein moieties of these forty fusion proteins. The majority of them (29) migrated as a full-length fusion, since the M2 antibody can also recognize these forms (Fig. 2C, lanes 7 and 8; e.g. 2Ce and 2Cf). The random peptides of these fusion proteins were unusually resistant to proteolysis, suggesting that the peptides assumed a stable, folded conformation that evades destruction by cellular proteases. Eleven fusion proteins exhibited multiple bands, with varying lengths ranging from full-length fusion to approximately the size of wild-type invertase (Fig. 2C, lanes 5 and 6; e.g. 3Ab and 2Cd). The protein forms with molecular weights smaller than the intact fusion most likely represent proteolytic products of a cellular degradation process that recognizes the random peptides as being misfolded. However, the stable folded invertase molecule was not degraded and migrates at approximately the same molecular weight as endo H-digested wild-type invertase or cleaved invertase fusions to λ repressor domains (Fig. 2C, lanes 1-4). The mode of degradation for these peptides will be discussed below along with other proteolyzed invertase fusions.

Analysis of fusion proteins that were not transported to the cell surface in an intact form

From the original screen for full-length fusion proteins able to be secreted to the cell surface, the vast majority of transformants (98.4%) did not exhibit an M2 epitope signal on the nitrocellulose filters. There are several possible explanations that could account for this lack of secreted, intact fusion protein. These fusions could have been retained in the ER, where an ER proteolytic system degraded the unfolded random peptide and the M2 epitope. The fusion proteins may have undergone transport from the ER lumen out to the cytoplasm for degradation of the peptide domain by the proteasome, leaving the invertase moiety intact (Hiller et al., 1996; Wiertz et al., 1996). However, since the proteasome has not been described to be able to mediate partial

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degradation of substrate, this possibility is unlikely. A second explanation is that the fusion proteins may have been targeted to the vacuole for degradation of the random peptide, as in the case of Inv-(N)cl$_{L57A}$. Degradation may also have been mediated by cell surface proteases upon transport of the fusion protein to the plasma membrane or by other unknown proteases localized in the secretory pathway. Finally, a stop codon may have been incorporated into the random sequence, resulting in a truncated protein that would not bear an M2 epitope.

We examined a total of 162 fusion proteins, 54 each from the 50-mer, 75-mer and 100-mer peptide lengths, that weren't detected as being secreted out to the cell surface as an intact fusion. Whole cell extracts were examined by SDS gel electrophoresis and western blot using both M2 and invertase antibodies. The random peptides of 86 out of the 162 fusions were oriented in the the opposite direction and were not studied any further. The remaining 76 fusions experience a number of different fates that could be deduced by analyzing the westerns of whole cell extracts and extracts treated with endo H.

Fourteen of the 76 fusions were strongly retained in the ER, as evidenced by their appearance as solely core-glycosylated fusions (Fig. 3A, lane 5; e.g. 3-7). Upon endo H digestion, the protein moiety migrated with a molecular weight close to what would have been expected for a full-length fusion (Fig. 3B, lane 5; e.g. 3-7). However, the endo H-digested protein of 13 out of 14 fusions was not recognized by M2 antibody, suggesting either the M2 epitope of these fusions was clipped off by a protease, leaving the rest of the random peptide intact, or a stop codon was inserted at the very end of the random peptide, resulted in a slightly truncated protein. Only one out of the ER-retained, core-glycosylated 14 fusions (3-50) was recognized by M2 antibody, and its random peptide sequence is shown in Figure 1. Since the only fusions that migrated as core-glycosylated forms were also the ones that were expressed as full-length or nearly full-length fusions, we suspected that these random

peptides were causing retention of the fusions in the ER and yet, were not susceptible to extensive degradation themselves.

The remaining 62 out of 76 fusions migrated as mature-glycosylated forms or a combination of both mature and core-glycosylated forms that were recognized by invertase antibody, but not M2 antibody (Fig. 3A, lanes 3-4; e.g. 1-20 and 2-6). Upon endo H digestion, the protein moieties migrated in a range between the molecular weights of full-length fusions and wild-type invertase (Fig. 3B, compare lanes 3 and 4 with 5). The varying lengths of the fusion proteins suggest that either the random peptides were differentially degraded during transport through the secretory pathway or stop codons were inserted in the middle of the random sequences, yielding truncated protein. In any case, since undegraded, full-length fusions were completely retained in the ER, there appeared to be a correlation between fusion proteins that are able to be secreted and processing of their random peptides.

Degradation of random peptides mostly occur in a post-ER compartment

To localize where the degradation of random peptides was taking place in the cell, 56 fusion proteins were expressed in temperature-sensitive *sec* mutants: *sec12-4* and *sec18-2* mutants are defective in the transport of cargo protein from the ER to the cis-Golgi, while *sec 1-1* mutants fail to fuse post-Golgi vesicles with the plasma membrane at the restrictive temperature. If degradation was taking place in the ER, then the random peptides would be expected to be proteolyzed since the fusions are retained at the site of proteolysis. However, if degradation occurred following transport of fusions to the Golgi, then proteolysis of the random fusions would be blocked in both *sec12* and *sec18* mutants. Proteolysis of random peptides in a *sec1* mutant would signify that degradation took place before the transport to the cell surface. Both types of proteolyzed fusion proteins, those identified as being secreted to the cell surface intact (6 total) and those that weren't (50 total) were analyzed in these mutants.

A wild-type (CKY406) and the mutant strains expressing the fusion proteins were induced for invertase expression at the restrictive temperature, protein extracts were made, and endo H-digested extracts were examined by Western blot using invertase antibody. 21 out of 56 fusion proteins migrated as a higher molecular weight protein in the *sec12* and *sec18* strains compared to a wild-type strain, implying that proteolysis of the random peptides normally occurred in a post-ER compartment (Fig. 4A and B, compare lanes 1 with 2 and 3; e.g. 2-6, 3Ab). Fusion 3Ab was also shown to be proteolyzed in a *sec1* strain, placing the site of degradation in either the vacuole or in the Golgi compartments (Fig. 4B, lane 4). These findings are reminiscent of the behavior of the invertase fusion fused to the thermally unstable N-terminal domain of λ repressor discussed in chapters 2 and 3.

Out of the remaining 35 fusion proteins, 31 did not exhibit any difference in the molecular weight of the endo H-digested fusion protein between the *sec* mutants and a wild-type strain. To test whether these fusions underwent proteolysis in the ER, short labeling experiments were performed to try and detect a higher molecular weight form that would represent the full-length fusion protein. A wild-type strain (CKY406) expressing these fusions were labeled for 5 min, lysed with glass beads, and the lysates immunoprecipitated with invertase antibody. The processed samples were digested with endo H, resolved on SDS-polyacrylamide gels, and analyzed using the phosphorimager. All of the fusions examined did not express a protein that migrated at a higher molecular weight than the forms seen in the *sec* or wildtype strains. Since there wasn't any evidence that these fusion proteins were created by degradation of a larger precursor, we surmise that they contained a stop codon in their random sequences, which led to the expression of fusions bearing shorter random sequences that were transported to the cell surface.

Degradation of the fusion peptides occurs in both a pep4-dependent and pep4-independent manner

In our previous study of degradation of an unfolded domain appended to invertase, Inv-(N)cl$_{L57A}$ was targeted by a receptor-mediated process to the vacuole for proteolysis (Hong, et al., 1996). To determine whether the fusion proteins bearing random peptides in this study were also delivered to the vacuole for proteolysis, fusions were expressed in a *pep4Δ* mutant, which exhibits greatly reduced activity for most of the vacuolar proteases (Jones, 1991). Disruption of the *PEP4* gene (in CKY414) largely blocked proteolysis in 9 out of the 21 fusions examined (Fig. 4A, lane 4; e.g. 2-6). However, the random peptides of 10 fusions were clearly degraded to the same extent when expressed in either a *pep4Δ* mutant or a wild-type strain (Fig. 4B, lane 4; e.g. 3Ab). Expression of these 10 fusions in a mutant deleted for *PRB1*, another vacuolar protease, also resulted in degradation as in a wild-type strain. These findings suggested that at least two types of proteolysis were degrading the peptide sequences: one dependent on *pep4Δ*, and the other not dependent on *pep4Δ* function.

Taking into account the different categories of secretory fates experienced by the fusion proteins that weren't detected at the cell surface and subtracting out the number of fusions that were expressed as truncated forms, we have calculated that ~40% are retained in the ER in a mostly undegraded form, with the remaining ~60% undergoing some form of proteolysis of their random peptides. This 60% was split fairly evenly between the the *pep4*-dependent and *pep4*-independent processing. From this analysis, we surmise that a secretory protein becomes exposed to a number of different quality control events during transport through the secretory pathway.

A search to identify the responsible protease(s) that proteolyze the *pep4Δ*-independent degraded fusion proteins

Since the *pep4Δ*-independent degraded fusions were shown earlier not to be subjected to proteolysis in the ER or at the cell surface, we suspected that degradation may be taking place in the Golgi compartments. Presently, there aren't any known proteases in the Golgi that non-specifically degrade misfolded protein. However, it may be possible that some of the random peptides are being cleaved at specific sites to yield the observed shorter fusion protein, rather than a more non-specific degradation in which proteolysis clips away at an exposed, unfolded site and only stops upon reaching an inaccessible folded domain.

There are several specific endoproteases in the cell that have been shown to cleave on the carboxyl side of dibasic and monobasic residues. The best-studied specific protease in the Golgi is Kex2p, which has been characterized as a serine endoprotease in the trans-Golgi that cleaves the precursors of secreted peptides, such as α-factor and K1 killer toxin, on the carboxyl side of Lys-Arg and Arg-Arg (Julius et al., 1984). Two aspartyl proteases have been identified in yeast that can partially substitute for Kex2p function. *YAP3* was identified as a multicopy suppressor of both the pro-α-factor processing defect in a *kex2Δ* strain (Egel-Mittani et al., 1990) and of anglerfish prosomatostatin processing in *sex1* (somatostatin-28 expression) mutants when heterologously expressed in yeast (Bourbonnais et al., 1993). Since processing of prosomatostatin requires cleavage at monobasic sites, Yap3p appears to possess an overlapping, but distinct, proteolytic specificity compared to Kex2p. Yap3p has been localized to the plasma membrane; however, its ability to process pro-α-factor suggests that this enzyme displays some proteolytic ability while localized in the Golgi compartments (Ash et al., 1995). The second aspartyl protease, encoded by *MKC7*, was isolated as a multicopy suppressor of the cold-sensitive growth phenotype of a *kex2* mutant (Komano and Fuller, 1995). Mkc7p and Yap3p share 53% sequence identity and 73% similarity. *mkc7yap3* double mutants exhibited impaired growth at

37°C, whereas the single mutants were not temperature-sensitive, suggesting that Mkc7p and Yap3p perform redundant functions.

We investigated whether Kex2p, Yap3p or Mkc7p played a role in the proteolysis of random peptides from *pep4Δ*-independent degraded fusions by expressing the fusion proteins in *kex2Δ*, *yap3Δ*, and *mkc7Δ* strains. However, the pattern and extent of degradation in these mutant strains remained unchanged when compared to a wild-type strain.

A search of the yeast protein database identified several novel proteins bearing potential signal sequences that exhibited sequence identity to aspartic and serine proteases, suggesting that other as-yet-uncharacterized proteases may function to degrade immature protein in the secretory pathway. To try and globally inactivate some of these proteases, we took advantage of the observation that Mkc7p, Yap3p, and some of these predicted protease sequences possess a glycosyl-phosphatidylinositol (GPI)-linked anchor attachment site. We reasoned that crippling the ability of GPI-linked proteases to anchor to membranes may lead to mislocalization and subsequent inactivation of the proteases. To this end, we expressed the *pep4Δ*-independent degraded fusions in a *gaa1* mutant, which is defective in the attachment of a complete GPI anchor onto proteins in the ER (Hamburger et al., 1995). However, similar to the finding for the *yap3Δ* and *mkc7Δ* strains, the random peptides were still degraded in a *gaa1* mutant.

Attempts to find the responsible protease are complicated by the possibility that several proteases may possess the ability to proteolyze the *pep4Δ*-independent degraded fusions. Demonstration that both Mkc7p and Yap3p are able to cleave pro-α-factor in a *kex2Δ* mutant supports a model in which proteases in the secretory pathway exhibit at least some partial overlapping function. From a technical point of view, it may be difficult to construct yeast strains that contain multiple deletions of proteases. As mentioned earlier, the *yap3Δmkc7Δ* double mutant was sickly at 37°C,

while a *yap3Δmkc7Δkex2Δ* triple mutant resulted in an even more profound temperature-sensitive and cold-sensitive growth phenotype, indicating that function of at least one of these proteases was required for normal growth.

In addition, further difficulties may arise if proteases in different cellular locations recognize the same substrate. For example, if a strain can be constructed in which all possible Golgi proteases that process a specific substrate are deleted, intact substrate that would then be secreted out to the cell surface may still be degraded by a cell surface protease that performs the same processing event as the Golgi proteases. From our studies of invertase fused to the destabilized mutant of λ repressor, we have already observed the activity of an unidentified cell surface protease on Inv-(N)cI$_{L57A}$ upon overexpression. In both the protease deficient strain and a comparable wild-type strain under steady state conditions, the substrate would appear to be transported to the cell surface in a processed form. This particular problem may be addressed by including a *sec1* mutant in the strain background to observe whether processing actually occurs before transport to the cell surface.

Acknowledgements

Figure legends

Figure 1: Amino acid sequences of the random peptides appended to invertase fusions that were detected to be secreted to the cell surface intact and of fusion 3-50, the only isolated fusion protein that was completely retained in the ER and still possessed the M2 epitope.

Figure 1

3-50:
KISTVSGSGSGARSLIRARSVTVLIKFQVRTLFKTVSQIVALSLIRWIKVQYEAQT
RARTGDGTQSQNEWIGEDL

Sequences of 50-mers (Two cassettes):

1Ab:
KISALNGSLNQVQIRAQVV<u>I</u>LVGLIKDLTLAEIQTQTQSQSEALTRWIREDL

1Af:
KISARVRTLYEAEDEAVDESGAELIKTQSRSQAQYQYLSGAQTLYVWIREDL

1Bg:
KISFGAVSGFRYLA<u>V</u>IGVRTRIVLIK<u>D</u>QTLAGNGSGTQARAEILTQWIREDL

1Ca:
KISS<u>G</u>IGARFLIQVVVGNRSGSGLIKSQAV<u>D</u>LARSRTQAGALSGSGWIREDL

1Ce:
KISTLAQALIGAGTGVQNEARAGLIKDLTRSLNQVLTE<u>D</u>RVLSQIQWIREDL

1Da:
KISDGIRVEIVVRVRFQFESGSVLIKSQTLTLSQ<u>I</u>QAVILAQ<u>D</u>LTQWIREDL

1Ee:
KIS<u>I</u>RTETRTEAGFQSGDVVGSVLIKSQIVAQIRAQNGAGFQAQFQWIREDL

1Ga:
KISTGAGVQSVSPTRVEYRVLDVLIKVGNQSVSLTLDLAQSRTQFGWIREDL

1Ge:
KISAE<u>I</u>EYVARIRVVVQFGDVTLLIKTL<u>D</u>LSQNQTQSLTQNRAQTEWIREDL

Sequences of 75-mers:

3Ab:
KISTEAVILVGNGYGSVTRDVHGLIKTVSLSQNRSLTVSGASSGFEWIKTGNQA
EAGSESLDQAQDLNQWIREDL

3Af:
KISARSRAQSGDVSEVRAGTLAGLIKAGGVFQAQIQAVNRALALYQWIKSQS
QNQAQNVNQTQTETQIVWIREDL

3Bn:
KISNRDRAVIVNGTQFRVGSGVVLIKDLDGSQAGTQIRIQNRTQAQWIKARTG
NETLDVVVIVSLTQSRWIREDL

3Bp:
KISAEDRTGVESEDGSGVQDGTGLIKVVYSI[PIPGPVPNSVDQVRISDPVPDPD
RNCNSIPIPV]IREDL

3Cm:
KISTLSQSQTQDGSGVQALAEVELIKTQAEVGARVQTLNQSQTQTQWIKSRTG
TGARALTVSLSGSLTGWIREDL

3Dm:
KISAGTRDGDRIEDLSRDGSGAQLIKSENRNQTRALTGSQARFESEWIKSLTLD
GTQVQYRALTVSGFEWIREDL

3Ea:
KISSEYEARDEYQSEALTETGFLLIKALSRNGTGNQALDETRTVNLWIKAENQT
LSLTQTQDVIRYVFEWIHEDL

3El:
KISYGARTRSLSQIVFEVRVGVLLIKTQFQNRSVTQVGNQASRVAMDQDPNPS
PKLTQVPTQAKSQFQWIREDL

3En:
KISDLNRTDSGTRNVSLARNVALLIKSQTQFLIVARTGDGALFQTGWIKNLNQ
TRSRYGARILDQPLNQWIREDL

3Eu:
KILMDPGFSLGPGPRPEIRSDPADQDPISNSDPISSPNPNSGRDPVDQGSNSNPV
PVPDPIPSSTQTQWIREDL

3Fl:
KISDVSGSEIQFVAGIEDGARSGLIKDLSGTQALNVALTRAQVQIRWIKDVSLA
QYRDQTVAQSRTLTQWIREDL

Sequences of 100-mers (Four cassettes)

2Ak:
KISFVSVSRALAGNRAGTLVGALLIKNVDLTETESRIETQTRSQTRWIVRDGAR
DRAQIRVGSQFQSGLIK[SGPSPVPVPGPIRS]LARSQWIREDL

2Cc:
KISSQDGVEVGIGTGAGAEAGFVLIKNVTRDQALDQSLVLTLNQTLWISVRAG
FLDRILTESQTVILVLLIKIQNVDQAQARTRSQARTVTQWIREDL

2Cd:
KISSRDRFQYQTGAGYESQYESGLIKSRDQVQNRDQVRALTVNQVQWISVRVL
SESQVAVVNGSEDQAVLIKTEVRIGNQSRALNGTQIRALWISEDL

2Ce: (last 3 of 4 cassettes)
LIKSQTLTQTQAQTRYQALSGNVWISDEDRTEARAGTGTGTQSVIELIKVRFQE
FQVQNLTQTRAEVEWIREDL

2Db: (last 3 of 4 cassettes)
LIKSLAQNVTQSGFQDLIQDQVGWISVGVGSRSLFGDGYVNRFQSELIKIQSLT
GSRTESRSQTQFGSEWIREDL

2Dd:
KISNENGNGAGARFGARNESESVLIKIRDEDQTEVLILDRILVLTGWISVEYGTL
VVVDVDGAVARLIKTQSRAQAQTQSRSQSLTQAQWIREDL

2Ec: (last 2 of 4 cassettes)
WISYQA[LCVWERSCIPKC]VLDELIKTGTRDVTLFQTQAVTEARTQWIREDL

2Ei:
KISDGAVSRTAYRTLTVDVSRFLLIKAQTESRVQFQSLTLTGSLTRWISILVGVG
ARDGTGTEDHSGAELIKTRNLTRSRNQTRTLSQVRSGWIREDL

2Fa: (last 3 of 4 cassettes)
LIKAEVLTQTLTRSLTQNEFQTLWISFGNGNLAQVVAVDEAGARDGLIKDRDR
NVTQVQTQAEDVYRALWIEDL

2Ge:
KISNENGNGAGARFGARNESESVLIKFRDEDQTEVLILDRILVLTGWISVEYGT
LVVVDVDGAVARLIKTQSRAQAQTQSRSQSLTQAQWIREDL

2Gc:
KISNQFETVTLVETQVVNLNETELIKTETQTRSQAQAQIQSLVLAQWISFGVGN
GVRNGTLVGTE[RECADQEWESSPIPS]RFRDGNVTQTEWIREDL

Figure 2: Invertase fusions detected at the cell surface are mostly retained in the cell and some undergo processing of the random peptide.

A wild-type strain (CKY406) was induced for expression of wild-type invertase (lane 1), Suc2-s11 (lane 2), Inv-(N)cl$_{wt}$ (lane 3), Inv-(N)cl$_{L57A}$ (lane 4), 3Ab (lane 5), 2Cd (lane 6), 2Ce (lane 7), or 2Cf (lane 8). Protein extracts were resolved on an 8% SDS-polyacrylamide gels, and the fusion proteins visualized by Western blotting using invertase antibody (A), M2 antibody (B), and invertase antibody combined with endo H treatment of the protein extracts (C).
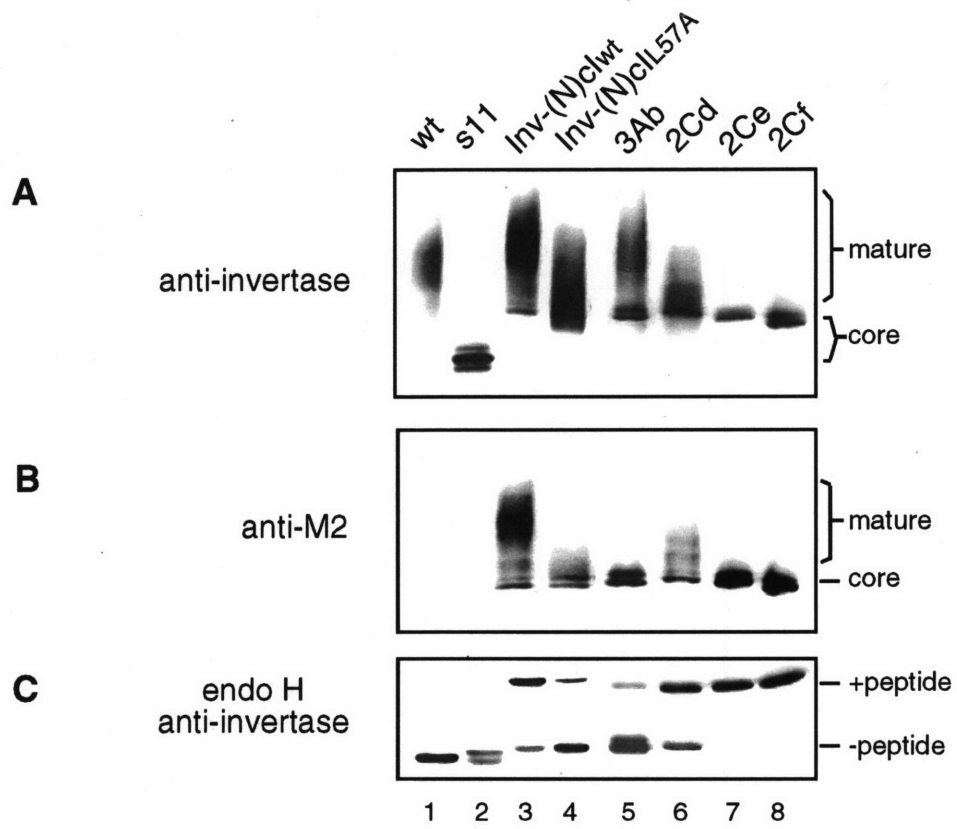
Figure 2

Figure 3: Invertase fusions not detected at the cell surface undergo processing of the random peptide.

A wild-type strain (CKY406) was induced for expression of wild-type invertase (lane 1), Inv-(N)cl$_{L57A}$ (lane 2), 1-20 (lane 3), 2-6 (lane 4), or 3-7 (lane 5). Protein extracts were resolved on an 8% SDS-polyacrylamide gels, and the fusion proteins visualized by Western blotting using invertase antibody (A) and invertase antibody combined with endo H treatment of the protein extracts (B).
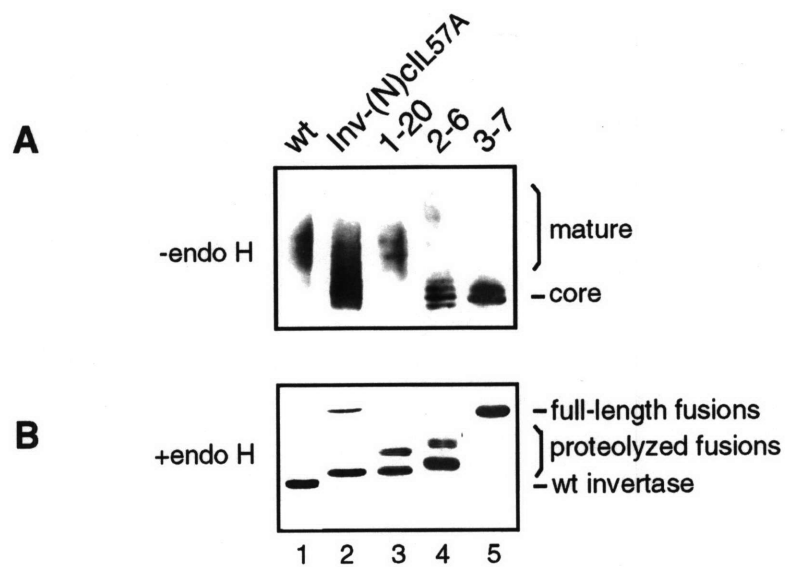
Figure 3

Figure 4: The random peptides are proteolyzed by either a *pep4*-dependent or *pep4*-independent degradation process.

(A) The expression of fusions 2-6 was induced in CKY406, wild-type (lane 1); CKY407, *sec12-4* (lane 2); CKY420, *sec18-2* (lane 3); and CKY414, *pep4Δ* (lane 4). Protein extracts were digested with endo H, resolved on 8% SDS gels, and the fusion proteins visualized by invertase antibody.

(B) The fusion protein 3Ab was examined as in (A) except CKY413, *sec1-1* (lane4) and CKY414, *pep4Δ* (lane 5).

Figure 4

A   fusion 2-6

        1   2   3   4

B   fusion 3Ab

        1   2   3   4   5
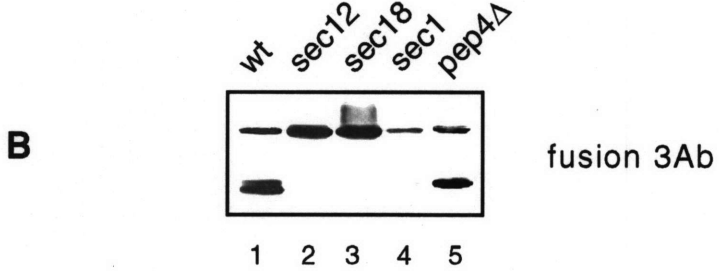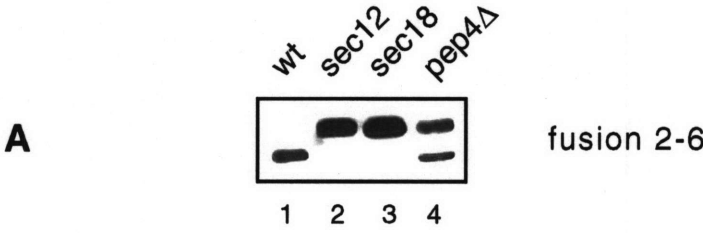
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Chapter 6

Prospectus

In this dissertation, fusion proteins between invertase and either thermally-unstable folding mutants of the N-terminal domain of λ repressor (chapters 2 and 3) or random peptides from 50 to 100 residues in length (chapter 5) were analyzed to determine how the yeast quality control system responded to the presence of these unfolded domains. Since the ER has generally been considered the site where misfolded proteins are bound by molecular chaperones and subsequently degraded, we expected that the invertase fusions would be recognized as mutant protein and retained in the ER. Instead, we found that the invertase fusions undergo a number of different secretory fates in post-ER compartments.

The invertase fusion to the L57A folding mutant of λ repressor, Inv-(N)cI$_{L57A}$, underwent two quality control events in the Golgi: (1) Vps10p receptor-mediated targeting from the trans-Golgi to the vacuole and subsequent degradation of the repressor moiety by vacuolar proteases, and (2) recycling from the cis-Golgi back to the ER in a COPI and Kar2p-dependent manner. Delivery to the vacuole had been described previously for soluble resident vacuolar hydrolases, such as CPY and PrA, which bear a propeptide targeting sequence that is recognized by the receptor Vps10p (Marcusson et al., 1994; Cooper and Stevens, 1996). In addition, the vacuole is considered to be the default pathway for mutant Golgi membrane proteins bearing a non-functional Golgi localization signal (Roberts et al., 1992; Wilcox et al., 1992) and other mutant membrane proteins (Gaynor et al., 1994; Chang and Fink, 1995). Our demonstration that vacuolar targeting of the soluble invertase fusions to mutant λ repressor is also dependent on Vps10p implies that a sorting mechanism exists in the trans-Golgi that segregates mutant protein from mature cargo heading to the cell surface. Most likely, detection of this vacuolar sorting process was only made possible by using the stable and protease-resistant invertase as the carrier protein.

Analysis of Inv-(N)cI$_{L57A}$ transport also revealed that the fusion protein was subjected to recycling from the cis-Golgi back to the ER, presumably to re-expose the

mutant domain to the luminal chaperones and folding enzymes. Although the recycling process was originally described for the retrieval of soluble and membrane ER resident proteins bearing the C-terminal retrieval signals -KDEL (-HDEL) and -KKXX, respectively, that had escaped to the cis-Golgi (Munro and Pelham, 1987; Nilsson et al., 1989), a few examples of misfolded protein being recycled have also been reported (Hsu et al., 1991; Hammond and Helenius, 1994; Knop et al., 1996). Our studies of Inv-(N)cI$_{L57A}$ recycling led to the identification of protein components that play a role in this process, putting forth a model in which Kar2p binds to the mutant repressor domain in the cis-Golgi and recycles the fusion proteins back to the ER through its association with the Kar2p receptor, Erd2p, via COPI-coated retrograde vesicles.

Finally, the invertase fusions to the 50, 75, and 100-mer random peptides appear to undergo several secretory fates, including retention of intact fusion in the ER, vacuole-dependent degradation of the random peptides, and a post-ER degradation process that functions independent of the vacuole or transport to the cell surface. Possibly, this latter process may represent a Golgi-localized degradative system that monitors for unfolded protein. Thus, our investigation of invertase fusions to different types of unfolded domains has uncovered multiple quality control processes that function in the Golgi compartments.

To gain further insight into the mechanisms of these various Golgi quality control processes, more detailed investigation of the protein components that mediate these processes will have to be performed. In the case of Vps10p, this receptor has been shown to bind to resident vacuolar hydrolases via their pro-regions, which by structural studies, assume a partially-folded conformation with very little tertiary structure (Sorensen et al., 1993). Based on our finding that vacuolar targeting of the mutant λ repressor fusions is also dependent on functional Vps10p, we speculated

that Vps10p may be recognizing an extended polypeptide chain in an unfolded state rather than a specific peptide sequence. To provide biochemical evidence for Vps10p binding to immature domains, co-immunoprecipitation or cross-linking experiments could be performed to detect a stable physical interaction between Vps10p and either the mutant λ repressor fusions or the 50-100 residue random peptides that exhibited *pep4*-dependent proteolysis. Since this complex would be expected to form during transport from the trans-Golgi to the pre-vacuolar compartment and before proteolysis, Vps10p should be found to associate with an intact, full-length fusion protein.

To gain an idea of how well Vps10p binds to unfolded substrate, binding affinities could be measured between Vps10p and either the mutant λ repressor domains or the random peptides in in vitro binding experiments. Since Vps10p is a transmembrane protein, it will be necessary to produce a soluble fragment of the receptor containing the large luminal domain that presumably interacts with substrate to use in the binding experiments. Such a truncated receptor has been expressed as a secreted protein in the methylotropic yeast *Pichia pastoris* in other studies (Cooper and Stevens, 1996). Although the mutant λ repressor fragments can be expressed and purified from bacteria, generation of random peptides may prove to be difficult since expression of unfolded sequences in bacteria often result in rapid proteolysis (Parsell and Sauer, 1989; Davidson and Sauer, 1994). One possibility would be to express the fragments in a bacterial strain containing multiple deletions for cellular proteases.

The binding studies can be performed using the Biacore set-up, which allows for the calculation of apparent binding constants, $K_{d\ app}$. Purified Vps10p luminal domain would be cross-linked onto a chip, over which buffer containing either the mutant λ repressor or random peptide domains would flow. The rate of association and dissociation of complex formation can then be determined and the $K_{d\ app}$ calculated from these measurements. Obtaining binding constants for the various

Vps10p ligands would reveal whether interactions occur with a high or low affinity. Possibly, examination of ligands that exhibit stronger binding may yield more structural information on what constitutes a target site for Vps10p binding. Similarly, the $K_{d\,app}$ of Vps10p binding to the pro-regions of vacuolar hydrolases CPY and PrA can be determined and compared to that of the unfolded domains.

A fluorescence equilibrium assay has been described for determining the stoichiometry of binding by measuring the change in tryptophan fluorescence signal intensity upon association of two proteins (Cooper and Stevens, 1996). The concentrations of the two proteins which produce the greatest amount of signal quenching corresponds to maximal binding conditions. Thus, in addition to providing evidence for in vitro binding, this technique would allow us to determine the number of binding sites that Vps10p possesses for unfolded substrate molecules.

To detemine what regions in the luminal domain of Vps10p are critical for ligand binding, vacuolar targeting of the λ repressor and random peptide fusions could be monitored in strains expressing various mutant forms containing deletions or amino acid substitutions in the luminal domain of Vps10p. It may be possible to isolate deletion mutants that affect either the sorting of CPY or unfolded protein, but not the other, which would suggest the existence of different binding sites on Vps10p. By analogy with other receptor proteins, the structure of the Vps10p luminal domain most likely contains a binding pocket that accepts the unfolded ligand. Mutational studies on the cytoplasmic tail of Vps10p have demonstrated that this domain mediates receptor stability, localization and, recycling from the trans-Golgi to the prevacuolar compartment (Cereghino et al., 1995). Although the luminal binding domain was kept intact in these mutants, CPY was still missorted to the cell surface, implying that a functional cytoplasmic tail is required for vacuolar sorting of cargo protein. Presumably, targeting of the λ repressor and random peptide fusions to the vacuole would similarly be impaired in Vps10p cytoplasmic tail mutants.

A second area of research that can be further explored is the characterization of the *pep4*-independent proteolysis exhibited by some of the invertase fusions to random peptides. We had speculated that the degradation event may be taking place in the Golgi compartments since proteolysis occurred in a post-ER compartment but before transport of the fusion protein to the cell surface. There are basically two different strategies to try and identify the responsible proteases: (1) testing known proteases that reside in the secretory pathway by determining whether inactivation of these proteases results in a loss of degradation, and (2) performing a de novo screen to isolate mutants of this degradation process and then clone the proteases.

We have explored the first strategy to some extent. Although there aren't any known non-specific proteases that function in the Golgi to degrade unfolded substrate, there are several specific endoproteases that cleave on the carboxyl side of dibasic and monobasic residues. These include Kex2p, which resides in the trans-Golgi, and Yap3p and Mkc7p, which were identified as suppressors of Kex2p mutant phenotypes. We also included in our examination the vacuolar proteinase B (PrB). However, in *yap3Δ*, *mkc7Δ*, and *prb1Δ* deletion strains, the random peptides of the fusion proteins were still degraded, suggesting that the absent proteases were not responsible for the proteolysis effects.

A complication in assaying for proteolysis of the random peptides is that multiple proteases may be able to perform the degradation; for example, Kex2p, Yap3p and Mkc7p are all able to process α-factor. Deletion of one protease may not have a noticeable effect because other proteases with overlapping function will process the substrate. To try and address this problem, we have taken advantage of the observations that Yap3p and Mkc7p both are attached to membranes by a GPI-linked anchor and that a database search looking for homologies to known classes of proteases identified several open reading frames whose predicted protein sequences

bore a signal for attachment of GPI-linked lipid anchors. We expressed the mutant fusions in a *gaa1* mutant, which is defective in the attachment of GPI-linked anchors to protein, with the hope that secretory proteases would be mislocalized and thus inactive for processing the random peptides. However, processing still occurred in a *gaa1* mutant.

In addition to proteases of overlapping function in the secretory pathway, cell surface proteases may also degrade the random sequences. We've already observed a processing event of the repressor domain of Inv-(N)cI$_{L57A}$ that takes place only upon secretion to the cell surface. Thus, it may be necessary to introduce a *sec1* mutation into any protease deficient strains to rule out the possibility that cell surface proteases are causing the degradation. A *vps10Δ* mutation can also be added to prevent the targeting of the invertase fusion to the vacuole if proteolysis of the random peptides does happen to be blocked in any protease deficient strain. Similarly, a *pep4Δ* mutation in the strain would inactivate vacuolar proteases, so that an intact fusion can be detected in the cell.

A likely next step in this strategy is to construct strains deleted for multiple proteases. This may prove difficult for some mutant combinations, since a *kex2Δyap3Δmkc7Δ* strain exhibits a severe growth defect. However, preliminary experiments suggest that a double mutant combination between *pep4Δ* and *prb1* may be defective in the processing of some of the random peptides. This finding brings up the possibility that vacuolar proteases may be proteolytically active while in the Golgi compartments. More work will need to be performed before the implication of PrA and PrB in the observed degradation process of the random peptides.

An alternative method to try and identify the responsible proteases would be to incubate cells with various protease inhibitors during growth and examine whether there is an effect on random peptide degradation. In order to be able to inactive proteases, the inhibitors would have to be able to enter into the cell and be at low

enough concentrations not to compromise cell viablitity. In addition, like with the *gaa1* mutant, this method may have other secondary effects on cell metabolism that would be difficult to identify. We have attempted to incubate strains expressing the invertase fusions with PMSF (a serine protease inhibitor), pepstatin A (aspartic protease inhibitor), or iodoacetamide (a thiol protease inhibitor). However, processing of the random peptides still proceeded in the presence of these protease inhibitors under our experimental conditions.

Finally, the identity of these proteases may be determined by performing a de novo genetic screen. The basic strategy would be to mutagenize cells that are expressing an invertase fusion bearing a random peptide that undergoes *pep4-*independent proteolysis and isolate mutants that secrete full-length intact fusion protein to the cell surface. However, many of the complications discussed above can also be applied to this method. For example, if the responsible protease is mutagenized in the screen, the invertase fusion may then be re-directed to the vacuole for proteolysis instead of to the cell surface. Possibly, using a *vps10Δ* starting strain would impair targeting of the invertase fusion to the vacuole. A more difficult possibility to contend with is if several proteases are able to act on the random peptide. Mutagenesis of only one of the proteases would not be detectable by the screen, since the other proteases will be functional.

Instead of detecting full-length protein at the cell surface, a morphological screen could be performed to detect intact fusion protein in the cell by immunofluorescence. The starting strain should be both *sec1*, to prevent the transport of fusion protein to the cell surface, thus avoiding the possibility of cell surface proteases acting on the random peptide, and *pep4Δ*, to inactivate vacuolar proteases in case the intact fusion protein is transported to the vacuole. Thus, intact protein present in the cell because of mutagenesis of the responsible protease would be able

to be detected. As a control, the invertase fusion would not be visualized in the starting strain because the protease would have degraded the epitope tag.

For the multiple processes in the secretory pathway that mediate quality control, the protein components will have to be identified to understand the mechanisms of action. In some cases, such as vacuolar receptor Vps10p and the Kar2p/Erd2p retrieval system, we were able to ascribe novel functions to known proteins . However, as in the case of the *pep4*-independent processing of the random peptides, additional work will need to be performed to identify the responsible proteases in this degradation process.

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