A Pathway for Targeting Soluble Misfolded Proteins to the Yeast Vacuole

Elizabeth Hong,* Alan R. Davidson,† and Chris A. Kaiser*

*Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139; and†Department of Medical Genetics and Department of Biochemistry, University of Toronto, Toronto, Ontario, Canada M5S1A8

Abstract. We have evaluated the fate of misfolded protein domains in the Saccharomyces cerevisiae secretory pathway by fusing mutant forms of the NH2-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. (a) The invertase–repressor fusions, like wild-type invertase, behave as soluble proteins in the ER lumen. (b) 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. (c) 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 nonnative luminal proteins by receptor-mediated transport to the vacuole.

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 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 Saccharomyces cerevisiae secretory pathway. The idea is to direct a test polypeptide domain into the lumens 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 NH2-terminal DNA-binding domain of the phage λ repressor. Crystallographic and biochemical analyses have shown that the NH2-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 NH2-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 Leu77 to Ala mutation that reduces the thermal denaturation temperature of 50% (Tm)1 from 54 to 20°C for the purified NH2-terminal domain (Parsell and Sauer, 1989).

Wild-type and mutant λ repressor domains were introduced into the secretory pathway by fusion to the COOH

1. Abbreviations used in this paper: CPY, carboxypeptidase; DAPI, 4′,6-diamidino-2-phenylindole; PrA, proteinase A; SD, minimal medium; Tm, the temperature of 50% thermal denaturation; YEP, yeast rich medium.
term 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 COOH terminus of invertase does not interfere with folding of the active enzyme, allowing enzymatic assays to be performed to quantify 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.

Materials and Methods

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 Laboratories Inc., 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 antiserum was elicited in rabbits to purified cytoplasmic invertase (gift of D. Botstein, Stanford University, CA). Recombinant lyticase was prepared by expression of β-glucanase from Oerskovia sphaeroides in Escherichia 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, Inc. (Beverly, MA).

Construction of Invertase and Invertase Fusion Plasmids

The SUC2 gene on a 4.3-kb EcoRI fragment from pRB55 (Carlson and Botstein, 1982) was inserted into the centromeric vector pRS161 to create pEBH2. pEBH29 is pRS161 containing a mutant form of invertase (SUC2-s11) in which the signal sequence cleavage site has been altered from alanine to isoleucine at position 16 (Bohni et al., 1988). A vector suitable for fusions to the COOH terminus of invertase (pEBH9) was constructed by introduction of BglII and NheI restriction sites at the COOH terminus of the invertase coding sequence in pEBH2 by site-directed mutagenesis (Kunkel et al., 1987). The mutagenic oligomer consisted of 17 nucleotides of noncomplementary sequence containing the BglII (bold) and NheI (underlined) sites, flanked by 16 and 18 nucleotides complementary to the SUC2 sequence on the 5' and 3' ends, respectively. (5'-TAA GGT TTA TAA CCT CTA GCT AGC TGC TGA)

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 × 10⁷ cells/ml. Invertase was induced by vigorous agitation with acid-washed glass beads, breaking cells by vigorous agitation with acid-washed glass beads, and diluting to 0.1 ml with sample buffer.

To spheroplasts, induced cultures of 6 × 10⁷ cells were washed in distilled water, incubated in 0.3 ml of 100 mM Tris-SO₄, pH 9.4, 50 mM β-mercaptoethanol for 10 min, washed in 1.2 M sorbitol, resuspended in 60 µl of spheroplasting buffer (1.2 M sorbitol, 10 mM Tris-HCl, pH 7.5) containing 60 U of recombinant lyticase, and incubated for 30 min at 30°C. The endpoint for complete spheroplasting was determined by >85% cell lysis upon dilution into 1% Triton X-100. Centrifugation at 500 g for 5 min 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, 1 mM PMSF and 0.5 µg/ml leupeptin (both Boehringer Mannheim Corp., 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 antin invertase at 1:1,000 dilution, mouse anti-M2 (Eastman Kodak Co., Rochester, NY) at 1:10,000 dilution, HRP-coupled protein A (Organon Teknika-Cappel, Durham, NC) 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 was diluted with 2 vol of 50 mM sodium citrate, pH 5.1, containing 100–250 U of Endo H (New England Biolabs) and incubated at 37°C for 2–16 h. Samples were boiled before gel electrophoresis.

Invertase Assays

To quantify the invertase activity secreted to the cell surface and retained in the cell, spheroplasts and spheroplast supernatants were diluted to 1 ml with 50 mM Tris-HCl, pH 7.5, 10 mM sodium azide. Invertase activity was assayed (Goldstein and Lampeu, 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)c1,-s11 were made to ensure that the assay was always conducted in its linear range.
Table I. Saccharomyces cerevisiae Strains

| Strain | Genotype | Source or reference |
|--------|----------|---------------------|
| CKY406 | MATa ura3-52 leu2-3, 112 suc2-Δ9 | This study |
| CKY407 | MATa sec 12-4 ura3-52 leu2-3, 112 suc2-Δ9 | This study |
| CKY408 | MATa sec 13-1 ura3-52 leu2-3, 112 suc2-Δ9 | This study |
| CKY409 | MATa 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 | MATa sec1-1 ura3-52 ade2-101 suc2-Δ9 | This study |
| CKY414 | MATa pep4::LEU2 ura3-52 leu2-3, 112 suc2-Δ9 | Marcusson et al., 1994 |
| SEY6210 | MATa leu2-3, 112 ura3-52 his3-Δ200 trpl-Δ901 lys2-801 suc2-Δ9 | Marcusson et al., 1994 |
| EMY3 | MATa Δps 10::HIS3 leu2-3, 112 ura3-52 his3-Δ200 trpl-Δ901 lys2-801 suc2-Δ9 | Marcusson et al., 1994 |

Indirect Immunofluorescence

Indirect immunofluorescence was performed essentially as described (Pringle et al., 1991). CKY414 (pep4Δ) cells expressing pH44 [Inv-(N)Clwt] were fixed in a final concentration of 3.7% formaldehyde for 1 h at 25°C, collected by centrifugation, and spheroplasted in 0.1 M potassium phosphate, pH 7.5, 2 μM β-mercaptoethanol, 800 U lyticase at 30°C for 30 min. Incubations in primary and secondary antibody were performed for 1 h each on a coverslip in a dark, humid chamber at 25°C. Cells were fixed in a final concentration of 3.7% formaldehyde for 1 h at 25°C, collected by centrifugation, and spheroplasted in 0.1 M potassium phosphate, pH 7.5, 2 μM β-mercaptoethanol, 800 U lyticase at 30°C for 30 min. Incubations in primary and secondary antibody were performed for 1 h 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 Corp.). Invertase antisera was affinity-purified by adsorption to nitrocellulose strips bearing concentrated amounts of deglycosylated invertase, followed by elution of bound antibodies with 0.2 M glycine, pH 2.3, 1 mM EGTA. Cells were mounted in medium containing 1% p-phenylene-diamine and 4'6-diamidino-2-phenylindole (DAPI), photographed on an axioscope (Carl Zeiss, Inc., Thornwood, NY) with film (T-Max 400; 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 × 10⁶ cell equivalents of spheroplast pellets were resuspended in 20 μl cell lysis buffer (200 mM sorbitol, 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM PMSF), lysed with acid-washed glass beads by vigorous agitation 8 x 30 s with cooling on ice, and the lysate was centrifuged at 100,000 g at 4°C for 10 min in an ultracentrifuge rotor (model TLA 100.3; Beckman Instruments, Fullerton, 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 NH₂-terminal Domain of A Repressor Is Secreted to the Cell Surface

The COOH-terminal end of the SUC2 gene was modified by the addition of restriction sites, and the NH₂-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)Clwt, which consists of invertase with a total of 111–amino acid residues appended to the COOH terminus (Fig. 1).

To examine whether this fusion protein was secreted to the cell surface, wild-type cells (CKY406) expressing Inv-(N)Clwt were converted to spheroplasts, and cell bod-
compare lanes 1 and 6). This protein was not recognized by antibody to the M2 epitope, suggesting that the COOH-terminal portion of the fusion protein had been proteolytically removed. In summary, fusion of the NH2-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 fusions to thermally unstable repressor mutants. For this analysis, we examined a set of related mutant repressors: Leu57 to Ala (L57A); Leu57 to Gly (L57G); and the triple mutant Leu57 to Ala, Gly46 to Ala, Gly48 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 \( T_m \) of the wild-type repressor and each of the mutants has been determined for purified NH2-terminal domains and is given in Fig. 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 wild-type 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)Clwt, which gave mostly secreted fusion protein, the three hybrids bearing unstable repressor domains expressed <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. 2 a, lanes 8, 10, and 12). This behavior can be contrasted to that of the SUC2-sll 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. 2 a, 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. 2 b, lanes 8, 10, and 12). The truncated intracellular protein was not recognized by antibody to the M2 epitope, indicating that the COOH-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...

Table II. Distribution of Periplasmic and Intracellular Activity for Invertase-repressor Fusions

| Invertase construct | Invertase activity | Percent secreted to the cell surface | \( U/O/D^* \) |
|---------------------|-------------------|-------------------------------------|--------------|
|                     | 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)Clwt         | 0.75 ± 0.06 | 0.49 ± 0.04 | 67                       |              |
| Inv-(N)Cl57A        | 0.29 ± 0.06 | 1.08 ± 0.18 | 23                       |              |
| Inv-(N)Cl57G        | 0.27 ± 0.06 | 1.03 ± 0.18 | 23                       |              |
| Inv-(N)ClLAGA (30°C)| 0.26 ± 0.02 | 1.01 ± 0.13 | 23                       |              |
| Inv-(N)ClLAGA (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.

*1 U of invertase releases 1 µmol of glucose from sucrose per min at 37°C.

\( \text{Percent secreted} = \frac{\text{periplasmic activity}}{\text{total activity} - \text{cytoplasmic activity (for WT inv.)}} \).
increasing the temperature of induction for Inv-(N)ClL57A brings the measured Tm of the triple mutant from 20°C to between 26 and 28°C (Reidhaar-Olsen et al., 1990). Decreasing the temperature of induction to 25°C partially stabilized the (N)ClLAGA 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)clL57A secreted to the cell surface (data not shown). Together these results showed that transport to the Golgi, but not to the plasma membrane, was required for degradation of Inv-(N)clL57A.

The possibility that degradation of Inv-(N)clL57A 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)clL57A, implicating vacuolar proteases in the degradation of the repressor moiety (Fig. 3, lane 4). Similarly, the pep4Δ mutation blocked degradation of Inv-(N)clL57G, Inv-(N)clLAGA, and the intracellular form of Inv-(N)clLwt (data not shown). Targeting of Inv-(N)clL57A to the vacuole was further supported by cytological examination of the intracellular location of the fusion protein. Immunofluorescent staining of Inv-(N)clL57A with antiinvertase antibody located the protein to large intracellular bodies, distinct from the DAPI-stained nuclei, that appeared to be vacuoles (Fig. 4).

**Degradation of the Repressor Domain of Inv-(N)clL57A Occurs in the Vacuole**

We used the Inv-(N)clL57A fusion to determine where degradation of the mutant hybrids was taking place in the cell. When Inv-(N)clL57A 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, and sec23) similarly prevented degradation of the repressor moiety of Inv-(N)clL57A (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)clL57A 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)clL57A.

**Figure 3.** The repressor domain of Inv-(N)clL57A is degraded in the vacuole. Inv-(N)clL57A 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 and resolved on an 8% SDS-polyacrylamide gel, and invertase was visualized by Western blotting with invertase antiserum.
Inv-(N)cIwt and Inv-(N)cIL57A 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 nonnative membrane proteins are segregated to the vacuole (Gaynor et al., 1994; Chang and Fink, 1995). Although the sequence of the NH₂-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)cIL57A and Inv-(N)cIwt 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)cIL57A 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 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)cIwt and Inv-(N)cIL57A 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)cIL57A 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)cIL57A 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)cIL57A 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 pro-
teinase A (PrA); in wild-type cells, 95% of both proteins reach the vacuole, but upon overexpression, >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)clL57A was overexpressed from the high-copy plasmid pRS306-2μ in a wild-type strain (CKY406). Upon overproduction, 33% of Inv-(N)clL57A 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)clL57A expressed in high copy 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)clL57A was present in two forms: a form that comigrated with the degraded 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)clL57A in low copy (Fig. 7, lane 3). The small amount of partially truncated Inv-(N)clL57A 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)clL57A was being exposed to periplasmic proteases once the fusion protein 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)clL57A was blocked in a sec1 mutant at the restrictive temperature (Fig. 6, lanes 7 and 8). The only forms of Inv-(N)clL57A detected in the sec1 mutant were the full-length fusion protein and the truncated fusion protein produced by vacuolar proteolysis that comigrates with wild-type invertase (Fig. 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)clL57A 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)clL57A secreted to the cell surface during overexpression, most likely resulting from saturation of a receptor-mediated targeting system that sorts Inv-(N)clL57A to the vacuole.

Invertase activity Percent secreted to

Table III. Redistribution to the Cell Surface for Inv-(N)clL57A Overexpression and Mutant Repressor Fusions in vps10Δ

| Strain/Invertase fusion | Invertase activity | Percent secreted to the cell surface* |
|------------------------|-------------------|------------------------------------|
|                        | Periplasmic        | Intracellular                      |
| wildtype/Inv-(N)clL57A (CEN) | 0.29 ± 0.07       | 1.31 ± 0.08                        | 20 |
| wildtype/Inv-(N)clL57A (2μ) | 0.21 ± 0.24       | 5.25 ± 0.44                        | 33 |
| vps10Δ/Inv-(N)clL57A (CEN) | 0.69 ± 0.06       | 0.48 ± 0.03                        | 66 |
| vps10Δ/Inv-(N)clL57A (2μ) | 0.56 ± 0.06       | 0.41 ± 0.02                        | 66 |
| vps10Δ/Inv-(N)clL57A (2μ) | 0.66 ± 0.07       | 0.44 ± 0.03                        | 67 |

*1 U of invertase releases 1 μmol of glucose from sucrose per min at 37°C.

1Percent secreted = total activity - cytoplasmic activity (for WT inv.)

Figure 6. Overexpressed Inv-(N)clL57A is secreted to the cell surface. CKY406 (wild type) (lanes 1 and 2) and CKY414 (pep4Δ) (lanes 3 and 4) expressing pEHB44 [Inv-(N)clL57A-CEN] and CKY406 (wt) (lanes 5 and 6) and CKY413 (sec1-1) (lanes 7 and 8) expressing pEHB55 [Inv-(N)clL57A-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 and resolved on an 8% SDS-PAGE gel, and invertase was visualized by Western blotting using invertase antiserum. The protein amount in the fractions derived from expression of Inv-(N)clL57A-2μ, lanes 5–8, represent one-fifth of the protein loaded in lanes 1–4.

Inv-(N)clL57A 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)clL57A, Inv-(N)clL57G, and Inv-(N)clLAGA partitioned with the spher-
Figure 7. Vps10p is required to target Inv-(N)Cl\textsubscript{L57A} to the vacuole. CKY406 (wt) (lanes 1 and 2), CKY414 (Δpep4) (lanes 3 and 4), EMY3 (Δvps10) (lanes 5 and 6) expressing pEHB44 [Inv-(N)Cl\textsubscript{L57A}], EMY3 (Δvps10) expressing pEHB51 [Inv-(N)Cl\textsubscript{L57G}], (lanes 7 and 8), and pEHB53 [Inv-(N)Cl\textsubscript{LAGA}] (lanes 9 and 10) were treated as described in the legend of Fig. 6. A wild-type strain (SEY6210), isogenic to EMY3, expressing pEHB44 exhibitd the same pattern of cleaved Inv-(N)Cl\textsubscript{L57A} in the pellet fraction as seen in CKY406 (lanes 1 and 2).

Discussion

A large body of both published experimental data and unpublished observations 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 NH\textsubscript{2}-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 NH\textsubscript{2}-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 (Fig. 8 a). In contrast, mutations that reduce the T\textsubscript{m} of the NH\textsubscript{2}-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 char-
mediates much of the protein turnover in a yeast cell (Teichertz 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., 1993; 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 and 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), a hybrid protein composed of the transmembrane domain of the ER membrane protein Wbp1p fused to invertase (Gaynor et al., 1994), and mutants of the late-Golgi protease Kex2, which disrupt the Golgi retention signal located in the cytosolic tail (Wilcox et al., 1992). 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 sub-units 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 (Fig. 8 b).

Vps10p is a 178-kD type 1 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 Q24-R-P-L27 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 Q24 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 R25 and P26, 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 (Cooper and Stevens, 1996; Westphal et al., 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 sorting 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 Leu57 to Ala mutation is partially suppressed by second-site mutations at two Gly residues that have been shown to increase the Tm of the NH2-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 Leu57, we tested another destabilizing repressor mutation that contains an amino acid change at a different position, Leu69 to Gly, which similarly lowers the Tm of the NH2-terminal domain measured in vitro (Sauer, R.T., unpublished observations). The corresponding invertase fusion of this mutant was also targeted to the vacuole, em-
phasing 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 COOH terminus also specify targeting of the fusion protein to the vacuole (Hong, E., 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)clL57A binds to the same site on Vps10p as proCPY by determining whether overexpression of Inv-(N)clL57A can cause secretion of proCPY into the extracellular medium. No effect on CPY secretion was seen when Inv-(N)clL57A was expressed from a 2μ plasmid. Similarly, in the converse experiment, overexpression of CPY did not result in an increased level of Inv-(N)clL57A 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 NH2 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 that assume an unfolded conformation appended to the COOH 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.

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