Single, context-specific glycans can target misfolded glycoproteins for ER-associated degradation

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The endoplasmic reticulum (ER) maintains an environment essential for secretory protein folding. Consequently, the premature transport of polypeptides would be harmful to the cell. To avert this scenario, mechanisms collectively termed "ER quality control" prevent the transport of nascent polypeptides until they properly fold. Irreversibly misfolded molecules are sorted for disposal by the ER-associated degradation (ERAD) pathway. To better understand the relationship between quality control and ERAD, we studied a new misfolded variant of carboxypeptidase Y (CPY). The molecule was recognized and retained by ER quality control but failed to enter the ERAD pathway. Systematic analysis revealed that a single, specific N-linked glycan of CPY was required for sorting into the pathway. The determinant is dependent on the putative lectin-like receptor Htm1/Mnl1p. The discovery of a similar signal in misfolded proteinase A supported the generality of the mechanism. These studies show that specific signals embedded in glycoproteins can direct their degradation if they fail to fold.

Introduction

The maturation of newly synthesized proteins entering the secretory pathway is monitored by mechanisms collectively termed ER quality control (for reviews see Ellgaard and Helenius, 2003; McCracken and Brodsky, 2003; Trombetta and Parodi, 2003). Proteins in the midst of folding are retained in the ER until the process is completed. Irreversibly misfolded species are sorted from normal proteins and targeted for degradation. Because aberrant proteins are not benign, elimination provides the most effective means of abrogating potential toxicity. The best-described route is the ER-associated degradation (ERAD) pathway, with its basic tenets conserved among all eukaryotes. In ERAD, misfolded proteins are translocated from the ER lumen to the cytosol (termed "dislocation"), most likely through a pore complex composed of Sec61 subunits and/or Der1p (Pilon et al., 1997; Plemper et al., 1999; Lilley and Ploegh, 2004; Ye et al., 2004). On the cytosolic side, the substrate is ubiquitylated by ER localized ubiquitin conjugating enzymes and ligases. The driving force for substrate extraction from the ER comes from the AAA-ATPase Cdc48p/Ufd1p/Npl4p complex (Shamu et al., 2001; Ye et al., 2001; Jarosch et al., 2002). Finally, the substrate is deglycosylated by protein *N*-glycanase (if applicable) and degraded by the 26S proteasome (Suzuki et al., 2000; Hirsch et al., 2003).

The upstream events of substrate sorting and targeting to the translocation pore are less clear. In higher eukaryotes, folding and quality control functions of glycoprotein synthesis are integrated within the calnexin/calreticulin lectin cycle. A third ER lectin, EDEM (Htm1/Mnl1p in yeast), is used to direct misfolded proteins off-cycle and into the ERAD pathway (Molinari et al., 2003; Oda et al., 2003). The mechanism is not universal because many proteins (e.g., nonglycosylated) use other pathways that are less defined. In budding yeast, the process is murkier with the absence of a typical calnexin/calreticulin cycle. However, the requirement of Htm1/Mnlp for ERAD reflects its functional conservation and emphasizes that general strategies of protein quality control are shared among all eukaryotes (Jakob et al., 2001; Nakatsukasa et al., 2001).

The determinants used for sorting and targeting substrates have not been fully characterized. At first glance, tackling the problem seems simple. However, the range of possible substrates illustrates the enormity of the task. Trafficking through the ER includes soluble proteins, single and multi-spanning integral membrane proteins, and lipid anchored proteins. For their maturation, additional steps may include glycosylation, prolyl hydroxylation, disulfide bond formation, and assembly into complex oligomers. Within this backdrop is the vast number of conformations and configurations that the cell must determine are unfolded (in the process of folding), folded, and misfolded. Although the prevailing evidence indicates that chaperones recognize and bind unfolded and misfolded proteins, how the cell arrives at the decision to degrade individual

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Abbreviations used in this paper: CPY, carboxypeptidase Y; ERAD, ER-associated degradation; GT, glucosyltransferase; UPR, unfolded protein response.

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molecules remains unknown. Defining the nature of substrate determinants is key to this understanding.

Previously, we reported that cytosolic and lumenal surveillance mechanisms coexist to monitor the range of proteins trafficking through the ER (Vashist and Ng, 2004). Because the pathways converge at the ubiquitylation/degradation step of ERAD, they were designated ERAD-C (cytosolic) and ERAD-L (lumenal). In this study, we focused our attention on glycoprotein substrates of ERAD-L. By systematically analyzing a series of substrate variants, we discovered that determinants used for sorting/retention (from folded proteins) could be distinguished from those used to target substrates to ERAD. Initially, the unfolded polypeptide alone suffices for efficient substrate recognition and retention. The decision to terminate the molecule, however, requires an additional structural determinant embedded in the substrate.

Results

CPYΔ1, a misfolded protein variant recognized and retained by ER quality control but not by ERAD

The most extensively studied model ERAD substrate is CPY*, a mutant of the vacuolar protease carboxypeptidase Y (CPY). CPY* is a product of the *prc1-1* allele (glycine to arginine replacement at position 255) that misfolds irreversibly (Finger et al., 1993). To extend its versatility, we intended to create a substrate that permitted simultaneous monitoring of endogenous CPY as an internal control for secretory function and gel loading. A CPY variant designated CPYΔ1 was constructed that differs by a 154–amino acid deletion near the COOH terminus and migrates distinctly on SDS gels (Fig. 1, A and B). Guided by the crystal structure, we predicted that the deletion would disrupt folding due to the extensive loss of intramolecular interactions (Endrizzi et al., 1994). Three lines of evidence supported this view.

By pulse-chase analysis, CPYΔ1 was not modified by compartmentalized enzymes that report on the transit state of CPY (Fig. 1 B). This result suggested that CPYΔ1 might be recognized and retained by ER quality control. Cell localization experiments confirmed this notion. As visualized by indirect immunofluorescence, CPYΔ1 accumulated intracellularly at sites precisely coincident with the ER marker Kar2p (Fig. 1 C; Normington et al., 1989; Rose et al., 1989). Correspondingly, this pattern was indistinguishable to that observed for CPY* (Fig. 1 C). We next tested whether CPYΔ1 expression activates the unfolded protein response (UPR). The UPR is an ER-to-nucleus signal transduction pathway sensitive to the presence of misfolded proteins the ER lumen (for reviews see Patil and Walter, 2001; Spear and Ng, 2001; Rutkowski and Kaufman, 2004). By monitoring the activity of the UPR-specific reporter gene *UPRE-LacZ* (Cox et al., 1993), cells expressing CPYΔ1 activated the UPR to the same extent as cells expressing CPY* (Fig. 1 D). Together, these experiments demonstrated that CPYΔ1 is a bona fide misfolded protein recognized by ER quality control and the UPR.

Unexpectedly, metabolic pulse-chase experiments showed that CPYΔ1 turned over poorly by comparison to CPY* (Fig. 1,

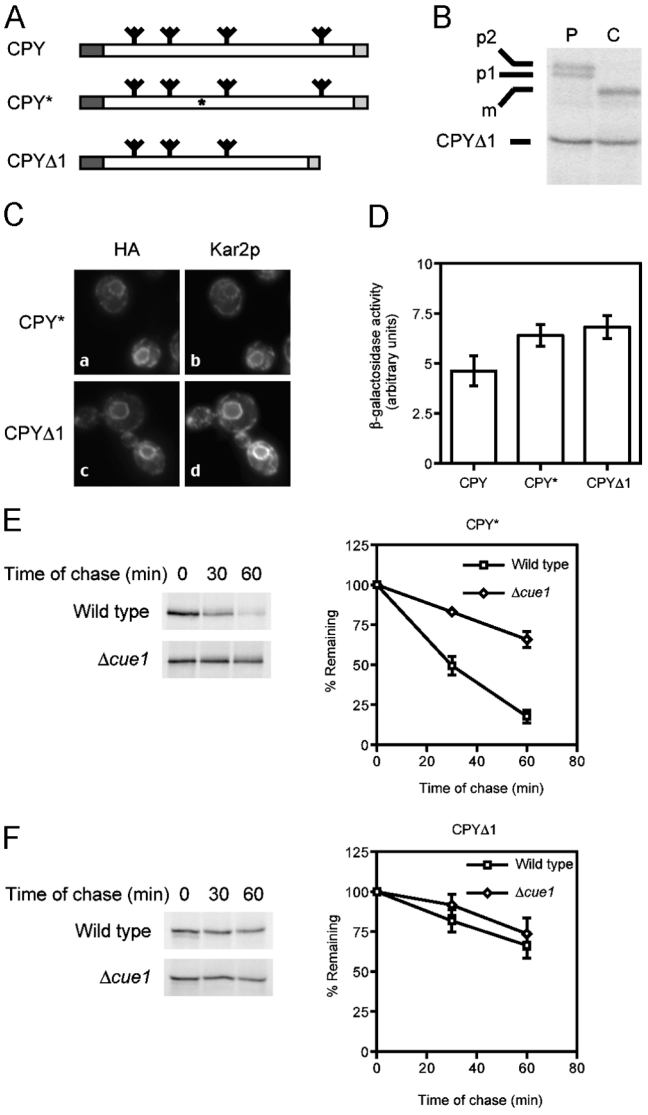

Figure 1. **CPYΔ1 is a misfolded protein recognized by ER quality control but poorly degraded by ERAD.** (A) Schematic representation of CPY, CPY*, and CPYΔ1. Carbohydrates are represented by branched symbols, asterisk indicates the CPY* G255R mutation, dark gray boxes indicate signal sequences, and light gray boxes represent HA-epitope tags. (B) CPYΔ1 remains unmodified by Golgi and vacuolar enzymes. Wild-type cells expressing CPYΔ1 (pES57) were pulsed labeled for 10 min and chased for 0 (lane P) or 30 min (lane C). Immunoprecipitated CPY and CPYΔ1 were resolved by SDS-PAGE and visualized by autoradiography. CPYΔ1, ER proCPY (p1), Golgi carbohydrate-modified proCPY (p2), and vacuolar protease-processed mature CPY (m) are indicated. (C) Intracellular localization of CPYΔ1. CPY* and CPYΔ1 were localized by indirect immunofluorescence as described in Materials and methods (C, panels a and c, respectively). Simultaneous localization of Kar2p was performed as a marker of the ER (C, panels b and d). (D) CPYΔ1 induces the UPR. Wild-type cells carrying an integrated UPRE-*LacZ* reporter gene (ESY39) and expressing HA epitope-tagged CPY, CPY*, or CPYΔ1 were assayed for β-galactosidase activity. The data reflect three independent experiments with the SD of the mean indicated. (E) Wild-type and Δ*cue1* cells expressing CPY* were pulse labeled for 10 min and chased for the times indicated. CPY* was immunoprecipitated from detergent lysates and resolved by SDS-PAGE. CPY* decay was quantified by phosphorimager analysis and plotted to the right of autoradiograms. The data reflect three independent experiments with the SD of the mean indicated. (F) Wild-type and Δ*cue1* cells expressing CPYΔ1 were analyzed by pulse-chase analysis as described in E.

compare E with F). This indicated that CPYΔ1, though retained by ER quality control, is a poor substrate for ERAD. This view was confirmed by its failure to be further stabilized in the Δ*cue1* ERAD mutant (Fig. 1 F). Cue1p is a critical component for CPY* degradation as it anchors the ubiquitin conjugating enzyme Ubc7p to the ER membrane (Biederer et al., 1997). The residual turnover was likely through alternative pathways that degrade misfolded proteins when ERAD is disrupted or saturated (Haynes et al., 2002; Spear and Ng, 2003). Together, the data show that ER quality control and ERAD are mechanisms that can be uncoupled at the substrate level. This raised the intriguing possibility that ER retention and entry into ERAD use distinct substrate determinants.

Nonetheless, we envisioned other equally plausible explanations that could account for CPYΔ1's unusual behavior. Because the deletion is large, the severity of the lesion might increase the tendency of the remaining polypeptide to aggregate. Substrate solubility is an important prerequisite for ERAD. CPY* aggregates caused by faulty chaperone function were shown to degrade inefficiently (Nishikawa et al., 2001). To determine whether aggregate formation contributes to CPYΔ1 stability, microsomal membranes containing CPY* or CPYΔ1 were prepared from logarithmically growing cells. The membranes were solubilized in nonionic detergent under physiological conditions and subjected to centrifugation. Under these conditions, large protein aggregates sediment rapidly and separate from soluble proteins remaining in the supernatant. Detergent-insoluble (pellet) and detergent-soluble (supernatant) fractions were collected, proteins resolved by SDS-PAGE, and analyzed by immunoblotting. To control for the procedure, we specifically generated CPY* aggregates from cells severely limiting for ER chaperones (Δ*ire1*; Spear and Ng, 2003). This species was analyzed in parallel. As shown in Fig. 2, CPY* and CPYΔ1 were both recovered entirely from the supernatant fraction (B and C), whereas CPY* aggregates from Δ*ire1* cells were found predominantly in the pellet fraction (A). In every case, the ER integral membrane protein Sec61p was recovered from the soluble fraction showing that membranes were completely solubilized. This experiment showed that the formation of detergent-insoluble aggregates was not a root cause of the CPYΔ1 ERAD defect. Furthermore, CPYΔ1 puncta, which would be characteristic of intracellular aggregates, were never observed in immunolocalization experiments (DePace et al., 1998). Instead, CPYΔ1 was always found to be evenly distributed throughout the ER in patterns indistinguishable from CPY* and Kar2p (Fig. 1 C).

We next tested a second possibility. Inspection of the CPYΔ1 sequence revealed a striking consequence of the deletion. A lysine rich domain was eliminated, leaving behind a 116–amino acid stretch devoid of lysines at the COOH terminus. Because CPY* ubiquitylation is required for its dislocation (Shamu et al., 2001; Jarosch et al., 2002), we wondered whether loss of putative ubiquitylation sites crucial for ERAD could explain the defect. To test the possibility, nine lysine residues corresponding to the CPYΔ1 deleted region were changed to arginine in full-length CPY*. The resulting molecule, K(9)R-CPY*, was degraded as efficiently as CPY* demonstrating that the COOH-terminal lysines are not required for ERAD (Fig. 3 A). Conversely, the addition of lysine residues to the COOH-terminal domain of CPYΔ1 failed to destabilize it (Fig. 3 B). Together, these data show that the COOH-terminal lysine residues of CPY do not constitute critical determinants for its turnover by ERAD.

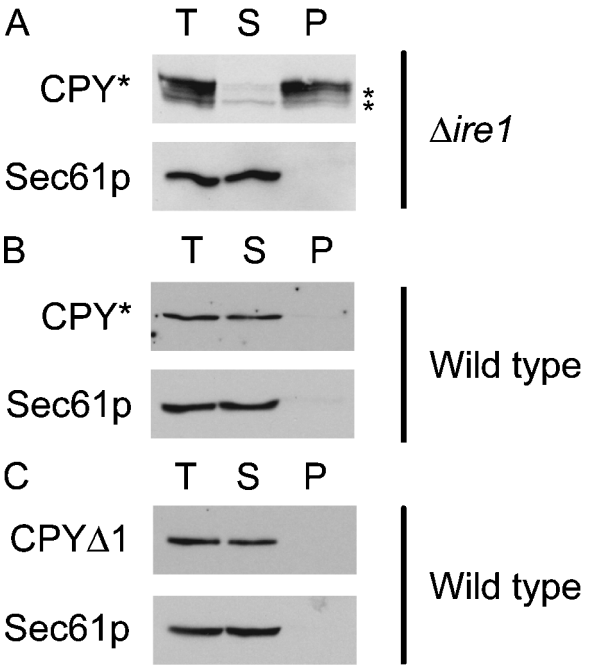

Figure 2. **CPYΔ1 does not form detergent insoluble aggregates.** Microsomes were prepared from Δ*ire1* cells overexpressing CPY* (A), wild-type cells expressing CPY* (B), and wild-type cell expressing CPYΔ1 (C). Membranes were solubilized in 1% Triton X-100 and separated into pellet and supernatant fractions by centrifugation at 100,000 *g*. Detergent-soluble (S), detergent-insoluble (P), and total (T) fractions were resolved by SDS-PAGE, followed by immunoblotting to detect CPY* and CPYΔ1 using anti-HA antibodies. The extent of membrane solubilization was determined by reprobing blots for Sec61p, an integral membrane protein control. Asterisks indicate underglycosylated and cytosolic CPY* that form when overexpressed in Δ*ire1* cells (Spear and Ng, 2003).

A context-dependent glycan signal for ERAD

Another outcome of the CPYΔ1 deletion was the elimination of the N-linked glycosylation site nearest the COOH terminus (Fig. 4 A, glycan D). Because CPYΔ1 retains three other N-linked glycans (Fig. 4 A, sites A–C), it was not apparent how its loss alone could so severely disrupt ERAD. To test whether the carbohydrate is a critical determinant, a CPY* glycan D mutant (Fig. 4 A, ABCd-CPY*) was created and its turnover analyzed. Indeed, ABCd-CPY* was degraded poorly compared with CPY* (Fig. 4 C). This result showed that glycan D is an important determinant of CPY* degradation and explains, at least in part, why CPYΔ1 is not a substrate for ERAD.

In considering glycan D's role in ERAD, three potential mechanisms were formulated to explain its requirement. The first envisions a carbohydrate threshold that demands a minimum number for each substrate. This requirement could be related to molecular weight, with the similarly sized CPY* and

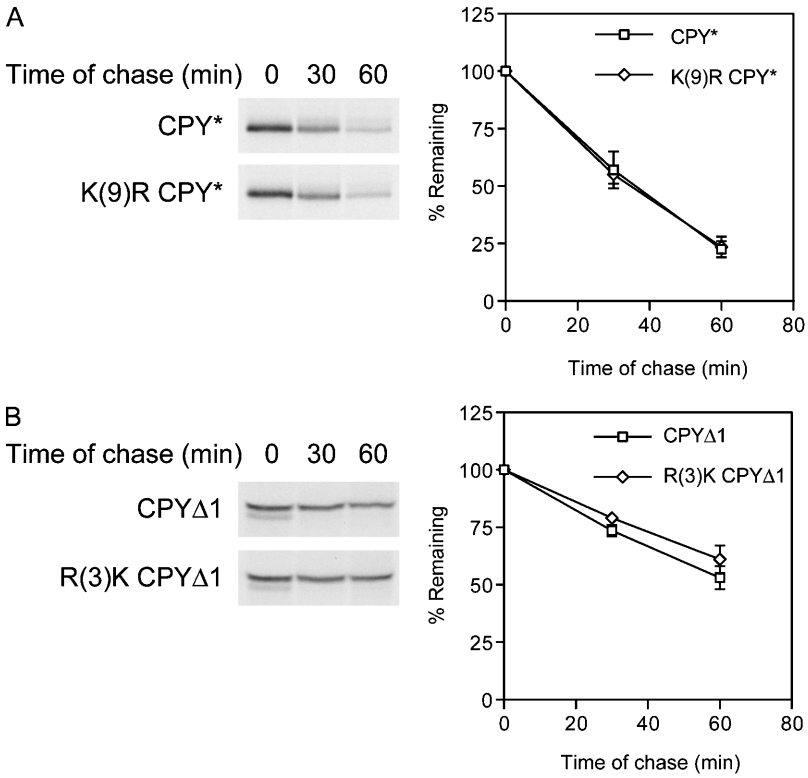

Figure 3. **COOH-terminal lysines are not required for CPY* degradation.** (A) CPY* (pDN436) or K(9)R CPY* (pES115) degradation in wild-type cells was determined by pulse-chase analysis performed in Fig. 1 E. (B) Turnover of CPYΔ1 (pES57) or R(3)K CPYΔ1 (pES95) was determined using wild-type cells as in A. Plots reflect two independent experiments with the SD of the mean indicated.

KHN (misfolded simian virus 5 HA-neuramindase) substrates requiring four glycans, whereas the smaller PrA* (misfolded proteinase A) requires only two (Finger et al., 1993; Vashist et al., 2001). The second is a positional model that requires a carbohydrate at a fixed point in the polypeptide, perhaps as a "molecular handle" to orient its transfer to the translocon after lectin binding. In line with this view, both KHN and PrA* contain glycosylation sites at roughly the same position to their COOH termini as glycan D of CPY*. For the first two models, the protein content apart from maintaining glycosylation site(s) is irrelevant. In the third model, glycan D forms part of an ERAD-specific signal that is recognized only when the protein is unfolded. Here, the signal would be comprised of the carbohydrate and a protein determinant. The last model is compatible with a wider range of proteins because it requires only a single carbohydrate and no positional requirement.

To distinguish the models, we generated the remaining CPY* single-site glycosylation mutants to test if ERAD efficiency is related to carbohydrate density. By contrast to the D glycan, eliminating any of the other three carbohydrates had no effect on CPY* degradation (Fig. 4 C). These data show that only glycan D is essential for efficient degradation of misfolded CPY and rule out the notion of a carbohydrate threshold.

The findings were intriguing because Htm1/Mnl1p was proposed to be an ERAD-specific lectin (Jakob et al., 2001; Nakatsukasa et al., 2001). However, little was known regarding how it recognizes substrate. If Htm1/Mnl1p acts specifically through glycan D, the stability of ABCd-CPY*, which bears the other three glycans, should be unaffected in cells lacking *HTM1/MNL1*. To test the assertion, we measured CPY* and ABCd-CPY* turnover in wild-type and Δ*htm1/mnl1* cells. In metabolic pulse-chase experiments, CPY* degradation was dependent on *HTM1/MNL1* as previously reported (Fig. 4 D; Jakob et al., 2001; Nakatsukasa et al., 2001). By contrast, ABCd-CPY* was degraded poorly in wild-type cells with no further stabilization in the Δ*htm1/mnl1* cells (Fig. 4 E). Interestingly, the residual degradation of ABCd-CPY* in the presence or absence of Htm1/mnl1p required Cue1p (Fig. 4 E). This likely reflects a lectin-independent mode of ERAD revealed only upon simultaneous disruption of the lectin and its substrate determinant. We next assessed whether glycan D is sufficient to direct CPY* to ERAD in an Htm1/Mnl1p-dependent manner. For this, a mutant variant glycosylated only at site D was constructed (abcD-CPY*). When expressed in wild-type cells, abcD-CPY* was degraded efficiently (Fig. 4 F). In Δ*htm1/mnl1* cells, however, abcD-CPY* was stabilized to the same extent as CPY* (Fig. 4 F). Together, these data show that a single, specific carbohydrate is necessary and sufficient in directing substrate into ERAD by way of Htm1/Mnl1p.

We wondered if the specificity of the glycan was unique to CPY* or a general feature of lectin-dependent ERAD. For this, we analyzed the ERAD substrate PrA* (Finger et al., 1993). PrA* is a mutant version of the endogenous vacuolar enzyme, proteinase A. PrA* contains two N-linked glycans, one near its NH_2 terminus and the other near the COOH terminus at a position similar to CPY*'s glycan D. Each site was disrupted singly by replacing asparagine codons with glutamine. The mutant variants, Ab-PrA* and aB-PrA* (Fig. 5 A, follows the nomenclature of CPY* glycan mutants), were expressed in wild-type cells (deleted of endogenous *PEP4* gene for detection of PrA*) and their turnover measured. As shown in Fig. 6, the Ab-PrA* was degraded indistinguishably from PrA*. Because

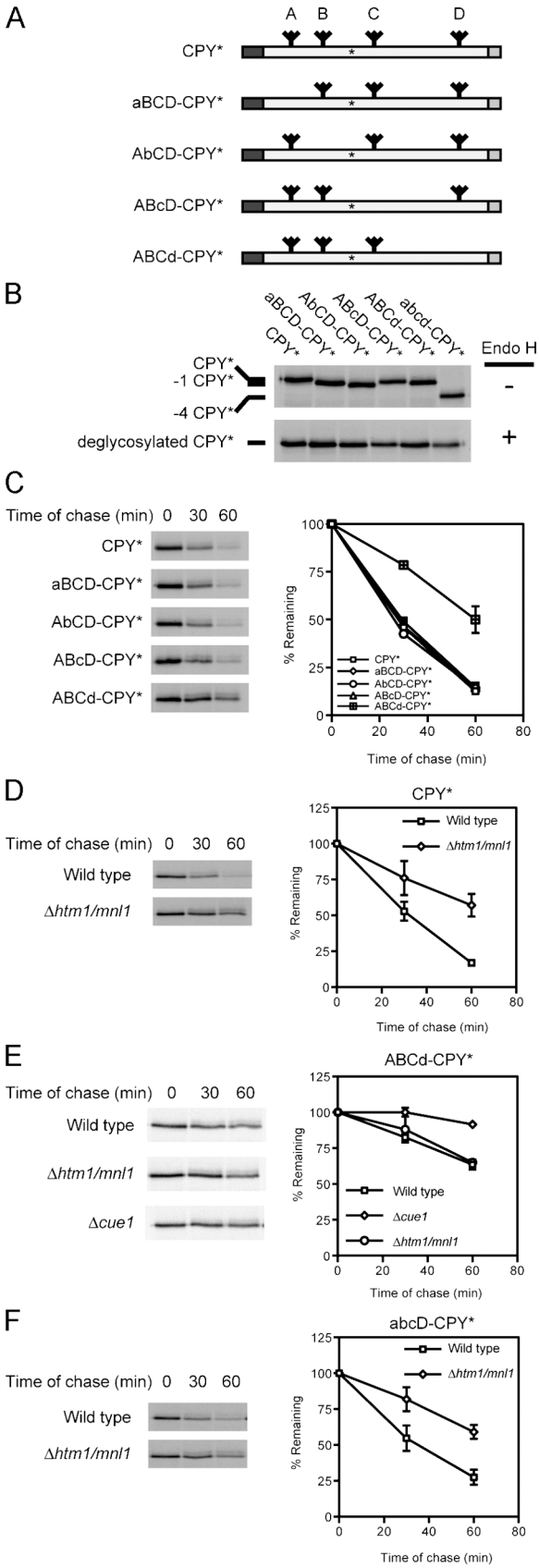

Figure 4. **A single, specific N-linked oligosaccharide is required for CPY* degradation.** (A) Schematic representation of CPY* N-linked glycosylation mutants. Glycans at positions 124, 198, 279, and 479, are denoted by the upper case letters A, B, C, and D, respectively. Lower case letters designate mutant sites. (B) CPY* and mutant variants were pulse labeled for 10 min, immunoprecipitated, and treated (or mock treated) with Endo H to remove N-linked oligosaccharides. Proteins were resolved by SDS-PAGE and visualized by autoradiography. Positions of CPY*, −1 CPY* (altered at one of four glycosylation sites), −4 CPY* (all four glycosylation sites altered), and deglycosylated CPY* are indicated. (C) Degradation of CPY* glycosylation mutants analyzed by metabolic pulse chase. Cells were pulse labeled for 10 min with [^{35}S]methionine/cysteine and chase for times indicated. Proteins were resolved by SDS-PAGE and quantified by phosphorimager analysis. The data reflect two independent experiments with the SD of the mean indicated. Representative autoradiograms are shown for each experiment. (D–F) Turnover of CPY* glycosylation mutants in wild-type and Δ*htm1/mnl1* cells performed as in C. ABCd-CPY* turnover was also analyzed in Δ*cue1* cells for comparison (E).

PrA* is glycosylated at only two sites, its degradation depends on a single, specific glycan signal like CPY* or is carbohydrate independent. The question was answer by the results of two experiments. First, Ab-PrA* degradation is dependent on Htm1/Mnl1p to a similar extent as PrA* (Fig. 6, A and B). Second, turnover of reciprocal mutant, aB-PrA*, was strongly defective and little affected by the loss of Htm1/Mnl1p (Fig. 6 C). These data support the idea that single ERAD glycan determinants are preembedded in glycoproteins. However, a wider range of substrates must be tested to determine whether other configurations are used. Serendipitously, analysis of the PrA* model also ruled out COOH-terminal positioning being a requirement because its sole determinant is closer to the NH_2 terminus.

Together, these data show that determinants for recognition and retention of misfolded proteins are distinct from signals used for targeting to the ERAD pathway. For entry into the Htm1/Mnl1p-dependent arm of ERAD, specific N-linked glycans preencoded in glycoproteins form essential determinants. The signals are not positionally constrained but are nevertheless context dependent because glycans at other positions cannot substitute.

Discussion

Cells deploy an array of mechanisms devoted to the detection and disposal of aberrant proteins. In the secretory pathway, checkpoints posted at the ER, Golgi apparatus, and plasma membrane monitor the state of newly synthesized proteins and the integrity of folded proteins (Arvan et al., 2002; Ellgaard and Helenius, 2003). Irreversibly misfolded proteins detected at any of these sites are sorted and targeted for degradation. In the ER, multiple quality control pathways are needed to monitor the topologically diverse molecules that traffic through the organelle. Two distinct pathways, acting as sequential checkpoints, were previously defined through analysis of their client substrates (Vashist and Ng, 2004). The first, termed ERAD-C monitors the cytosolic domains of membrane proteins. Membrane proteins passing the ERAD-C checkpoint and all soluble proteins are next examined by ERAD-L. Molecules with lumenal lesions are retained and degraded.

Misfolded proteins recognized by ERQC are not always inevitably degraded by ERAD. Among a group of misfolded Ste6p mutants retained in the ER, some variants are degraded by ERAD, whereas others are stable (Loayza et al., 1998). Al-

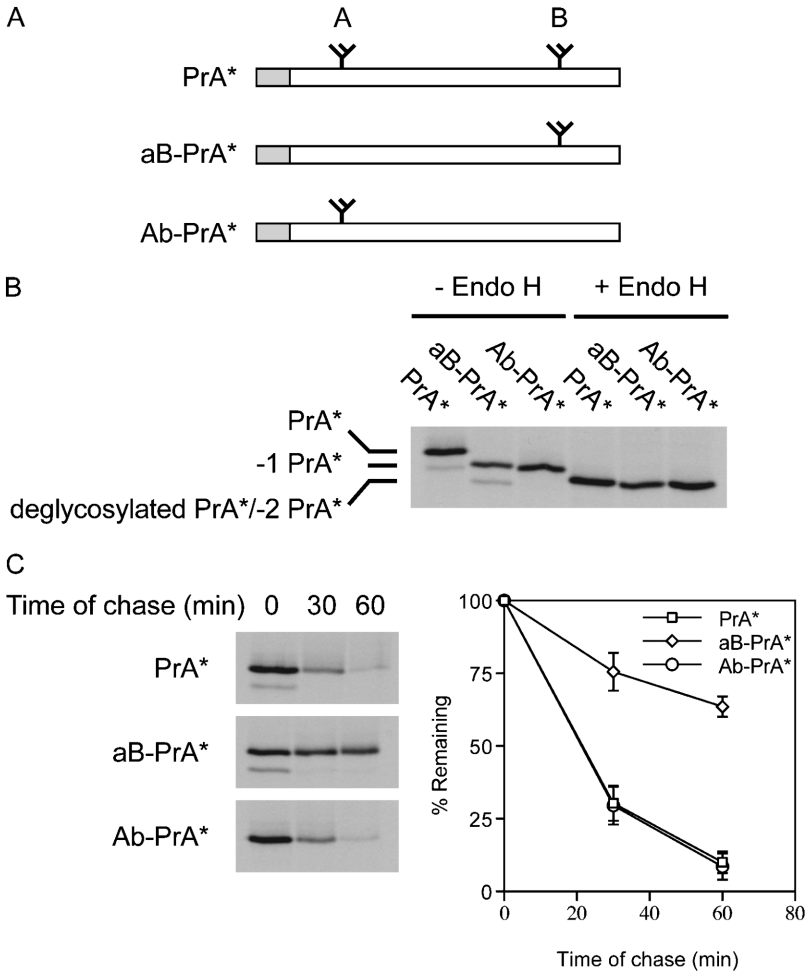

Figure 5. **Degradation of the misfolded protein PrA* requires a single NH_2-terminal glycan.** (A) Schematic representation of PrA* and glycosylation site mutant derivatives. Asparagines modified by glycosylation at positions 107 and 308 are indicated with A and B, respectively. Lower case designations indicate mutant sites. (B) Cells expressing PrA*, aB-PrA*, or Ab-PrA* were pulse labeled for 10 min with [^{35}S]methionine/cysteine. Proteins were immunoprecipitated from detergent lysates using polyclonal anti-PrA antibodies, mock treated (−) or treated (+) with Endo H, and resolved by SDS-PAGE. Positions of PrA*, −1 PrA*, and deglycosylated PrA* are indicated. (C) Wild-type cells expressing PrA*, aB-PrA*, or Ab-PrA* were analyzed by pulse-chase analysis as in Fig. 1 E. The data reflect two independent experiments with the SD of the mean indicated.

though the reasons for the difference are unknown, these studies demonstrated that retention and degradation are separable mechanisms. In the ERAD-L system, the theme is similar. Although both CPY* and CPYΔ1 are retained by ERQC, only CPY* is degraded efficiently by ERAD (Fig. 1). Through systematic analysis of these substrates, we discovered that the deleted glycan of CPYΔ1 is a critical determinant for targeting CPY to ERAD when misfolded. Subsequent analysis using PrA* revealed an analogous signal demonstrating the generality of the mechanism. In either case, the determinant is used exclusively as other glycans naturally positioned along the molecules cannot substitute. By contrast, ERAD-C substrates are degraded independent of glycosylation state (Vashist and Ng, 2004).

N-linked carbohydrates perform many functions and their participation in ERQC is well established. In higher eukaryotes, a subset of glycoproteins relies on the ER lectins calnexin and calreticulin for folding (for reviews see Ellgaard and Helenius, 2003; Sitia and Braakman, 2003). They work by binding trimmed, monoglucosylated N-linked glycans of newly synthesized proteins and provide a platform for the participation of accessory folding enzymes. Removal of the remaining glucose residue by glucosidase II frees the substrate from the lectin. Should the substrate remain unfolded, UDP-glucose/glycoprotein glucosyltransferase (GT) reglucosylates the glycan for another round of lectin binding. In this mode, GT is the folding sensor and substrates remaining in the cycle are retained in the ER as a consequence. Proteins that cannot fold properly go off-cycle and enter an ERAD pathway. Although used by many organisms, this mechanism is absent in yeast due to the lack of GT.

Even if the calnexin cycle is not universally conserved, current evidence indicate that all eukaryotes have adapted N-linked glycans for use in ERAD. For example, eliminating the N-linked glycosylation sites of CPY* disrupted its degradation in yeast cells (Knop et al., 1996). Although their role in ERAD was unclear, it was suggested that the carbohydrates were needed to maintain substrate conformations favorable for ERAD. A different view emerged from genetic and pharmacological studies that assessed the effects of compromised carbohydrate processing. The trimming of protein-linked $Glc_3Man_9GlcNAc_2$ core carbohydrate(s) to the $Man_8GlcNAc_2$ form (Glc, glucose; Man, mannose; GlcNAc, *N*-acetylglucosamine) was shown to be required for efficient substrate degradation (Jakob et al., 1998; Tokunaga et al., 2000). This led to the proposal that N-linked glycans can function as signals for targeting ERAD substrates. The failure of $Man_9GlcNAc_2$, $Man_7GlcNAc_2$, and $Man_6GlcNAc_2$ glycoforms to substitute for $Man_8GlcNAc_2$ implied a degree of specificity expected of a ligand–receptor interaction (Jakob et al., 1998). This model is particularly appealing because the crucial mannose trimming step was found to be much slower than the preceding process-

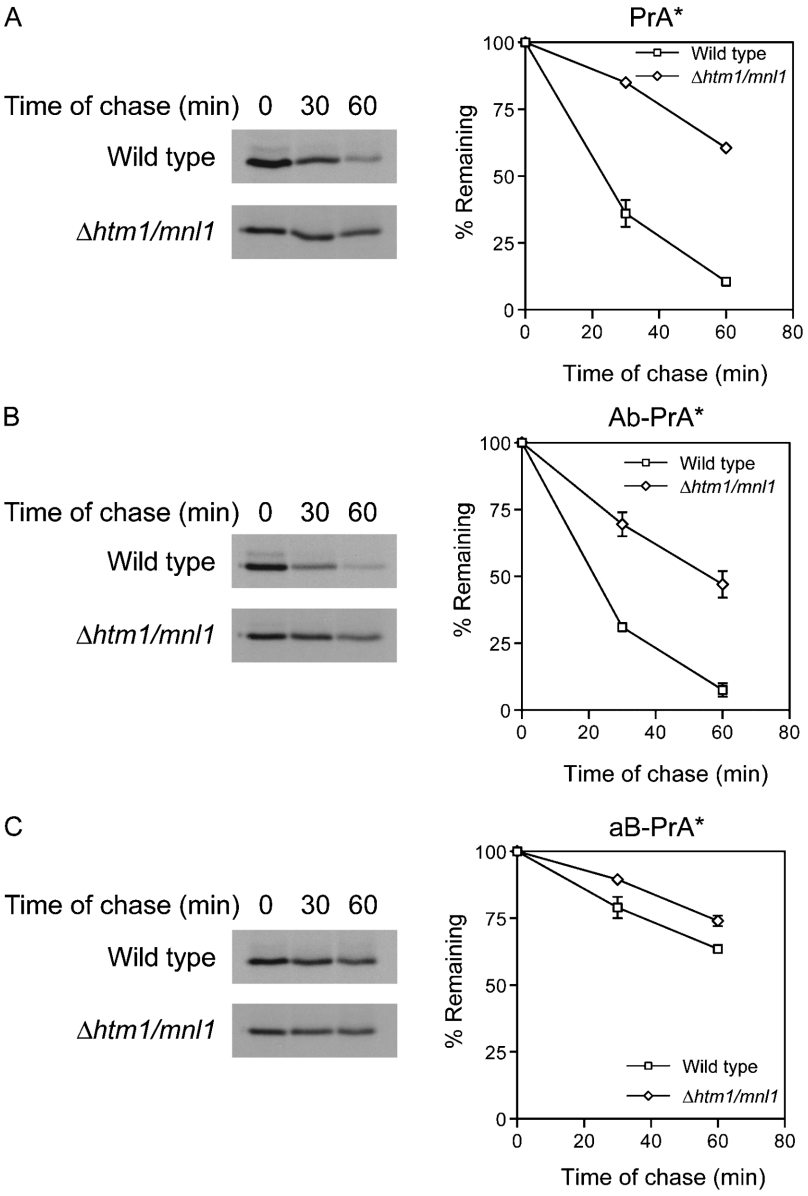

Figure 6. **The ER lectin Htm1p/Mnl1p mediates PrA* degradation through the NH_2-terminal glycan.** Wild-type or Δ*htm1/mnl1* cells expressing PrA* (A), Ab-PrA* (B), or aB-PrA* (C) were analyzed by pulse-chase analysis. The data reflect two independent experiments with the SD of the mean indicated.

ing steps. This led to the idea of a mannosidase I–dependent mechanism that provides newly synthesized proteins a fixed window of time for folding. Should the protein remain unfolded by the time the enzyme acts, a lectin receptor was hypothesized that targets $Man_8GlcNAc_2$-containing substrates to ERAD (Jakob et al., 1998).

The discovery of an ER lectin-like protein required for ERAD provided support for the proposed mechanism. Known variously as Htm1p1, Mnl1p, or EDEM, the results of four independent groups indicate that glycoprotein substrates are directed into ERAD by way of this membrane bound factor (Hosokawa et al., 2001; Jakob et al., 2001; Nakatsukasa et al., 2001; Molinari et al., 2003; Oda et al., 2003). The genetic experiments that showed the importance of $Man_8GlcNAc_2$ would also suggest that Htm1/Mnl1p plays a role in recognizing the glycan (Jakob et al., 1998). Even as the Htm1p/Mnl1p/EDEM lectin filled a gap in the model, other important questions remained. Most importantly, how can substrates be distinguished by this mechanism when properly folded glycoproteins also bear $Man_8GlcNAc_2$ (Gemmill and Trimble, 1999)? The discovery that ERAD glycan determinants are constrained to single, specific sites helps address the conundrum.

The preference for specific carbohydrates reveals new insight into how substrates are recognized by the glycan-dependent ERAD system. The simple explanation of a general positional constraint along any polypeptide chain was ruled out because respective signals were found in different positions within PrA* and CPY*. This finding, together with the inability of other glycans to substitute, suggest that a yet uncharacterized component of the signal is located somewhere along the polypeptide chain. Either of two scenarios could account for how the determinant would act. In one, it directs modifications to specific glycans much like the enzyme UDP-GlcNAc/lysosomal enzyme *N*-acetylglucosamine-1-phosphotransferase that participates in the addition of mannose 6-phosphate to prelysosomal hydrolases (Baranski et al., 1990). The modified glycan

would then serve as the ligand for the lectin receptor. Recent experiments from the Jakob group have ruled out this possibility. In agreement with substrate requirements from their previous genetic studies, direct analysis of CPY* glycans purified from wild-type cells showed that they are primarily $Man_8GlcNAc_2$ (C. Jakob, ETH Switzerland, personal communication).

If CPY*'s four N-linked carbohydrates share the same structure and yet only the COOH-terminal glycan can function to signal ERAD, it can be deduced that the signal also contains a specific peptide component. By extension, we propose a model by which the lectin binds $Man_8GlcNAc_2$ carbohydrates only in conjunction with a specific protein determinant in the unfolded state. This would explain why only single, specific glycans in CPY* and PrA* are used to target the substrates to the ERAD pathway. This simple mechanism would provide the means to distinguish folding proteins ($Man_9GlcNAc_2$/unfolded determinant: no binding), folded proteins ($Man_8GlcNAc_2$/folded determinant: no binding), and "misfolded" proteins ($Man_8GlcNAc_2$/unfolded determinant: binding). Operationally, the cell need not define "misfolded" in structural terms. Any molecule exceeding its time limit to fold is simply degraded whether it could eventually fold or not. In line with the proposed mechanism, our preliminary studies have determined a short peptide segment from CPY* that fulfills the criteria for an additional determinant. It is required for lectin-mediated ERAD and is functionally transposable to other parts of the polypeptide (unpublished data).

This study shows that determinants for ER quality control (sorting and retention) and ERAD (targeting and degradation) can be modular in their function. In the Htm1/Mnl1p arm of ERAD, single, specific carbohydrates are required to target substrates for degradation but are dispensable for their recognition and retention as misfolded proteins (e.g., CPYΔ1, ABCd-CPY*). As these substrates represent but a small fraction of all classes of misfolded proteins, our understanding of ERQC/ERAD determinants is far from complete. With the list of ERQC/ERAD factors rapidly expanding, it has never been more important to uncover the nature of their substrates to understand how aberrant proteins are sorted for degradation.

Materials and methods

Strains and antibodies

Saccharomyces cerevisiae strains used in this study are listed in Table I. Anti-HA (HA.11) was purchased from Covance Research Products. Anti-Kar2p and anti-Sec61p antibodies were provided by P. Walter (University of California, San Francisco, San Francisco, CA). Anti-PrA antiserum was a gift from B. Jones (Carnegie Mellon, University, Pittsburgh, PA).

Plasmids used in this study

CPY* and CPYΔ1 expression vectors. Plasmids used in this study are listed in Table II. Plasmids were constructed using standard cloning protocols. pDN436, containing the gene for HA epitope-tagged CPY* was described previously (Ng et al., 1990). pES57, carrying the CPYΔ1 variant that lacks amino acids 369–522 of CPY was constructed in several steps. The HA tag encoded in the reverse primer was introduced into *PRC1* as a COOH-terminal 0.6-kb fragment by PCR amplification and digested with XbaI and BglI. A 1.1-kb *PRC1* NH_2-terminal fragment was purified as AccI (end blunted by T4 DNA polymerase) to BglI digest from plasmid pTS3 (provided by T. Stevens, University of Oregon, Eugene, OR). The two fragments were ligated into pDN201 (Ng et al., 1996), creating plasmid pDN301 (CPY_{HA} driven by the strong constitutive promoter *TDH3*). A 1.1-kb fragment was released from plasmid pDN301 by digesting with HpaI and EcoRV, and was religated to create plasmid pES9 ($CPY\Delta1_{HA}$ driven by the *TDH3* promoter). Plasmid pES57 ($CPY\Delta1_{HA}$ driven by the endogenous *PRC1* promoter) was created by ligating a ClaI–NgoMIV fragment from pTS3 with an NgoMIV–SalI fragment from pES9 into the ClaI–SalI sites of pRS316.

PrA* expression vector. The PrA* expression vector, pES142 (*LEU2* marked) was constructed by ligating BamHI–ScaI and SspI–EagI fragments released from plasmid BX33 (provided by B. Jones, Carnegie

Table I. **Strains used in this study**

Strain	Genotype	Source
W303a	*MAT***a** *leu2-3, 112, his3-11, trp1-1, ura3-1, can1-100, ade2-1*	P. Walter
ESY258	*MAT***a**, pDN436, W303 background	Spear and Ng, 2003
ESY259	*MAT***a**, *cue1::TRP1*, pDN436, W303 background	Spear and Ng, 2003
ESY262	*MAT***a**, pES57, W303 background	This study
ESY263	*MAT***a**, *cue1::TRP1*, pES57, W303 background	This study
ESY410	*MAT***a**, pES95, W303 background	This study
ESY578	*MAT***a**, pES115, W303 background	This study
ESY587	*MAT***a**, pES121, W303 background	This study
ESY593	*MAT***a**, pES123, W303 background	This study
ESY611	*MAT***a**, pES129, W303 background	This study
ESY617	*MAT***a**, pES132, W303 background	This study
ESY673	*MAT***a**, *htm1::KANMX*, pDN436, W303 background	This study
ESY676	*MAT***a**, pES147, W303 background	This study
ESY677	*MAT***a**, *htm1::KANMX*, pES147, W303 background	This study
ESY661	*MAT***a**, *pep4::HIS3*, pES163, W303 background	This study
ESY665	*MAT***a**, *pep4::HIS3*, pES159, W303 background	This study
ESY669	*MAT***a**, *pep4::HIS3*, pES170, W303 background	This study
ESY708	*MAT***a**, *pep4::HIS3*, *htm1::KANMX*, pES163, W303 background	This study
ESY709	*MAT***a**, *pep4::HIS3*, *htm1::KANMX*, pES159, W303 background	This study
ESY710	*MAT***a**, *pep4::HIS3*, *htm1::KANMX*, pES170, W303 background	This study
ESY714	*MAT***a**, *leu2-3,112, his3-11::HIS3-UPRE-LacZ, trp1-1, ura3-1, can1-100, ade2-1*, pDN437	This study
ESY715	*MAT***a**, *leu2-3,112, his3-11::HIS3-UPRE-LacZ, trp1-1, ura3-1, can1-100, ade2-1*, pDN436	This study
ESY721	*MAT***a**, *leu2-3,112, his3-11::HIS3-UPRE-LacZ, trp1-1, ura3-1, can1-100, ade2-1*, pES57	This study

Table II. **Plasmids used in this study**

Plasmid	Protein	Primers used	Vector	Source
pDN436	CPY*	–	pRS315	Ng et al., 2000
pDN437	CPY	–	pRS315	Ng et al., 2000
pES115	K(9)R-CPY*	N325, N326, N335, N336, N337, N338, N435, N436, N437, N438, N439, N440	pRS315	This study
pES129	aBCD-CPY*	N491, N492	pRS315	This study
pES121	AbCD-CPY*	N493, N494	pRS315	This study
pES123	ABcD-CPY*	N495, N496	pRS315	This study
pES132	ABCd-CPY*	N441, N442	pRS315	This study
pES147	abcD-CPY*	N493, N494	pRS316	This study
pES57	CPYΔ1	–	pRS316	This study
pES95	R(3)K-CPYΔ1	N339, N340	pRS315	This study
pES163	PrA*	–	pRS316	This study
pES159	aB-PrA*	N527, N528	pRS316	This study
pES170	Ab-PrA*	N537, N538	pRS316	This study

Mellon University; Woolford et al., 1986) into the BamHI–EagI sites of pRS315 (Sikorski and Hieter, 1989). A *URA3* marked version, pES163, was created by subcloning a NotI–XhoI fragment from pES142 into pRS316.

Site-directed mutagenesis of CPY* and CPYΔ1. Site-directed mutagenesis was performed using a PCR-based approach as described previously (Ng et al., 1996). Primers used for site-directed mutagenesis are listed in Table III. All mutants were confirmed by DNA sequence analysis performed by the Penn State DNA sequence core facility.

Cell labeling and immunoprecipitations

Metabolic pulse-chase analysis and immunoprecipitations were performed as described previously (Vashist et al., 2001). Immunoprecipitates in Fig. 6 were treated with 300 U endoglysosidase H (New England Biolabs, Inc.) to aid in the quantification of hyperglycosylated PrA* species.

Indirect Immunofluorescence

Indirect immunofluorescence experiments were performed as described previously (Spear and Ng, 2003). Polyclonal rabbit anti-Kar2p and HA.11 mAb (Covance Inc.) were used as primary antibodies and Alexa Fluor 488 goat anti–rabbit and Alexa Fluor 546 goat anti–mouse (Molecular Probes, Inc.) were used as secondary antibodies. Formaldehyde-fixed cells were visualized using an Axioplan epifluorescence microscope (Carl Zeiss MicroImaging, Inc.) with a Plan-Neofluar 100× objective (1.3 NA; Carl Zeiss MicroImaging, Inc.) and immersion oil (Carl Zeiss MicroImaging, Inc.). Image acquisition was performed using a SPOT 2 cooled CCD camera using SPOT v. 3.5.5 software (Diagnostic Instruments, Inc.). Images were archived and converted to gray scale using Adobe Photoshop 4.0 (Adobe Systems).

Analysis of protein aggregates

Wild-type cells expressing CPY* (pDN436) or CPYΔ1 (pES57) were grown in synthetic complete media supplemented with 2% glucose and lacking leucine and uracil, respectively, to logarithmic phase. Control Δ*ire1* cells containing the *GAL-CPY** gene (plasmid pES67; Spear and Ng, 2003) were grown to logarithmic phase in synthetic complete containing 3% raffinose and 50 μg/ml myo-inositol. CPY* overexpression was induced for 7 h by the addition of galactose to 2%. Cells (5.0 OD_{600} units) were collected by centrifugation and washed once with ice-cold water. The cell pellet was resuspended in 500 μl TNE (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA) containing protease inhibitor cocktail (Sigma-Aldrich) and 1 mM PMSF, and transferred to a 1.5-ml screw-cap tube on ice. Zirconium beads (0.4 ml of 0.5-mm diam) were added and cells were homogenized using a vortex mixer at full speed for 1 min, followed by 1 min on ice. This cycle was repeated 8–10 times in the cold. The lysate was cleared by centrifugation at 750 *g* for 5 min and repeated. Triton X-100 was added to 1% vol/vol and incubated for 5 min at RT. A 50 μl portion (T) was saved before ultracentrifugation at 100,000 *g* for 15 min at 4°C. The supernatant (S) was removed and the pellet was resuspended in 450 μl 3% SDS, 50 mM Tris, pH 7.5, and incubated at 100°C for 5 min. Total (T), supernatant (S) and pellet (P) fractions were resolved by SDS-PAGE, transferred to nitrocellulose, and probed using specific antisera (1:10,000 anti-HA; 1:5,000 anti-Sec61) and HRP-conjugated secondary antibodies. Proteins were visualized by ECL (Pierce Chemical Co.).

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Table III. **Oligonucleotide primers used in this study**

Primer	Mutation	Sequence (5′ → 3′)
N323	R361K CPYΔ1	gccggtcttttggtaaggagccaa
N324	R364K CPYΔ1	aagaacgtttacgataacgaatgg
N325	K493R CPY*	gtaggatctgacttcaccagcgac
N326	K496R CPY*	agacacttcacctatttgagagtc
N335	K448R CPY*	acagatgaaatctctatcgcctgc
N336	K458R CPY*	aactggttgggtaatcgggcgtgg
N337	K467R CPY*	ttcgtcgtaccgccatggcaagac
N338	K476R CPY*	gaatttgcaagccaacgagtacgt
N339	R276K CPYΔ1	gtccttgtgagacaaaatttcaga
N340	R276K CPYΔ1	aaaaacttcaacttaacctccgtc
N435	K371R CPY*	cctgatatcgtaaacgtttctgcc
N436	K371R CPY*	agagattgtgaaggtggcaatttg
N437	K396R CPY*	gacgtagtcctggtttaagtagtc
N438	K396R CPY*	agagaagctgtcggtgcggaggtt
N439	K425R CPY*	catccaatcacccgcaaacaggaa
N440	K425R CPY*	agaccttaccacaccgccgtaaca
N441	N479Q CPY*	acgtacttttggcttgcaaattc
N442	N479Q CPY*	cagtggactgcttctatcaccgac
N491	N124Q CPY*	tgggtcaatgcccaggattttagg
N492	N124Q CPY*	caggtcacacagtacacgggttac
N493	N198Q CPY*	gctgttccaagagtaagggttccc
N494	N198Q CPY*	caggccaccgtgatcttccttgac
N495	N279Q CPY*	gaagtttctgtccttgtgagacaa
N496	N279Q CPY*	cagttaacctccgtcttgatcgga
N527	N107Q PrA*	agctttgtagcttgatgaagcttc
N528	N107Q PrA*	cagggtactgaatttgccattcaa
N537	N308Q PrA*	attgaagttgaaaattagat
N538	N308Q PrA*	ggctaccagttcactattgg

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