Importance of carbohydrate positioning in the recognition of mutated CPY for ER-associated degradation

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Summary

In the endoplasmic reticulum (ER), *N*-linked glycans (N-glycans) function as signals to recruit the lectin chaperones involved in protein folding, quality control and ER-associated degradation. We undertook a systematic study of the four N-glycans of mutated carboxypeptidase yscY (CPY*) to determine whether there are positional differences between the glycans in ER-associated degradation. We constructed hypoglycosylated CPY* variants containing one, two or three N-glycans in various combinations and studied their degradation kinetics. We found that the four carbohydrate chains on CPY* are not equal in their signaling function: presence of the Asn368-linked glycan is necessary and sufficient for efficient degradation of CPY*. We also analysed the involvement of the ER lectins Htm1p and Cne1p (yeast calnexin) in the glycan-based recognition process with respect to number and position of N-glycans. We observed that Htm1p function depends on the presence of N-glycans in general but that there is no positional preference for a particular glycan. Cne1p, however, is selective with respect to substrate, and participates in the quality control only of some underglycosylated variants. For cases in which both lectins are involved, Cne1p and Htm1p play competing roles in targeting the substrate for degradation: loss of Cne1p accelerates degradation, whereas loss of Htm1p stabilizes the substrate.

Key words: Protein folding, ER-associated degradation, Chaperones, Carboxypeptidase yscY, N-Glycans

Introduction

Secretory proteins enter the endoplasmic reticulum (ER) through the Sec61 translocation channel in an unfolded state. In the ER, proteins are modified and folded to acquire their functional conformations (Haigh and Johnson, 2002; Rapoport et al., 1996). Major post-translational ER modifications include *N*-linked glycosylation, disulfide-bond formation and glycosylphosphatidylinositol (GPI)-anchoring (Ellgaard and Helenius, 2003; Helenius and Aebi, 2001). The folding process in the ER is controlled by a retention-based quality control system of ER-resident chaperones, protein disulfide isomerases (PDIs) and lectins. This system selectively distinguishes between properly folded proteins and incompletely folded, potentially cell-damaging conformers. Improperly folded or orphan proteins are trapped in the ER, retrograde transported back into the cytosol and degraded by the ubiquitin-proteasome machinery (Brodsky and McCracken, 1999; Kostova and Wolf, 2003; Plemper et al., 1999; Sommer and Wolf, 1997; Wolf, 2004).

In recent years, the role played by *N*-linked glycans (N-glycans) in the recognition and retention of misfolded glycoproteins has received particular attention. Following co-translational addition to proteins, the $Glc_3Man_9GlcNAc_2$ glycan chains (where Glc represents glucose, Man represents mannose and $GlcNAc_2$ represents *N*-acetylglucosamine) are processed by the stepwise removal of the two glucose residues by α-glucosidases I and II. The resulting monoglucosylated glycan ($Glc_1Man_9GlcNac_2$) interacts with the lectins calnexin/calreticulin, which, together with Erp57p, assist in the folding process (Ellgaard and Helenius, 2003; Helenius and Aebi, 2004). Cleavage of the innermost glucose by α-glucosidase II releases the lectins and permits entry of the properly folded protein into the secretory pathway. In mammalian systems, incompletely folded proteins, by contrast, are recognized by UDP-glucose:glycoprotein glucosyltransferase, which adds back a single glucose residue, allowing binding of calnexin to initiate a new round of folding. Proteins unable to acquire their native conformation are targeted by ER α-mannosidase I, which cleaves off a mannose from the middle branch of the glycan. It is postulated that misfolded proteins bearing the trimmed $Man_8GlcNAc_2$ oligosaccharide are recognized by an ER-associated degradation (ERAD)-specific putative lectin (EDEM in mammalian cells, Htm1/Mnl1p in yeast), which marks the misfolded protein for retrograde transport into the cytosol (Jakob et al., 2001; Molinari et al., 2003; Nakatsukasa et al., 2001; Oda et al., 2003). Despite the absence of UDP-glucose:glycoprotein glucosyltransferase from the *Saccharomyces cerevisiae* genome, the basic machinery for recognition of improperly folded glycoproteins (ER α-

mannosidase I and Htm1/Mnl1p) functions similarly in this organism (Jakob et al., 2001; Jakob et al., 1998; Knop et al., 1996b; Nakatsukasa et al., 2001). Studies in yeast using a mutated carboxypeptidase yscY (CPY*) (Finger et al., 1993; Hiller et al., 1996; Knop et al., 1996a; Taxis et al., 2003) have contributed significantly to our understanding of protein quality control and degradation of malfolded secretory proteins in eukaryotic cells (Kostova and Wolf, 2003). Just like wild-type CPY, CPY* becomes glycosylated at four positions (Asn13, Asn87, Asn168, Asn368) during translocation into the ER. The N-glycans are necessary for efficient ER-associated degradation: an unglycosylated version of CPY*, although capable of eliciting the unfolded protein response (UPR), is stabilized in the ER (Knop et al., 1996b). To elucidate the distinct mechanistic steps taking place during glycoprotein quality control and to establish a relationship between the degree of glycosylation and efficiency of ER degradation, we undertook a systematic investigation into the function of carbohydrate positioning in CPY*. We constructed a series of hypoglycosylated CPY* mutants and quantified their degradation kinetics to determine whether the four carbohydrates on CPY* contribute equally to the discovery of improperly folded proteins and whether recognition by Htm1/Mnl1p is mediated by a specific glycan. We also addressed the relationship between Hmt1p and Cne1p with respect to the folding and degradation of particular CPY* variants.

Materials and Methods

Yeast strains and growth conditions

Molecular biological and genetic techniques were carried out using standard methods (Guthrie and Fink, 1991). Yeast cells were grown at 30°C to an optical density at 600 nm (OD_{600}) of 1-2 in YPD or SD medium supplemented as necessary.

All *S. cerevisiae* strains are based on W303 ΔC (*MATa*, *ura3-1*, *leu2-3,112*, *his3-11,15*, *ade2-1*, *trp1-1*, *can1-100*, *prc1Δ::LEU2*) described by Plemper et al. (Plemper et al., 1999). To disrupt *HTM1* and *CNE1* in the *Δprc1* (W303 ΔC) genetic background, we made use of the EUROSCARF Gene Deletion Bank (Frankfurt, Germany). Genomic DNA was prepared from the homozygous diploid strains BY4743*Δhtm1* (*MATa/α*, *his3Δ1/his3Δ1*, *leu2Δ0/leu2Δ0*, *lys2Δ0/LYS2*, *MET15/met15Δ0*, *ura3Δ0/ura3Δ0*, *htm1Δ::kanMX4/htm1Δ::kanMX4*) and BY4743*Δcne1* (*MATa/α*, *his3Δ1/his3Δ1*, *leu2Δ0/leu2Δ0*, *lys2Δ0/LYS2*, *MET15/met15Δ0*, *ura3Δ0/ura3Δ0*, *cne1Δ::kanMX4/cne1Δ::kanMX4*). Fragments comprising the *htm1Δ::kanMX4* and *cne1Δ::kanMX4* deletions were PCR amplified using the primer sets 5′ HTM1 (5′-GCGGTAG-GATAATCTCCTTGACGG-3′) and 3′ HTM1 (5′-GCGACCAGC-GAAATGGATGAGCTG-3′), and 5′ CNE1 (5′-GCTGAAAACCGT-GTGATGC-3′) and 3′ CNE1 (5′-GTGGTGCAATTATTGAGACC-3′), respectively. Each disrupting DNA fragment was transformed into W303 ΔC and 200 mg l^{-1} G418-resistant transformants were selected. Integration of the disrupting fragments to the correct chromosomal *HTM1* and *CNE1* loci, respectively, was verified by whole-cell PCR using a new set of primers annealing outside the region used to create the disruption. These manipulations gave rise to strains ZKy105 (*Δprc1Δhtm1*) and ZKy201 (*Δprc1Δcne1*). The *Δcne1 prc1-1* strain was obtained by crossing and sporulating *Δprc1Δcne1* to W303-1C (*prc1-1*) (Knop et al., 1996b).

Construction of CPY* glycosylation mutants

All CPY* (*prc1-1*) mutants were expressed from the centromeric plasmid pRS316 (Sikorski and Hieter, 1989). CPY* containing all four glycans was expressed from pRS316*prc1-1* (R. Hitt, Diploma thesis, Institut für Biochemie, Universität Stuttgart, 1997). For the glycosylation mutants, presence or absence of an N-glycan at a particular position (Asn13, Asn87, Asn168 or Asn368) is specified by the use of the numerals 1 and 0, respectively. The order reflects the position of a particular glycan in the primary sequence of CPY* (Fig. 1B). CPY*0011 and CPY*0001 were generated by cloning 2.8 kb *Sal*I-*Hin*dIII fragments containing the respective mutations from pRS306*prc1-1* (N. Hauser, Diploma thesis, Institut für Biochemie, Universität Stuttgart, 1995) into pRS316. Similarly, CPY*0000 was obtained by cloning a 2.6 kb *Sal*I-*Eco*RI fragment from pRS306*prc1-1*0000 (N. Hauser, Diploma thesis, Institut für Biochemie, Universität Stuttgart, 1995) into pRS316. CPY*1011 and CPY*0100 were generated as follows. Plasmids pJW21 and pJW233 (Winther et al., 1991) carrying, respectively, the wild-type *PRC1*(1011) and *PRC1*(0100) alleles were first converted into *prc1-1*(1011) and *prc1-1*(0100) by PCR mutagenesis. Then, 3.2 kb or 2.6 kb *Sal*I-*Pvu*II fragments containing *prc1-1*(1011) or *prc1-1*(0100), respectively, were ligated into the *Sal*I-*Sma*I sites of pRS316. To obtain CPY*1000 and CPY*0010, 3.2 kb or 2.6 kb *Sal*I-*Pvu*II fragments from plasmids pJW758 and pJW589 (Winther et al., 1991) carrying the wild-type *PRC1*(1000) and *PRC1*(0010) alleles, respectively, were ligated into *Sal*I/*Sma*I-digested pRS316. Then, *PRC1* was converted into *prc1-1* by replacing the *Bsu*36I-*Bgl*II fragment of *PRC1* with a corresponding fragment containing the *prc1-1* mutation G255R and Asn168, obtained from CPY*0000 (for CPY*1000) and CPY*0011 (for CPY*0010). CPY*1110 was constructed as follows: CPY*0100 was digested with *Bgl*II and *Aat*II to recover the 270 bp fragment containing Asn368. This fragment was used to replace the corresponding fragment in pRS316*prc1-1* treated with the same restriction enzymes. CPY*1100 was created similarly: CPY*1000 was digested with *Bsu*36I and *Bgl*II to recover the 650 bp fragment containing Asn168, which replaced the respective fragment in CPY*1110 also digested with *Bsu*36I and *Bgl*II. Construction of CPY*1001 and CPY*1101 followed a similar strategy: CPY*1000 and CPY*1100 were digested with *Sal*I and *Bgl*II to recover, respectively, fragments of 2.6 kb and 2.0 kb, carrying Asn13, Asn87 and Asn168. These fragments each replaced the corresponding *Sal*I-*Bgl*II fragment in pRS316*prc1-1*. Plasmids pJW21, pJW233, pJW589 and pJW758 were provided by J. R. Winther (Carlsberg Laboratory, Copenhagen, Denmark) (Winther et al., 1991).

Plasmid ZKb95 (pCTG*) expresses CTG* from the *PRC1* promoter. This construct was obtained by removal of the *TDH3* promoter sequence from pMA1 (Taxis et al., 2003) by digestion with *Sal*I and *Bsu*36I, and replacement with a similarly digested fragment containing the *PRC1* promoter originating from pRS316*prc1-1*. To obtain pCTG*0000, CPY*0000 was digested with *Sal*I and *Afl*II. The 2.3 kb fragment recovered from CPY*0000 contained all four mutated glycosylation sites and was ligated into the backbone of pCTG* digested with the same enzymes. Cloning details not included in the text are available upon request.

pRS316-P_{GAL4}-*sec61-2LEU2* was generated in three steps. First, the *URA3* fragment of a *sec61-2-URA3* fusion cloned in pRS406 was exchanged with a PCR-amplified *LEU2* gene digested with *Bcl*I and *Sac*I. Then, a *Hin*dIII-*Sac*I fragment comprising the *sec61-2-LEU2* fusion was transferred into pRS316. Finally, the *GAL4* promoter was PCR amplified and cloned into the *Hin*dIII-*Xba*I sites of pRS316*sec61-2-LEU2*, thus replacing the native *SEC61* promoter.

In vivo labeling and immunoprecipitation

Cells were grown to logarithmic phase at 30°C and pulse-chase experiments were performed as described previously (Taxis et al., 2002). Polyclonal antibodies against CPY (Finger et al., 1993) were used for immunoprecipitation of the CPY* and CTG* glycosylation mutants.

Results

The ER-associated degradation (ERAD) substrate CPY* is glycosylated at four positions (Asn13, Asn87, Asn168 and Asn368; Fig. 1A). Hypoglycosylated CPY* variants were constructed by site-directed mutagenesis of the N-glycan acceptor sites in a way that would result in minimal perturbations of the protein backbone (Thr15Ala, Asn87Ile, Asn168Gln, Asn368Gln) (Winther et al., 1991). The nucleotide changes introduced in each mutant were confirmed by sequencing of the respective plasmid DNA. Each CPY* glycosylation mutant was designated a four-digit code consisting of 1s and 0s, in which 1 indicates the presence and 0 the absence of a glycan chain at a given position (Fig. 1B). The hypoglycosylated mutants were expressed in a Δ*prc1* strain from a low-copy-number plasmid.

First, we investigated the degree of stabilization of fully unglycosylated CPY* (CPY*0000), described by Knop et al. (Knop et al., 1996b) as d4CPY*. By pulse-chase analysis, we determined that the total loss of carbohydrate resulted in considerable stabilization of CPY*0000 compared with CPY*, with >60% of the initial radioactivity remaining following a 90 minute chase (Fig. 2A). The stability of CPY*0000 was not altered in a strain deficient in the vacuolar proteases PrA and PrB (O. Fischer and D.H.W., unpublished). To determine the contribution of each glycan to this stabilization, we constructed individual CPY* variants each carrying only one glycan chain at Asn13 (CPY*1000), Asn87 (CPY*0100), Asn168 (CPY*0010) or Asn368 (CPY*0001). We found that CPY*0001 was degraded at a rate similar to fully glycosylated CPY*, whereas CPY*0100 was almost as stable as CPY*0000 (Fig. 2B). The other two mutants, CPY*1000 and CPY*0010, although less stable than CPY*0100, both exhibited considerable stabilization compared with CPY* carrying all four N-glycans (Fig. 2B). These data suggested that the glycan at Asn368 might act as a recognition determinant for CPY* degradation. Therefore, we constructed hypoglycosylated CPY* mutants bearing two or three glycans in various combinations to determine whether any hierarchy or co-operativity exists among the glycosylation sites on CPY*. We found that efficient CPY* degradation correlates with the position of glycans rather than their overall number. We determined that, whenever a glycan is present on Asn368 (CPY*0011, CPY*1001, CPY*1011, CPY*1101), the degradation kinetics of the mutant are undistinguishable from those of fully glycosylated CPY* (Fig. 3A,B). CPY*1110 and CPY*1100, which lack the Asn368 carbohydrate, by contrast, are degraded more slowly than CPY* over the 90 minute chase period (Fig. 3A,B).

Next, we examined the role of the putative ERAD-lectin Htm1p (EDEM in mammalian cells) in the degradation of CPY* with respect to number and position of N-glycans. The hypoglycosylated CPY* variants were expressed in a Δ*htm1*Δ*prc1* strain under the same conditions used for Δ*prc1*. We determined that, although lack of Htm1p partially

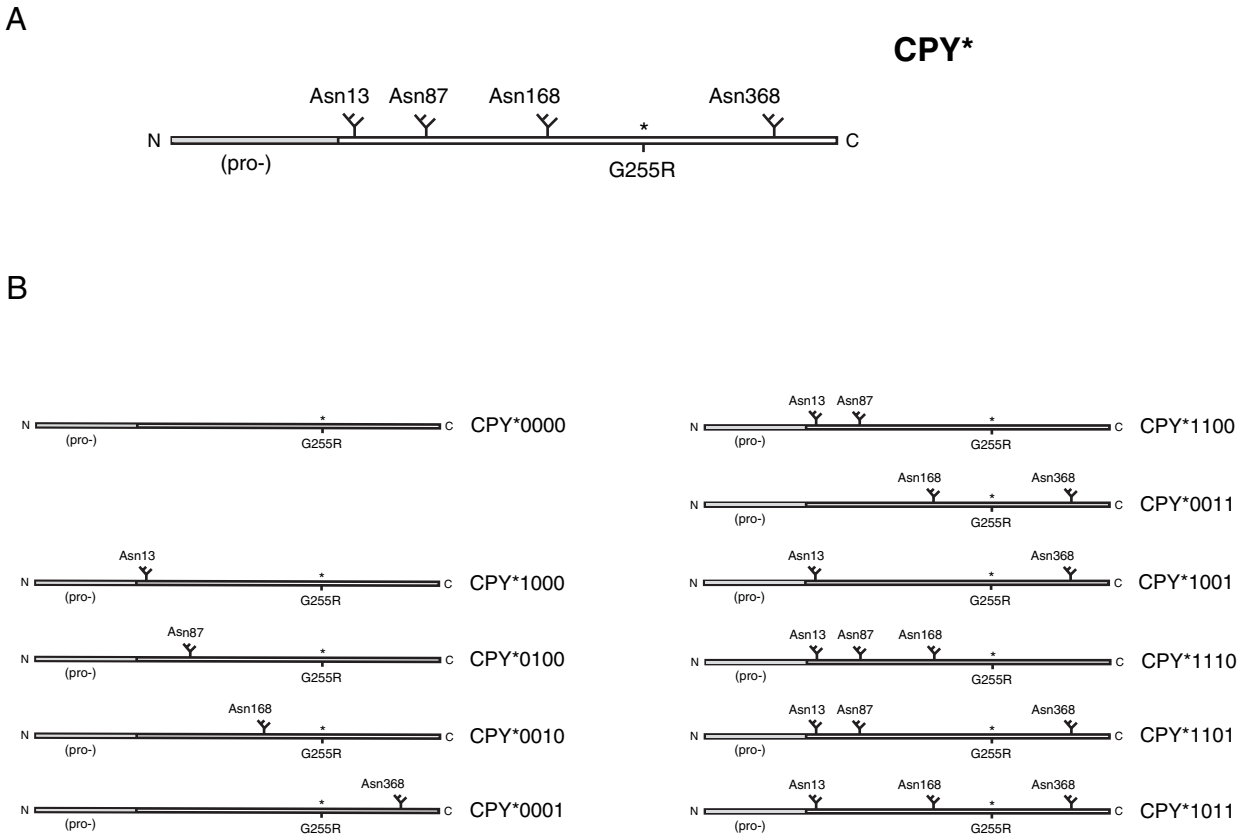

Fig. 1. Schematic representation of the hypoglycosylated CPY* variants used in this study. (A) The four glycosylation sites of CPY* are numbered according to their positions in the mature enzyme. G255R is the site of the CPY* mutation. (B) Hypoglycosylated CPY* variants are assigned a four-digit binary code, with 1 specifying the presence and 0 the absence of a glycan at a particular position (Asn13, Asn87, Asn168 or Asn368, respectively).

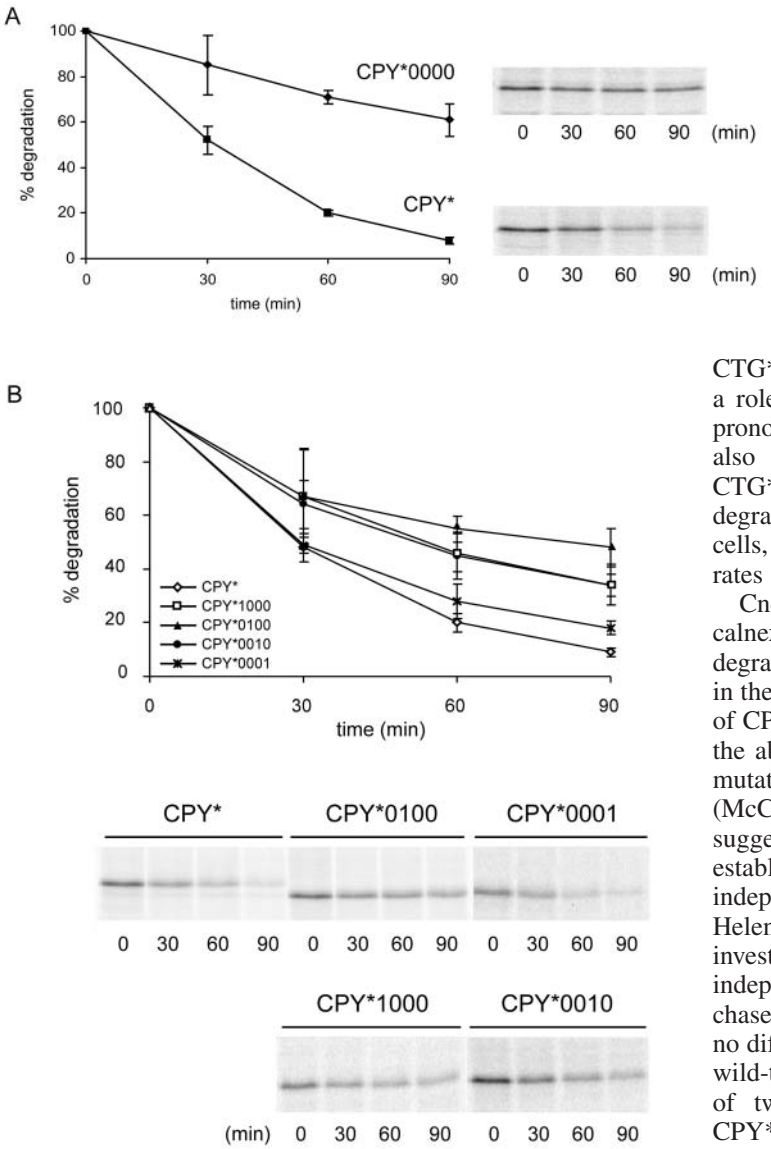

Fig. 2. Efficient degradation of CPY* depends on the position of N-glycans. (A) Unglycosylated CPY* is stabilized in wild-type cells. CPY* and CPY*0000 were expressed in a Δ*prc1* strain under identical conditions. Following pulse-chase, radiolabeled proteins were immunoprecipitated using anti-CPY antiserum. The graph represents the mean of three independent experiments. (B) Pulse-chase analysis of CPY* variants carrying only one N-glycan expressed in a Δ*prc1* strain. Graphs represent the mean of three independent experiments. A representative autoradiogram is shown for each mutant.

stabilized CPY* following a 90 minute chase, as previously reported (Jakob et al., 2001; Nakatsukasa et al., 2001), it did not lead to further stabilization of CPY*0000. This finding suggests that Htm1p action is solely carbohydrate based and independent of the misfolded protein backbone (Fig. 4A). We analysed the degradation profile of CPY* variants with one (CPY*0001), two (CPY*0011, CPY*1100) and three (CPY*1110) carbohydrate chains in Δ*prc1* and Δ*prc1*Δ*htm1* cells to explore any correlation between glycosylation sites and Htm1p action. We found that degradation of the hypoglycosylated CPY* variants in the Δ*htm1* background is delayed more than twofold compared with wild-type (*HTM1*) cells (Fig. 4B) in a manner independent of the number and position of N-glycans.

We have recently described membrane-bound CTG* as a bona-fide ERAD substrate consisting of malfolded, ER-lumenal CPY* fused to GFP in the cytoplasm via a single transmembrane domain (Taxis et al., 2003). To determine whether the carbohydrate moieties are equally important in this transmembrane substrate, we constructed the unglycosylated CTG*0000. We observed that, although carbohydrates do play a role in the degradation of CTG*, their function is not as pronounced as in CPY* (Fig. 5, CTG* and CTG*0000). We also analysed the effects of Htm1p on both CTG* and CTG*0000 degradation. In comparison to the wild type, the degradation kinetics of either substrate are altered in Δ*htm1* cells, with a broad range of variability in the initial degradation rates (Fig. 5).

Cne1p is the yeast homolog of the mammalian ER lectin calnexin. The involvement of calnexin in ER-associated degradation is still not fully understood. Calnexin is involved in the elimination of many mammalian proteins but not in that of CPY* (Fig. 6A) (Knop et al., 1996b). Interestingly, despite the absence of any *N*-linked oligosaccharides, elimination of mutated pro-α-factor depends on the presence of Cne1p (McCracken and Brodsky, 1996). Recent reports have suggested that mammalian calnexin, in addition to its well-established role as an ER lectin, also performs glycan-independent chaperone functions (Fontanini et al., 2004; Helenius and Aebi, 2004; Swanton et al., 2003). We investigated whether Cne1p is involved in the glycan-independent proportion of CPY*0000 degradation. Pulse-chase analysis of CPY* and CPY*0000 degradation revealed no difference in a Δ*cne1*Δ*prc1* background compared with the wild-type (Δ*prc1*) (Fig. 6A). However, pulse-chase analysis of two other hypoglycosylated mutants, CPY*0100 and CPY*0001, gave unexpected results: both variants were degraded more quickly and efficiently in a background lacking Cne1p (Fig. 6B,C). CPY*0001 degradation was analysed in parallel in wild-type (Δ*prc1*), Δ*cne1*Δ*prc1* and Δ*htm1*Δ*prc1* deletion strains to make comparisons possible. We found that absence of Htm1p inhibited degradation of CPY*0001, whereas absence of Cne1p increased the rate of degradation (Fig. 6C).

Discussion

We analysed the importance of carbohydrate positioning in the degradation of CPY*. We have previously reported stabilization of a CPY* variant (CPY*0000, formerly d4CPY*) containing no glycans (Knop et al., 1996b). By pulse-chase analysis, we now establish that, following a 90 minute chase, degradation of CPY*0000 is reduced by 50-60% compared with CPY* (Fig. 2A). This finding indicates that degradation of CPY* could be subdivided into a glycan-dependent and a glycan-independent pathway, as previously suggested by Jakob et al. (Jakob et al., 1998). We investigated whether any of the four glycans on CPY* is specifically needed for the glycan-dependent degradation. To determine the

influence of the number and position of glycans, we constructed CPY* variants bearing one, two or three glycans at various positions and in various combinations (Fig. 1B). Earlier studies by Winther et al. (Winther et al., 1991) indicate that intracellular transport and vacuolar sorting of hypoglycosylated CPY mutants is not affected by lack of glycosylation, although some mutants exhibit a decreased transport rate. Moreover, these glycosylation mutants, including CPY0000, retain intracellular and thermal stability, and carboxypeptidase activity, suggesting that the tertiary structure of the protein backbone is not greatly affected by the removal of glycans. Our analysis of CPY* variants bearing a single glycan (CPY*1000, CPY*0100, CPY*0010 and CPY*0001) revealed that not all glycans are equal in their signaling ability (Fig. 2B). Full glycan-dependent stabilization (50-60% compared with CPY*) was observed only in a CPY* mutant bearing a glycan at Asn87 (CPY*0100). By contrast, wild-type (CPY*) levels of degradation were almost fully restored in CPY*0001, carrying a single glycan at Asn368. CPY* species with one glycan each at Asn13 (CPY*1000) or Asn168 (CPY*0010) exhibited intermediate levels of stabilization. It is interesting that the glycan on Asn87 has been described as buried or inaccessible for digestion with EndoH (Trimble et al., 1983). Consistent with this, we observed that CPY*0100 is equivalent to CPY*0000 as far as carbohydrate-based ERAD signaling is concerned, probably because the N-glycan at Asn87 is not seen by the ER quality-control system. To clarify the importance of the carbohydrate on Asn368, we analysed CPY* mutants containing two (CPY*1100, CPY*1001 and CPY*0011) or three (CPY*1110, CPY*1011 and CPY*1101) carbohydrates. In every case, we can consistently link the degree of degradation primarily to position and then to the number of carbohydrates present on CPY* (Fig. 3A,B). Only the absence of the carbohydrate on Asn368 affects degradation (CPY*1100 and CPY*1110); absence of other glycans has no influence. Increasing the number of glycans from two (CPY*1100) to three (CPY*1110) partially compensates for the Asn368 glycan loss. These data indicate that the carbohydrate on Asn368 is the primary signaling glycan in CPY* degradation. Altogether, the consistent finding of a dominant role for the Asn368 glycan and a position-independent dosage effect of the first three glycans in the absence of Asn368 argue against the possibility that the observed effects are caused by random misfolded states of the individual glycosylation mutants. Structural analysis of the individual glycans might shed light for their preferential recognition in glycan-based ERAD signaling.

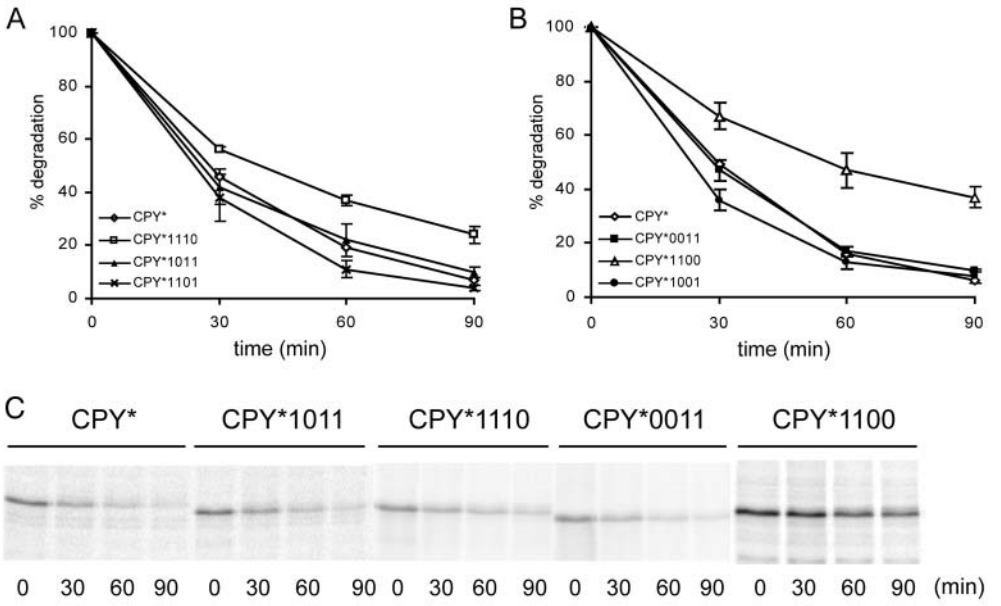

Fig. 3. Position rather than number of N-glycans determines the rate of CPY* degradation. Pulse-chase analysis of hypoglycosylated CPY* variants expressed in Δ*prc1* containing three (A) or two (B) carbohydrate chains shows that only variants lacking the Asn368 glycan (CPY*1100 and CPY*1110) are affected. Graphs represent average data from three independent experiments. (C) A representative autoradiogram is shown for each mutant.

Htm1p is a membrane-associated ER protein involved in the ER-associated degradation of glycosylated substrates such as CPY*, Pdr5*, Sst3-7p (Jakob et al., 2001; Nakatsukasa et al., 2001) and CFTR (Gnann et al., 2004). Its mode of action is not yet known. We investigated whether recognition by Htm1p is mediated solely via N-glycans or whether a protein determinant is also involved. Analysis of CPY*0000 degradation in a Δ*htm1*Δ*prc1* strain showed that unglycosylated CPY* is not further stabilized in the absence of Htm1p (Fig. 4A), suggesting that Htm1p action is carbohydrate driven and independent of a specific protein sequence or conformational feature. This is consistent with a report that degradation of lumenal, nonglycosylated BACE457ΔNOG is not affected by upregulation of EDEM, whereas normally glycosylated BACE457 and BACE457Δ exhibit significant reduction in half-life upon EDEM overexpression (Molinari et al., 2003). However, we do not exclude the possibility that contact with the protein backbone could be made during interaction of Htm1p with the substrate. Given the specific role of the Asn368 glycan, we investigated whether Htm1p interacted specifically or preferentially with the carbohydrate at this position. Therefore, we examined the degradation of various hypoglycosylated CPY* variants in the Δ*htm1*Δ*prc1* background. We did not find a correlation between Htm1p action and the number and/or position of N-glycans. Based on these data, we conclude that Htm1p does not have an affinity specific for a particular glycan on CPY* but one for glycans in general, reinforcing the idea that the Htm1p-substrate interaction does not depend on a predetermined protein conformation. About 50-60% of CPY* degradation is dependent on carbohydrate-based signaling events (Fig. 2A, Fig. 4A). However, fully glycosylated CPY*, CPY*0001 and CPY*0011 are only partially affected by lack

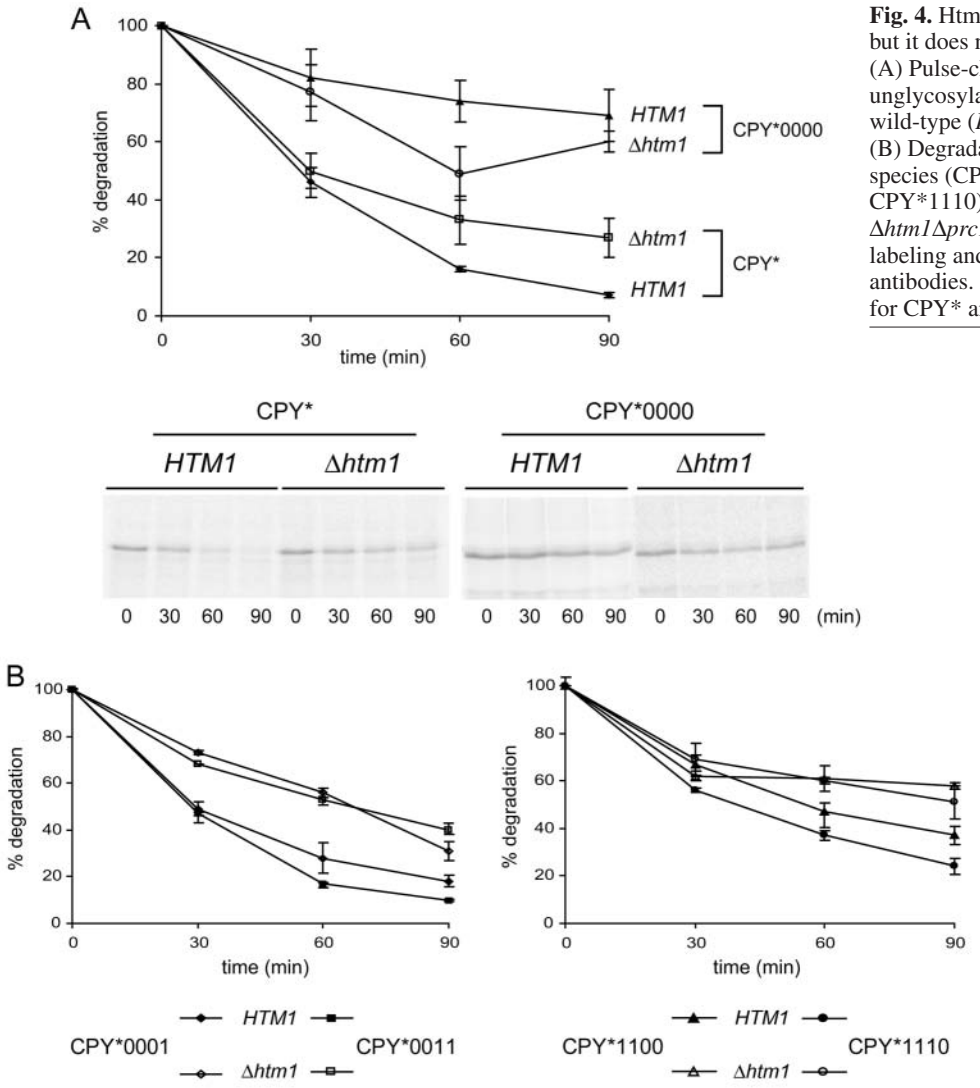

Fig. 4. Htm1p functions via the N-glycans of CPY* but it does not exhibit positional preference. (A) Pulse-chase analysis of CPY* and unglycosylated CPY*0000 expressed in isogenic wild-type (*HTM1*) and *Δhtm1Δprc1* (*Δhtm1*) strains. (B) Degradation of four hypoglycosylated CPY* species (CPY*0001, CPY*0011, CPY*1100 and CPY*1110) expressed in wild-type (*HTM1*) and *Δhtm1Δprc1* (*Δhtm1*) strains was analysed by pulse labeling and immunoprecipitation using anti-CPY antibodies. A representative autoradiogram is shown for CPY* and CPY*0000.

of Htm1p (Fig. 4A,B, compare *HTM1* and *Δhtm1*). This implies the existence of other lectin or lectin-like participants in the glycan-dependent ER quality control of CPY*. We have recently identified Yos9p (Buschhorn et al., 2004), previously involved in ER-to-Golgi transport of GPI-anchored proteins, as a factor participating in the ER-associated degradation of glycoproteins. Ongoing studies will shed light on the relationship between Yos9p and Htm1p, and the rest of the ER-lumen signal-recognition apparatus, and will also disclose the specific function of these ER-associated degradation components.

ER-associated degradation of CTG* follows the same requirements as for soluble CPY*, except for the need for the ER chaperone Kar2p and the transmembrane ER protein Der1p (Taxis et al., 2003). Membrane attachment of CPY* does not alter or restrict the protein's conformation, because a fusion protein containing wild-type CPY fused to GFP (CTG) is targeted and processed normally in the yeast vacuole and gives rise to an active enzyme (Taxis et al., 2003). Therefore, we created a glycan-free version of the membrane-bound ERAD substrate CTG* in order to analyse the Htm1p and glycan dependence of this CPY* derivative. Pulse-chase analyses showed that CTG* degradation was compromised in a *Δhtm1* strain. A comparable level (30% residual protein following a 90 minute chase, compared with the wild-type) of stabilization was obtained for CTG*0000 in both wild-type and *Δhtm1* cells, indicating that degradation of this substrate was less dependent on *N*-linked carbohydrates than is soluble CPY* (Fig. 5). If carbohydrate-based signaling events are part of the recognition process of glycosylated ERAD substrates and provide a way of recruiting the substrate to the ER membrane, it is not surprising that an already-membrane-bound substrate might not rely as heavily on this pathway. This variability in dependence on a particular recognition process by two proteins carrying the same degradation signal (CPY*) but diverging in their membrane attachment is yet another example of the differences that exist between the ERAD pathways of soluble and membrane-bound substrates. It also illustrates the flexibility of the quality-control and degradation system as a whole, in which decisions are made on an almost individual basis as substrates are encountered and disposed of in the most efficient way.

Mammalian calnexin is a well-studied ER lectin involved in protein folding and, via the calnexin cycle, ER quality control and degradation (Ellgaard and Helenius, 2003; Helenius and Aebi, 2004). At present, Cne1p's role in ERAD in yeast is not clear. The yeast homolog, Cne1p, is not involved in the degradation of CPY* but it has been implicated in the degradation of mutated, unglycosylated pro-α-factor (Knop et al., 1996b; McCracken and Brodsky, 1996). Mammalian calnexin has also been proposed to participate in the glycan-independent quality control of non-glycosylated misfolded proteolipid protein (Swanton et al., 2003) and misfolded transmembrane domains of Gas3/PMP22 (Fontanini et al., 2004). Recently, it has been reported that recombinant yeast Cne1p functions, in vitro, as a classical chaperone and a lectin in a manner similar to mammalian calnexin (Xu et al., 2004). We expressed CPY* and CPY*0000 in a *Δcne1Δprc1* strain to

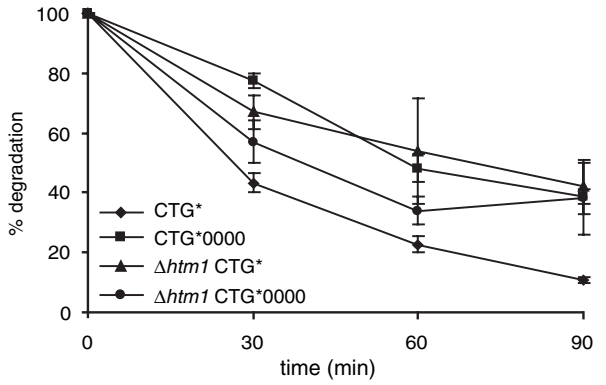

Fig. 5. CTG* degradation depends on N-glycans and Htm1p. CTG* and CTG*0000 were expressed from a low-copy-number vector in isogenic wild-type and Δ*htm1* strains. Proteins were labeled with [^{35}S]-methionine, chased at the indicated time points and immunoprecipitated using anti-CPY antibodies. Data are derived from four (CTG*, Δ*htm1*CTG*0000) or five (CTG*0000, Δ*htm1*CTG*) independent experiments.

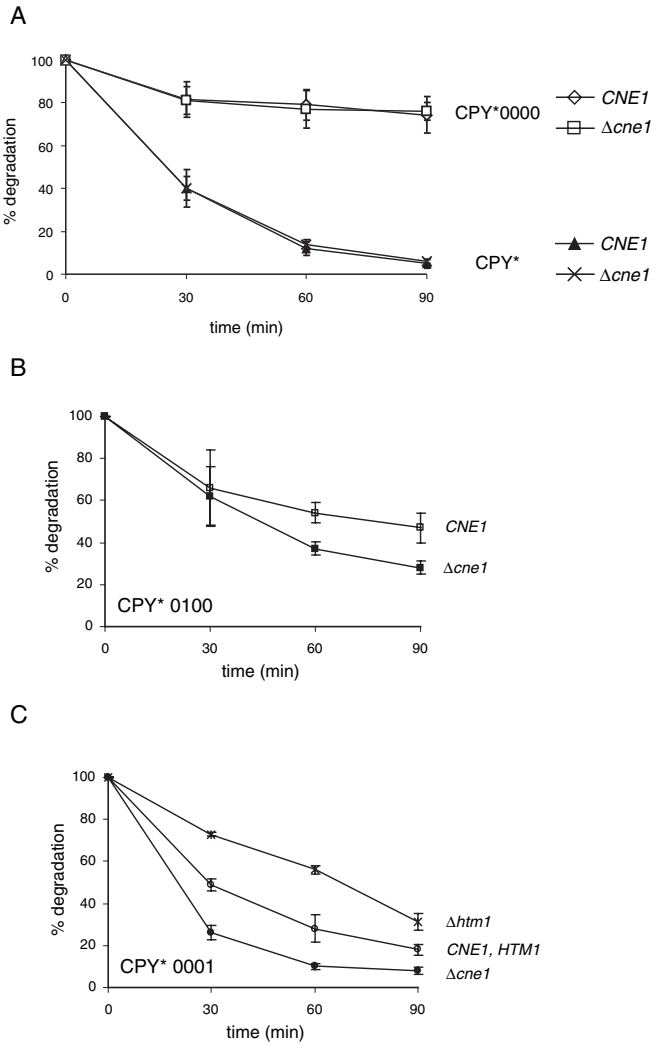

Fig. 6. The effects of Cne1p on CPY* degradation differ depending on the glycan present. (A,B) Pulse-chase analysis of CPY*, CPY*0000 (A) and CPY*0100 (B) expressed in isogenic wild-type (*CNE1*) and Δ*cne1*Δ*prc1* (Δ*cne1*) strains. (C) CPY*0001 degradation was analysed by pulse labeling in isogenic wild-type (*CNE1*, *HTM1*), Δ*cne1*Δ*prc1* (Δ*cne1*) and Δ*htm1*Δ*prc1* (Δ*htm1*) strains. Data are derived from four independent experiments.

assess whether Cne1p plays a role in the glycan-independent degradation of CPY*0000. No difference was detected in the degradation kinetics of either substrate in the presence or absence of Cne1p (Fig. 6A). We also analysed the degradation of CPY*0001 (bearing a single chain at Asn368 and degraded as efficiently as CPY*) and CPY*0100 (bearing a carbohydrate chain at Asn87 and stabilized similarly to CPY*0000) with respect to Cne1p involvement. Interestingly, degradation of both mutants was accelerated in the absence of calnexin (Fig. 6B,C). Comparison of the degradation kinetics of CPY*0001 in a wild-type background against strains missing either Htm1p or Cne1p showed that degradation is delayed in the absence of Htm1p but is enhanced in the absence of Cne1p (Fig. 6C). These data are in agreement with the finding that calnexin overexpression suppressed degradation of an α1-antitrypsin variant in mammalian cells (Oda et al., 2003). The mammalian homolog of Htm1p, EDEM, has been identified as the acceptor of terminally misfolded glycoprotein substrates (Molinari et al., 2003; Oda et al., 2003). Calnexin interacts with EDEM and this interaction might be the basis of substrate release from the calnexin cycle and transfer from one lectin (calnexin) to the other (EDEM) (Oda et al., 2003). In this scenario, Cne1p and Htm1p play opposite roles: Cne1p can be considered to be a pro-folding chaperone, with glycan-dependent or -independent functions, whereas Htm1p can be viewed as being pro-degradation, 'rescuing' glycoproteins that have fatally failed calnexin-mediated productive folding attempts. A competition between Htm1p and Cne1p for substrate is detected in the case of CPY*0001: when the pro-folding chaperone (Cne1p) is absent, Htm1p-mediated degradation proceeds more quickly, whereas, when the ERAD mediator (Htm1p) is missing, Cne1p-directed folding attempts take longer and degradation is inhibited. This is the first in vivo study connecting Cne1p to ER-associated degradation in yeast.

At the moment, we do not know the basis for substrate choice of Cne1p. Lectins show a preference for partially misfolded substrates. The misfolded structure of CPY* or CPY*0000 might be inaccessible to Cne1p, or the strong degradation signals on these substrates might permit only very weak and transient interactions with the pro-folding lectin. Introduction of a single glycan, by contrast, might result in a final structure that can be targeted by calnexin. The structural features of misfolded CPY* or any of the hypoglycosylated variants are not known. However, the consistency of the data presented and earlier studies by Winther et al. (Winther et al., 1991) suggest that the removal of glycans does not cause gross rearrangements of the peptide backbone. Furthermore, the mode of Cne1p action on CPY*0100 and CPY*0001 might be glycan dependent or independent. Finally, other components might be involved in situations in which Htm1p, but not Cne1p, action is necessary.

The picture that emerges from the growing number of studies focusing on ER quality control of a diverse range of substrates reveals the existence of a stepwise, and possibly hierarchical,

checklist for substrate recognition: membrane-bound vs soluble, glycosylated vs nonglycosylated, fatally misfolded vs slowly folding. At the same time, as expected from a system of such reach and complexity, we also find extensive flexibility to guarantee efficient disposal of unwanted and potentially harmful protein material.

This study shows that *N*-linked carbohydrate chains are not created equal in their ability to act as ER degradation signals. This finding is in agreement with our current knowledge of the variable role of N-glycans in processes such as protein folding, targeting, stability and function. Moreover, our data suggest that glycans might act as quite specific, rather than general, degradation signals. Whether a particular glycan structure is responsible for this phenomenon needs to be determined. The molecular basis of Htm1p-mediated recognition is still unknown. We find that Htm1p does not exhibit a preference for a particular glycan but rather for glycans in general. However, the existence of a 'primary recognition determinant' among glycans and the observation that Htm1p alone does not account for full glycan-dependent stabilization require the existence of other lectin-like ERAD participants. We have recently identified Yos9p as such a component and believe that more await discovery in the ER. We present the first in vivo evidence for the selective participation of Cne1p in ERAD in yeast and the competition between Cne1p and Htm1p for substrate. These results indicate that glycoprotein-ERAD in yeast follows the same principles as that, in higher eukaryotes, with the exception of the calnexin cycle. Further in-depth studies are necessary to uncover the interplay between the many components of this complicated system.

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