Degradation of Misfolded Endoplasmic Reticulum Glycoproteins in *Saccharomyces cerevisiae* Is Determined by a Specific Oligosaccharide Structure

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Abstract. In *Saccharomyces cerevisiae*, transfer of N-linked oligosaccharides is immediately followed by trimming of ER-localized glycosidases. We analyzed the influence of specific oligosaccharide structures for degradation of misfolded carboxypeptidase Y (CPY). By studying the trimming reactions in vivo, we found that removal of the terminal α1,2 glucose and the first α1,3 glucose by glucosidase I and glucosidase II respectively, occurred rapidly, whereas mannose cleavage by mannosidase I was slow. Transport and maturation of correctly folded CPY was not dependent on oligosaccharide structure. However, degradation of misfolded CPY was dependent on specific trimming steps. Degradation of misfolded CPY with N-linked oligosaccharides containing glucose residues was less efficient compared with misfolded CPY bearing the correctly trimmed $Man_8GlcNAc_2$ oligosaccharide. Reduced rate of degradation was mainly observed for misfolded CPY bearing $Man_6GlcNAc_2$, $Man_7GlcNAc_2$ and $Man_9GlcNAc_2$ oligosaccharides, whereas $Man_8GlcNAc_2$ and, to a lesser extent, $Man_5GlcNAc_2$ oligosaccharides supported degradation. These results suggest a role for the $Man_8GlcNAc_2$ oligosaccharide in the degradation process. They may indicate the presence of a $Man_8GlcNAc_2$-binding lectin involved in targeting of misfolded glycoproteins to degradation in *S. cerevisiae*.

Key words: protein degradation • endoplasmic reticulum • glycosylation • mannosidase • yeast

In *Saccharomyces cerevisiae*, as in other eukaryotes, the synthesis of asparagine-linked glycoproteins takes place in the ER. After transfer to protein, the N-linked oligosaccharide (NLO),[1] while present in the ER, is subject to trimming reactions (see Fig. 1) involving glucosidase I, glucosidase II, and mannosidase I (Herscovics and Orlean, 1993; Moremen et al., 1994; Roth, 1995). In higher eukaryotes, a specific role of the trimming intermediate $Glc_1Man_9GlcNAc_2$ oligosaccharide in the ER quality control process has been proposed (Helenius et al., 1997). An incorrectly folded glycoprotein bearing such an oligosaccharide structure is bound by specific ER resident proteins and retained in a folding competent environment. Correctly folded glycoproteins can exit the ER, enter the Golgi apparatus, and are delivered to their final destination. However, improperly folded glycoproteins are retained in the ER and are eventually degraded. In many cases, degradation occurs via the ubiquitin–proteasome pathway that requires their exit from the ER lumen to the cytosol as shown both in higher eukaryotic cells and in yeast (Jentsch and Schlenker, 1995; Bonifacino, 1996; Kopito, 1997; Sommer and Wolf, 1997; Varshavsky, 1997). For the export to the cytosol, constituents of the ER translocon play an important role (Pilon et al., 1997; Plemper et al., 1997). Additionally, ER proteins such as the chaperone Kar2p (Plemper et al., 1997), as well as the ubiquitin-conjugating proteins Ubc6p and Ubc7p, are thought to be involved in this process (Biederer et al., 1996; Hiller et al., 1996). In *Saccharomyces cerevisiae*, the proteolysis of nonglycosylated α-factor is ATP and cytosol-dependent (McCracken and Brodsky, 1996) and also mutated and therefore misfolded carboxypeptidase Y (*prc1-1*, CPY*; Wolf and Fink, 1975; Finger et al., 1993) has been shown to enter the ubiquitin–proteasome pathway (Hiller et al., 1996). The degradation of the misfolded protein appears to be glycosylation dependent, since nonglycosylated CPY* remains stable in the ER (Knop et al., 1996). Moreover, the degradation also appears to be mannosidase I–dependent

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1. *Abbreviations used in this paper*: CPY, carboxypeptidase Y; CPY*, mutated, misfolded CPY; endo H, endoglycosidase H; LLO, lipid-linked oligosaccharides; NLO, N-linked oligosaccharides.

Table I. Yeast Strains Used in This Study

Strain	Genotype	Reference
SS328	MATα *ade2-101 ura3-52 his3Δ200 lys2-801*	Vijayraghavan et al. (1989)
YG268	MATα *ade2-101 ura3-52 his3Δ200 lys2-801 Δalg3::HIS3*	Aebi et al. (1996)
YG414	MATα *ade2-101 ura3-52 his3Δ200 lys2-801 Δalg9*::KanMX	Burda et al. (1996)
YG427	MATα *ade2-101 ura3-52 his3Δ200 lys2-801 Δgls2*::KanMX	Jakob et al. (1998)
YG590	MAT**a** *ade2-101 ura3-52 his3Δ200 lys2-801 Δgls2*::KanMX *Δalg6::HIS3*	Jakob et al. (1998)
YG424	MAT**a** *ade2-101 ura3-52 his3Δ200 lys2-801 Δgls2*::KanMX *Δalg8::HIS3*	Jakob et al. (1998)
YG491	MAT**a** *ade2-101 ura3-52 his3Δ200 lys2-801 Δgls2*::KanMX *Δalg10::KanMX*	Jakob et al. (1998)
YG746	MAT**a** *ade2-101 ura3-52 his3Δ200 tyr1 Δmns1*::KanMX	This study
YG618	MATα *ade2-101 ura3-52 his3Δ200 lys2-801 prc1-1*	This study
YG619	MATα *ade2-101 ura3-52 his3Δ200 lys2-801 Δgls2*::KanMX *prc1-1*	This study
YG620	MAT**a** *ade2-101 ura3-52 his3Δ200 lys2-801 Δalg6::HIS3 prc1-1*	This study
YG623	MAT**a** *ade2-101 ura3-52 his3Δ200 lys2-801 Δgls2*::KanMX *Δalg6::HIS3 prc1-1*	This study
YG624	MAT**a** *ade2-101 ura3-52 his3Δ200 lys2-801 Δgls2*::KanMX *Δalg8::HIS3 prc1-1*	This study
YG696	MAT**a** *ade2-101 ura3-52 his3Δ200 lys2-801 Δgls2*::KanMX *Δalg10*::KanMX *prc1-1*	This study
YG796	MAT**a** *ade2-101 ura3-52 lys2-801 his3Δ200 Δalg9*::KanMX *prc1-1*	This study
YG797	MAT**a** *ade2-101 ura3-52 lys2-801 his3Δ200 Δalg3::HIS3 prc1-1*	This study
YG807	MATα *ade2-101 ura3-52 lys2-801 his3Δ200 Δalg12*::KanMX *prc1-1*	This study
YG777	MATα *ade2-101 ura3-52 lys2-801 his3Δ200 Δmns1*::KanMX *prc1-1*	This study
YG556	MATα *ura3-52 his3Δ200 lys2-801 leu2 sec18-50*	This study
YG557	MATa *ade2-101 ura3-52 his3Δ200 lys2-801 leu2 Δgls2*::KanMX *sec18-50*	This study
YG821	MATα *ade2-101 ura3-52 his3Δ200 lys2-801 prc1-1* YEp352	This study
YG822	MATa *ade2-101 ura3-52 lys2-801 his3Δ200 Δalg9*::KanMX *prc1-1* pALG9	This study
YG823	MAT**a** *ade2-101 ura3-52 lys2-801 his3Δ200 Δalg3::HIS3 prc1-1* pALG3	This study
YG824	MATα *ade2-101 ura3-52 lys2-801 his3Δ200 Δalg12*::KanMX *prc1-1* pALG12	This study

(Knop et al., 1996). Despite this, the molecular signals required for the initiation of ER glycoprotein degradation are not known.

We investigated the possible role of specific oligosaccharide structures in degradation of CPY* by genetic tailoring of the protein-bound oligosaccharide structure. We found that the $Man_8GlcNAc_2$ structure as the final product of the trimming reaction in the ER in yeast (Byrd et al., 1982) was mandatory for efficient degradation. Our results suggest that the ER α1,2-mannosidase represents a key enzyme for timing the onset of degradation. The period required for complete oligosaccharide trimming appears to be the time frame for glycoproteins to fold correctly.

Materials and Methods

Materials

Strains used are detailed in Table I. Wild-type denotes a strain with both normal biosynthesis of lipid-linked oligosaccharides and trimming of protein-bound oligosaccharides but harboring the *prc1-1* mutation. Oligonucleotides (Microsynth, Balgach, Switzerland) used for gene deletion and screenings are listed in Table II. The integrative plasmid *pRS306-prc1-1* containing the mutated CPY gene was provided by Dr. D.H. Wolf (University of Stuttgart, Germany). The antiserum against yeast hexokinase was provided by Dr. S. Schröder (Biozentrum, University of Basel, Switzerland).

Yeast Manipulations

Standard protocols were followed for growth of yeast, mating, sporulation, and ascus dissection (Guthrie and Fink, 1991). If not otherwise stated, the cells were grown at 30°C in either YPD medium (2% Bacto-Peptone, 1% Yeast extract [both from Difco Laboratories, Detroit, MI], 2% glucose) or for metabolic labeling experiments in MV medium (0.67% Yeast nitrogen base [Difco Laboratories], 2% glucose and the appropriate supplements).

Construction of Strains

Disruption of the MNS1 Locus. The *MNS1* locus ORF YJR131w (these data are available from GenBank/EMBL/DDBJ under accession number Z49631; Grondin and Herscovics, 1992) was inactivated by replacing a major part of the locus with the KanMX cassette (Wach et al., 1994). The

Table II. PCR Primers

Primer	Sequence (5′→3′)*
Knockout primers	
MNS1forKan	**aaacattgaaaaaggattctatgaagaactctgtcggtat**tcgatgaattcgagctc
MNS1revKan	**ccactatatagcacactaacctacaacgaccaacctgtgg**cgtacgctgcaggtcgac
MNS1-68u	**tgccaagaaacgaaagac**
MNS1+431L	**cggataataaaccaccta**
ALG12forKan	**aaaagagttgaataaagccattaaacaacgattcagttgaca**tcgatgaattcgagctc
ALG12revKan	**gctcgctatatattttattggaattgacgttagctattatca**cgtacgctgcaggtcgac
ALG12for	**caacctttttaccagccgg**
KanMXu	gtattgatgttggacgag
Primer for *prc1-1* screen	
CPY462u	ggatccggtcatcctttg
CPY885L	ctgagtcaatgggtcagt

*Bold face letters represent locus-specific sequence.

sequence of the kanamycin resistance gene was amplified by PCR by using the template pFA6a-KanMX4 plasmid (Wach et al., 1994) and the primers MNS1forKan and MNS1revKan (Table II). The resulting DNA was transformed into strain SS328 and the cells were selected on G418 plates (200 μg/ml). Transformants were analyzed for correct integration by whole cell PCR (Sathe et al., 1991) using KanMXu and the *MNS1*-specific MNS1-68u and MNS1 + 431L primers.

Disruption of the ALG12 Locus. The *ALG12* locus ORF YNR030w (these data are available from GenBank/EMBL/DDBJ under accession number Z71645; Lussier et al., 1997) was inactivated by the same procedure using the primers ALG12forKan and ALG12revKan for amplifying the KanMX cassette and KanMXu and ALG12for primers for verifying the correct gene deletion (Table II).

Replacement of the PRC1 Locus with prc1-1. The BglII-linearized plasmid *pRS306-prc1-1* (Knop et al., 1996) containing the mutated form (G255R) of CPY (Wolf and Fink, 1975) was integrated into the *PRC1* locus of various yeast strains, resulting in a duplication of the *PRC1* locus. Strains in which an excision of the duplication by homologous recombination had occurred were selected on 5-FOA plates and the resulting colonies screened by PCR for the *prc1-1* locus. A fragment of the *PRC1* locus was amplified by PCR using the primers CPY462u and CPY855L (Table II) giving raise to a product of 423 bp. Due to the *prc1-1* mutation, a BstXI restriction site is destroyed. Therefore, strains containing solely the *prc1-1* locus were identified by the resistance of the PCR fragment towards BstXI digestion. Western blot analysis confirmed that they only expressed mutant CPY*.

Metabolic Labeling and Immunoprecipitations

Stationary grown cells from a YPD overnight culture were inoculated in minimal medium and cultivated to an OD_{546nm} of 1.0. The cells were harvested by centrifugation, washed in minimal medium containing 0.1% glucose and then incubated in the same medium at 30°C for at least 3 h. For pulse–chase experiments, 2×10^7 cells per time point were labeled by the addition of 50 μCi [^{35}S]methionine (Tran^{35}S-label, 10 mCi/ml; ICN Pharmaceuticals) for 10 min and then chased with a 100-fold excess of nonradioactive methionine. The chase was terminated by the addition of NaN_3 (50 mM final concentration) and immediate freezing in liquid nitrogen. Protein extractions, immunoprecipitation, and SDS-PAGE were performed as described (Franzusoff et al., 1991; te Heesen et al., 1992). The dried gels were exposed and analyzed using a PhosphoImager. The kinetics of CPY* degradation were calculated by setting the counts of time point zero as 100%. For the studies of the transport kinetics of CPY in the strains with and without Mns1p, the cells were labeled for only 5 min at 26°C. The chase, protein extraction, and immunoprecipitation were performed as described above.

Assay for Degradation of CPY* by Western Analysis

Yeast strains were grown at 30°C in YPD or minimal medium containing the appropriate supplements into stationary phase. 3×10^8 cells were harvested and broken with glass beads in 50 mM Tris-HCl, pH 7.5, 1% SDS, 2 mM PMSF (Franzusoff et al., 1991; te Heesen et al., 1992). Protein extract equivalent to 7×10^6 cells was subjected to reducing SDS-PAGE, transferred to nitrocellulose membranes, and probed with specific antibodies. Binding was visualized by chemiluminescence (SuperSignal ULTRA; Pierce Chemical Co., Rockford, IL). The x-ray films were scanned and the intensity of the protein bands was determined. The antibody conjugates on the nitrocellulose membranes were stripped by treatment in 62.5 mM Tris-HCl, pH 6.7, 2% SDS, 100 mM β-mercaptoethanol at 65°C for 45 min, and the membranes were reprobed with another antibody. As an additional control for equal protein concentrations, protein contents were determined using the method of Sailer and Weissmann (1991).

Analysis of Lipid-linked and Protein-linked Oligosaccharides

The analysis of lipid- and protein-linked oligosaccharides has been described (Cacan et al., 1993; Zufferey et al., 1995; Jakob et al., 1998). For pulse–chase labeling of the oligosaccharides, typically 3×10^9 cells of a logarithmically growing culture were pelleted, washed with YP0.1D (2% Yeast extract, 1% Bactopeptone, 0.1% glucose), and resuspended in 450 μl YP0.1D containing 400 μCi 2-[^{3}H]mannose (30 Ci/mmol; ICN Pharmaceuticals). The oligosaccharides were labeled for 1 min at 26°C and the radioactivity was chased by adding nonradioactive (±)D-mannose (111 mM final concentration). At the given time points, 5×10^8 cells were removed, placed in 1 ml of CM 3:2 (chloroform/methanol 3:2 vol/vol) and mixed by vortexing. Extraction, work-up and analysis of lipid-linked oligosaccharides (LLO) and NLO was as described above. For detailed verification of oligosaccharide structure, endo H–released NLO were further digested with α1,2-specific mannosidase from *Aspergillus saitoi* (15 μU; Oxford Glycosystems, Abingdon, UK) in the supplied buffer. After the digest the NLO were separated by HPLC (see above).

Results

Trimming of Protein-bound Oligosaccharides In Vivo

To understand in more detail the role of NLO in glycoprotein degradation, we determined the kinetics of protein-bound oligosaccharide trimming in the ER in vivo. For this, two yeast strains that carry the *sec18-50* mutation were used. The *sec18-50* mutation results in a temperature-sensitive phenotype and prevents the fusion of ER-derived vesicles with the Golgi apparatus at nonpermissive temperature. No processing of protein-bound oligosaccharides by Golgi glycosyltransferases was observed in *sec18* mutant strains at nonpermissive conditions (Novick et al., 1980; Eakle et al., 1988). We performed the experiments at the permissive temperature for the *sec18-50* mutation, nevertheless, the export rate of secretory proteins to the Golgi apparatus was slower in *sec18-50* cells as compared with wild-type cells and we were able to analyze the trim-

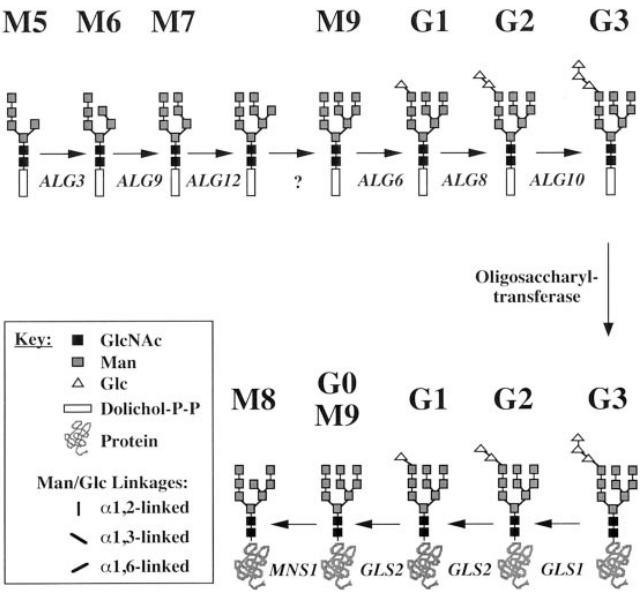

Figure 1. Biosynthesis and trimming of oligosaccharides in the ER lumen of *Saccharomyces cerevisiae*. The lipid-linked oligosaccharide precursors are synthesized at the ER membrane. Some of the involved mannosyltransferases (encoded by the *ALG3*, *ALG9*, and *ALG12* loci) and their products as well as the glucosyltransferases (encoded by the *ALG6*, *ALG8* and *ALG10* loci) and their respective products are depicted. The fully assembled $Glc_3Man_9GlcNAc_2$ oligosaccharide precursor is transferred to asparagine residues of the N-X-S/T sequence of polypeptides. This is followed by trimming involving glucosidase I (*GLS1*), glucosidase II (*GLS2*) and mannosidase I (*MNS1*) to yield the protein-bound $Man_8GlcNAc_2$ oligosaccharide.

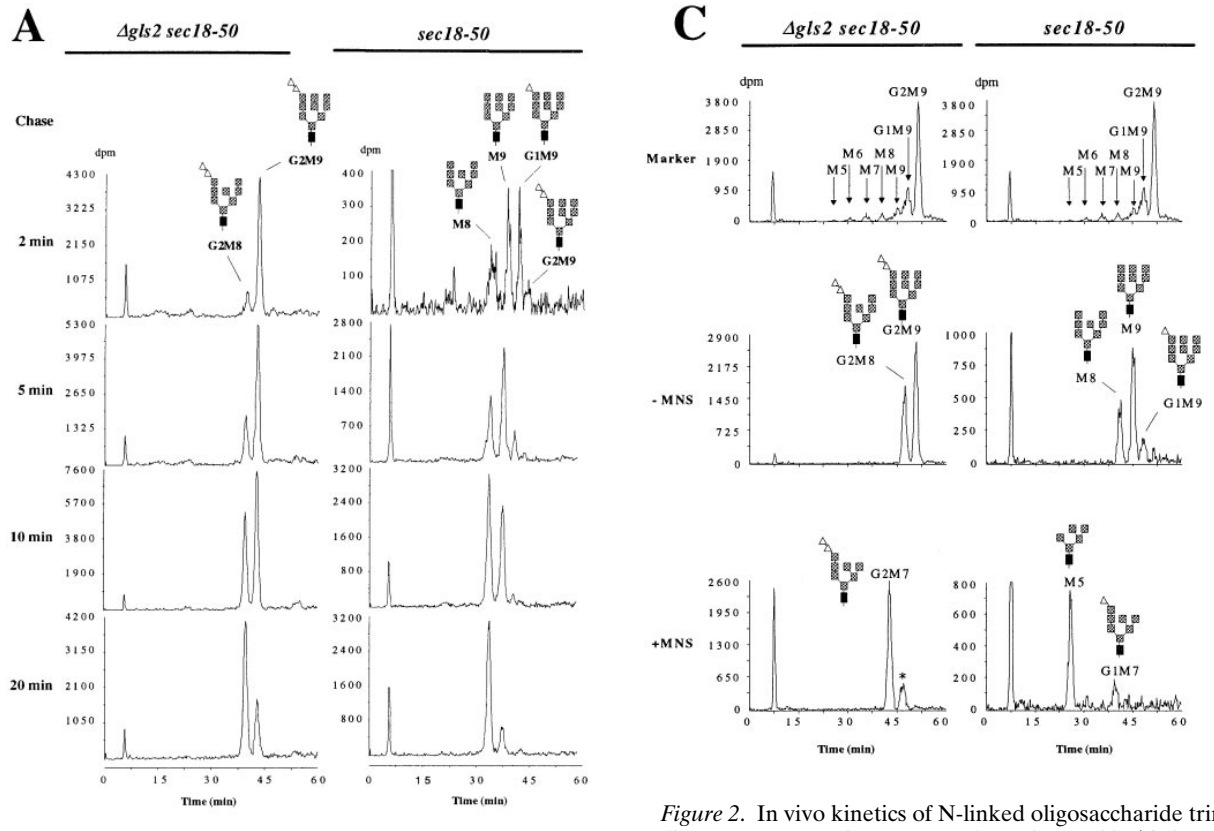

Figure 2. In vivo kinetics of N-linked oligosaccharide trimming. Two different yeast strains were used. Strain YG557 (*Δgls2 sec18-50*; *left*) carries a deletion of the glucosidase II–encoding locus and a mutation in the *SEC18* locus resulting in a temperature-sensitive protein transport from the ER to the Golgi compartment. Strain YG556 (*sec18-50*; *right*) is fully competent in oligosaccharide trimming in the ER. Cells were labeled with a 1-min pulse of ^{3}H-mannose, followed by a chase with an excess of unlabeled mannose. (*A*) Protein-bound oligosaccharides were isolated at the time indicated after initiation of the chase and analyzed by HPLC. The elution of radioactivity was monitored. The proposed oligosaccharide structure is given for the individual peaks. For explanation of the symbols, see Fig. 1. (*B*) Kinetics of maturation of N-linked oligosaccharides. The HPLC profiles shown in *A* were quantified using the FLO-ONE software (version 3.6; Packard Instrument Co., Meriden, CT). The amount of radioactivity present in each individual peak was determined, expressed as a percentage of total radioactivity present and plotted against time. The value for the G2M8 and the M8 oligosaccharide, respectively, was corrected for the absence of one mannose residue. (*C*) Verification of NLO species by exo-α1,2-mannosidase digestion. The NLO obtained from 5-min (*sec18-50*) and 10-min (*Δgls2 sec18-50*) chase periods (*A*) were treated with exo-α1,2-mannosidase from *A. saitoi* and separated by HPLC. Endo H–treated LLO, obtained from a *Δalg10 Δgls2* (G2) strain, was used as marker for the oligosaccharides. The proposed oligosaccharide structure is given for the individual peaks. For explanation of the symbols, see Fig. 1. *−MNS*, NLO without exo-α1,2-mannosidase treatment; *+MNS*, NLO after *A. saitoi* exo-α1,2-mannosidase cleavage.

ming of the NLO in the ER. One of the strains carried in addition a deletion in the *GLS2* locus inactivating glucosidase II. No growth phenotype was associated with the *Δgls2* mutation. Cells were labeled with ^{3}H-mannose for 1 min at 26°C and the radioactivity was chased by adding an excess of nonradioactive (±)D-mannose. At the given time points, the chase was terminated, NLO were released from protein by endoglycosidase H (endo H), and then analyzed by HPLC (see Materials and Methods section).

In the *Δgls2* strain, only two trimming events occur in the ER: the removal of the terminal α1,2-linked glucose residue by glucosidase I and the cleavage of an α1,2-linked mannose residue by mannosidase I. As expected, a protein-bound oligosaccharide with the putative structure $Glc_2Man_9GlcNAc_2$ (G2M9)[2] was found, which was slowly converted to $Glc_2Man_8GlcNAc_2$ (G2M8) with a half-life of ~10 min (Fig. 2, *A* and *B*, *left*). We were unable to detect protein-bound $Glc_3Man_9GlcNAc_2$.

We analyzed the structure of the different oligosaccharides in more detail. The endo H–released $Glc_2Man_9GlcNAc_2$ NLO comigrated with the $Glc_2Man_9GlcNAc_2$ oligosaccha-

2. Since the protein-bound oligosaccharides were released by Endo H cleavage, they are lacking one GlcNAc residue. For clarity, however, the NLO structure as found on protein is denoted.

ride obtained from endo H–treated LLO of a *Δalg10* strain (Fig. 2 *C*). This strain accumulates lipid-linked $Glc_2Man_9GlcNAc_2$ due to the inactivation of the α1,2 glucosyltransferase (Burda and Aebi, 1998). Digestion of the two major protein-derived oligosaccharides from a *Δgls2 sec18-50* strain by exo-α1,2-mannosidase (from *Aspergillus saitoi*) converted both of them to a single species (Fig. 2 *C*). Therefore, the two oligosaccharides found on protein in a *Δgls2 sec18* strain differed by one α1,2-linked mannose. This observation, the mobility of the exo-α1,2-mannosidase digestion product and the comigration of one oligosaccharide with the $Glc_2Man_9GlcNAc_2$ marker showed that protein bound $Glc_2Man_9GlcNAc_2$ and the corresponding mannosidase product $Glc_2Man_8GlcNAc_2$ were present in the *Δgls2 sec18-50* strain. The minor peak observed after exo-α1,2-mannosidase digestion (Fig. 2 *C*, *left*) was possibly due to incomplete digestion, since the presence of glucose residues on oligosaccharides reduced the efficiency of the enzyme (Burda, P., unpublished data). Taken together, the results confirmed that release of the α1,2-linked glucose from protein-bound oligosaccharide by glucosidase I was a rapid process in the *Δgls2* strain. However, trimming of the oligosaccharide by endogenous α1,2-mannosidase (Mns1p) occurred much slower.

When we analyzed the NLO processing in a strain fully competent for trimming (*sec18-50*; Fig. 2, *right*), we were again unable to detect the complete protein-bound $Glc_3Man_9GlcNAc_2$ oligosaccharide, even in preparations obtained shortly after the pulse (Fig. 2, *A* and *B*, *right*). The largest oligosaccharide, detected after 2 min of chase (1-min pulse), comigrated with the $Glc_2Man_9GlcNAc_2$ oligosaccharide but represented a minor fraction (<10%) of the total NLO. In contrast, significant amounts of $Glc_1Man_9GlcNAc_2$ were detected at this time point (the structural analysis of this oligosaccharide is described below). However, from this oligosaccharide one or two hexose units were rapidly trimmed. To determine whether this trimming was due to glucosidase II or mannosidase I activity, we analyzed the NLO preparation from the 5-min chase point by digestion with the exo-α1,2-mannosidase (Fig. 2 *C*). This preparation contained small amounts of oligosaccharides comigrating with the $Glc_1Man_9GlcNAc_2$ standard and significant levels of oligosaccharides that migrated as expected for $Man_9GlcNAc_2$ and $Man_8GlcNAc_2$ oligosaccharide. Indeed, digestion by exo-α1,2-mannosidase revealed that only the $Glc_1Man_9GlcNAc_2$ oligosaccharides contained a protective glucose residue and was converted to $Glc_1Man_7GlcNAc_2$, whereas the majority of the oligosaccharides was trimmed to $Man_5GlcNAc_2$. These results showed that the $Glc_1Man_9GlcNAc_2$ protein-bound oligosaccharide was converted primarily to $Man_9GlcNAc_2$ and that the peak representing this oligosaccharide contained no significant amounts of mannosidase I–trimmed, monoglucosylated oligosaccharide.

The analysis of the structure of protein-bound oligosaccharide species as well as their temporal appearance showed that the removal of the terminal α1,2-glucose on protein-bound oligosaccharides by glucosidase I was a rapid process in vivo. Similarly, since we observed only small amounts of diglucosylated oligosaccharides (Fig. 2, *A* and *B*, 2-min chase), the hydrolysis of the first α1,3-linked glucose by glucosidase II was a rapid process. We concluded that under our experimental conditions, the monoglucosylated oligosaccharide $Glc_1Man_9GlcNAc_2$ was converted to the $Man_9GlcNAc_2$ oligosaccharide with a half-life of ~2 min and that this occurred before processing by mannosidase I, which was a relatively slow process (half-life 10 min). Evidently, removal of glucose-linked residues was not a prerequisite for mannosidase I action because mannose hydrolysis occurred with approximately the same kinetics in both glucosidase II–proficient or –deficient strains (Fig. 2 *B*).

Role of N-linked Oligosaccharides in Glycoprotein Processing

Removal of a mannose residue by α1,2-mannosidase concludes the trimming of NLO in the ER of *S. cerevisiae* (Byrd et al., 1982). Since this cleavage occurred at a slow rate, we speculated that it represents a rate-limiting step and thus is important for efficient glycoprotein transport and maturation. Therefore, we analyzed this aspect in detail by studying the processing of vacuolar proteinase CPY. In the ER, CPY receives four N-linked oligosaccharides (p1CPY, glycosylated proCPY, 67 kD) that are modified in the Golgi apparatus (p2CPY, 69 kD). Upon reaching the vacuole, CPY maturates by proteolytic cleavage of the propeptide (mCPY, 63 kD). In a pulse–chase experiment, we compared the transport rates of CPY, from ER to Golgi and to vacuole in wild-type and *Δmns1* strains lacking α1,2-mannosidase activity. We observed that CPY was transported at the same rates (Fig. 3). This demonstrated that trimming of NLO by mannosidase I was not required for export of glycosylated CPY to the Golgi apparatus and the transport to the vacuole.

Role of N-linked Oligosaccharides in Degradation of Misfolded CPY

Previous studies have shown that a specifically mutated form of vacuolar proteinase CPY (CPY*) is retained in the ER and degraded by the proteasome (Hiller et al., 1996) in an oligosaccharide-dependent manner (Knop et al., 1996). Moreover, deletion of the *MNS1* locus affects the degradation of CPY*. These observations suggested that trimming of the oligosaccharides is required for processing

Figure 3. Maturation of CPY in wild-type and mannosidase-deficient mutant cells. Logarithmically growing cells were pulsed with ^{35}S-methionine for 5 min at 26°C and then chased for 30 min. The cells were broken, CPY immunoprecipitated, separated by SDS-PAGE and visualized by autoradiography. The processing of CPY in wild-type cells (wt, strain SS328) is shown in the top panel, that of mannosidase-deficient cells (mns1, strain YG746) in the bottom panel. The positions of the ER-modified form of proCPY (*p1CPY*); of the Golgi-modified form of proCPY (*p2CPY*) and of the vacuolar mature CPY (*mCPY*) are indicated.

Table III. Structure of N-linked Oligosaccharides in Mutant Yeast Strains

Genotype	NLO after transfer	Final NLO structure*		Underglycosylation	References
Δgls2	$Glc_3Man_9GlcNAc_2$	$Glc_2Man_8GlcNAc_2$	(G2)	No	This study
Δalg10 Δgls2	$Glc_2Man_9GlcNAc_2$	$Glc_2Man_8GlcNAc_2$	(G2)	Yes	Jakob et al. (1998)
Δalg8 Δgls2	$Glc_1Man_9GlcNAc_2$	$Glc_1Man_8GlcNAc_2$	(G1)	Yes	Jakob et al. (1998)
Δalg6 Δgls2	$Man_9GlcNAc_2$	$Man_8GlcNAc_2$	(G0)	Yes	Jakob et al. (1998)
Δmns1	$Glc_3Man_9GlcNAc_2$	$Man_9GlcNAc_2$	(M9)	No	Puccia et al. (1993)
Wild-type‡	$Glc_3Man_9GlcNAc_2$	$Man_8GlcNAc_2$	(M8)	No	Jakob et al. (1998), Verostek et al. (1991, 1993), Ziegler and Trimble (1991)
Δalg6	$Man_9GlcNAc_2$	$Man_8GlcNAc_2$	(M8)	Yes	Reiss et al. (1996)
Δalg12	$Man_7GlcNAc_2$	$Man_7GlcNAc_2$	(M7)	Yes	Burda, P., C.A. Jakob, J. Beinhauer, J.H. Hegemann, and M. Aebi, manuscript submitted for publication
Δalg9	$Man_6GlcNAc_2$	$Man_6GlcNAc_2$	(M6)	Yes	Burda et al. (1996)
Δalg3	$Man_5GlcNAc_2$	$Man_5GlcNAc_2$	(M5)	Yes	Aebi et al. (1996)

*Oligosaccharide structure on glycoproteins (NLO) after endogenous glycosidase trimming. The term in parenthesis indicates the abbreviations used throughout the figures.
‡The term wild-type is used with regard to oligosaccharide biosynthesis and trimming.

of misfolded CPY in the ER. To precisely define the NLO moieties important for proteasome-dependent degradation of CPY*, we generated yeast strains containing CPY* carrying defined NLO structures on glycoproteins (for details, see Fig. 1 and Table III).

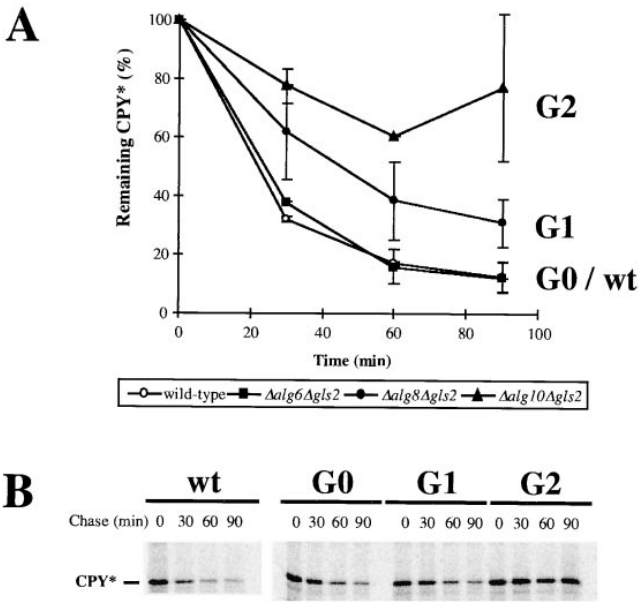

Figure 4. Degradation of misfolded CPY with glucosylated oligosaccharides. (*A*) Cells were labeled with a short pulse with ^{35}S-labeled methionine, chased with an excess of unlabeled methionine, lyzed at the time indicated after the chase and CPY* was precipitated using CPY-specific antiserum. Precipitated CPY* was resolved by SDS-PAGE, autoradiography was performed using a PhosphoImager System and the level of CPY* was determined. The CPY* level at initiation of the chase was taken as 100%. Degradation rates of misfolded CPY were calculated from two independent experiments. (*B*) Autoradiography of immunoprecipitated misfolded CPY resolved by SDS-PAGE. The time of the chase is indicated above the lanes. The following strains indicated on the left side of *A* and above the different autoradiographs (*B*) were used in the analysis: wt: *prc1-1*, YG618; G0: *Δalg6 Δgls2 prc1-1*, YG623; G1: *Δalg8 Δgls2 prc1-1*, YG624; G2: *Δalg10 Δgls2 prc1-1*, YG625.

In a first step, we investigated the influence of glucose residues of oligosaccharides on CPY* degradation (Fig. 4). For that purpose, we constructed mutant strains that produced the following NLO structures: $Glc_2Man_8GlcNAc_2$ (G2; *Δalg10 Δgls2*), $Glc_1Man_8GlcNAc_2$ (G1; *Δalg8 Δgls2*), $Man_8GlcNAc_2$ (G0; wild-type and *Δalg6 Δgls2*). It is important to note that α1,2-mannosidase can act on glucosylated oligosaccharides in vivo (Fig. 2). When we analyzed the processing of CPY* by pulse–chase experiments, a differential degradation of CPY* was observed depending on the number of glucose residues present on the NLO (Fig. 4). The G2 CPY* (*Δalg10 Δgls2*) was degraded at the slowest rate, whereas the G0 CPY* was degraded at the same rate as CPY* in a strain with normal oligosaccharide biosynthesis and trimming (Fig. 4, wild-type). By Western blot analysis, another G2 CPY*, *Δgls2*, behaved similarly as G2 CPY* (*Δalg10 Δgls2*, see below and Fig. 5 *C*). For the G1 CPY*, we observed an intermediate degradation rate (Fig. 4). From the initial degradation rates (time points 30 and 60 min), we calculated the half-life of CPY* in the various strains (Table IV). In the wild-type and the G0 cells it was 21 min, similar to published data (Hiller et al., 1996). In comparison, the half-life of CPY* in the G1 cells was 43 min and in the G2 cells 82 min under our experimental conditions. Thus, the larger the number of glucose residues the NLO of CPY* contained, the slower its degradation rate.

Next, we determined whether mannose residues of the NLO influenced degradation of CPY*. The core mannose structure of LLO is synthesized by a set of sequentially acting ER mannosyltransferases (Alg3p, Alg9p, Alg12p; see Fig. 1, Orlean, 1997). Due to the fact that incompletely assembled oligosaccharides can be transferred to protein, albeit with a reduced efficiency, we were able to obtain yeast strains with incomplete, but defined NLO structures, namely $Man_5GlcNAc_2$ (M5, *Δalg3*; Aebi et al., 1996), $Man_6GlcNAc_2$ (M6, *Δalg9*; Burda et al., 1996), $Man_7GlcNAc_2$ (M7, *Δalg12*, Burda and Aebi, manuscript in preparation), $Man_8GlcNAc_2$ (M8, wild-type with respect to NLO; M8, *Δalg6*; Reiss et al., 1996), and $Man_9GlcNAc_2$ (M9, *Δmns1*; Puccia et al., 1993; see also Table III).

When the half-lives of mutant CPY* bearing various oligosaccharide structures were analyzed, we found that CPY* containing $Man_8GlcNAc_2$ NLO was rapidly de-

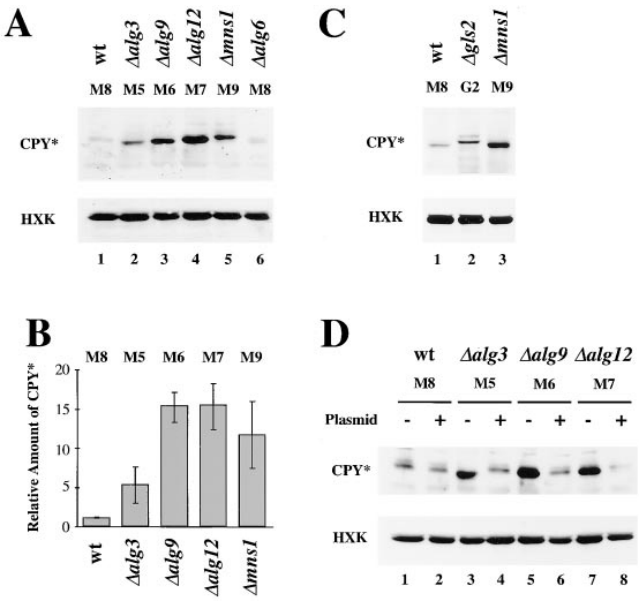

Figure 5. N-linked oligosaccharide structure affects degradation of misfolded CPY. Yeast cells were grown into stationary phase (*A* and *C*, cells grown in YPD; *D*, growth in minimal medium), equal cell numbers harvested and their proteins were extracted, separated by SDS-PAGE, transferred to nitrocellulose and probed with antiserum for CPY. The membranes were stripped and reprobed with antiserum directed against hexokinase (*HXK*) which served as internal standard. All strains analyzed in this figure contained the *prc1-1* mutation. The relevant genotype of the strains analyzed is given above each lane or below each column, respectively. Strains: YG618 (wt, wild-type, lanes *A1*, *C1*, and *D1*); YG797 (*Δalg3*, lanes *A2* and *D3*); YG796 (*Δalg9*, lanes *A3* and *D5*); YG807 (*Δalg12*, lanes *A4* and *D7*); YG777 (*Δmns1*, lanes *A5* and *C3*); YG620 (*Δalg6*, lane *A6*); YG619 (*Δgls2*, lane *C2*); YG821 (wild-type + YEp352, lane *D2*); YG822 (*Δalg3* + pALG3, lane *D4*); YG823 (*Δalg9* + pALG9, lane *D6*); YG824 (*Δalg12* + pALG12, lane *D8*); Abbreviations: *wt*, wild-type; *CPY**, misfolded CPY; *HXK*, hexokinase. (*A*) Degradation of misfolded CPY is dependent on core mannose residues. The position of malfolded CPY* is indicated. The mobility of CPY* in SDS-PAGE varied due to the different oligosaccharide structures. (*B*) Quantification of degradation of misfolded CPY. Protein amounts (mean ± SD) of three independent Western blot experiments as shown in *A* were quantified by using a CCD camera and the Wincam V2.1 software (Cybertech, Berlin, Germany) and normalized to the hexokinase levels. The amount of misfolded CPY of the wild-type strain was set as 1.0. (*C*) Degradation of misfolded CPY is reduced in *Δgls2* and *Δmns1* cells but not in wild-type cells. The mobility of CPY* in SDS-PAGE varied due to the different oligosaccharide structures. (*D*) Degradation of CPY* is dependent on the altered oligosaccharide structures. Wild-type and mutant strains with altered oligosaccharide biosynthesis (indicated above the lanes) with (+) or without (−) the corresponding complementing plasmid were analyzed for degradation of CPY* by Western blot analysis. The position of CPY* is indicated. Hexokinase served as a control protein.

graded (Fig. 5 *A*, lanes *1* and *6* and *B*). The $Man_5GlcNAc_2$ CPY* (Fig. 5, *A* lane *2* and *B*) was degraded at a reduced rate compared with the $Man_8GlcNAc_2$ CPY*. However, a greatly reduced rate of CPY* degradation was observed when the NLO were of $Man_6GlcNAc_2$ (Fig. 5 *A*, lane *3* and *B*), $Man_7GlcNAc_2$ (Fig. 5 *A*, lane *4* and *B*), and $Man_9GlcNAc_2$ (Fig. 5 *A*, lane *5* and *B*) structures. By Western blot analysis, CPY* with diglucosylated oligosaccharides (*Δgls2*) was also degraded at a slower rate than $Man_8GlcNAc_2$ CPY* (Fig. 5 *C*, compare with Fig. 4). However, the stabilization was not as prominent as for $Man_9GlcNAc_2$ CPY* (*Δmns1*; Fig. 5 *C*). Taken together, our results showed that efficient degradation of CPY* was dependent on the NLO structure. $Man_8GlcNAc_2$ CPY* was rapidly degraded, whereas the trimming intermediate $Man_9GlcNAc_2$ CPY* and the incompletely assembled $Man_{6-7}GlcNAc_2$ CPY* were inefficiently degraded.

To demonstrate that the differences in degradation rate of CPY* were due to the altered oligosaccharide structure, we transformed the yeast cells with plasmids complementing the deleted gene loci. The *Δalg3*, *Δalg9*, and *Δalg12* cells were transformed with plasmids containing the *ALG3* (pALG3), *ALG9* (pALG9) and *ALG12* (pALG12) locus. Further, the CPY* wild-type strain was transformed with the vector plasmid (YEp352). The NLO structure-dependent CPY* degradation phenotype could be reverted by complementing the deleted *alg* gene loci with the appropriate plasmids (Fig. 5 *D*, lanes *3–8*), but the degradation in wild-type cells was not influenced by the transformation with the empty plasmid (Fig. 5 *D*, lanes *1* and *2*).

Incomplete assembly of the LLO leads to a reduced oligosaccharide transfer to protein by the oligosaccharyltransferase (Sharma et al., 1981; Silberstein and Gilmore, 1996), which is then apparent by underglycosylation of glycoproteins (Huffaker and Robbins, 1981, 1983; Stagljar et al., 1994; te Heesen et al., 1994; Burda et al., 1996; Burda and Aebi, 1998). The equal degradation of $Man_8GlcNAc_2$ CPY* in glycosylation wild-type cells and in *Δalg6* cells (Fig. 5 *A*, lanes *1* and *6*) indicated that the degradation was not due to impaired synthesis of LLO, but rather to the altered oligosaccharide structure.

Also, in contrast to wild-type CPY, CPY* was efficiently glycosylated in both *Δalg3* ($Man_5GlcNAc_2$) and *Δalg9* ($Man_6GlcNAc_2$) cells under the conditions used. We observed a single CPY* glycoform in *Δalg3 prc1-1* and *Δalg9 prc1-1* cells (Fig. 6, lanes *1* and *3*), whereas correctly folded CPY was incompletely glycosylated in *Δalg3* and *Δalg9* cells (Fig. 6, lanes *7* and *9*). This hypoglycosylation of CPY was visualized by the distinct bands upon Western blot analysis representing different glycoforms of this protein (Stagljar et al., 1994). In addition, this experiment showed that such forms of CPY* did not reach the vacuole because endo H treatment resulted in the deglycosylated pro-form of CPY* in *Δalg9* cells (Fig. 6, lanes *3* and *4*, compare with lanes *9* and *10*). As expected, the oligosaccharides in *Δalg3* cells were resistant to endo H digestion,

Table IV. Calculated Half-life of Mutated CPY

Genotype of strains		Half-life*
prc1-1 (wild-type)		20.8 ± 3.7
Δalg6 Δgls2 prc1-1	(G0)	21.9 ± 1.0
Δalg8 Δgls2 prc1-1	(G1)	43.3 ± 0.3
Δalg10 Δgls2 prc1-1	(G2)	82.3 ± 1.1

*The values (mean ± SD) represent the calculated half-lives from time points 0, 30, and 60 min and were derived from the experiment shown in Fig. 2.

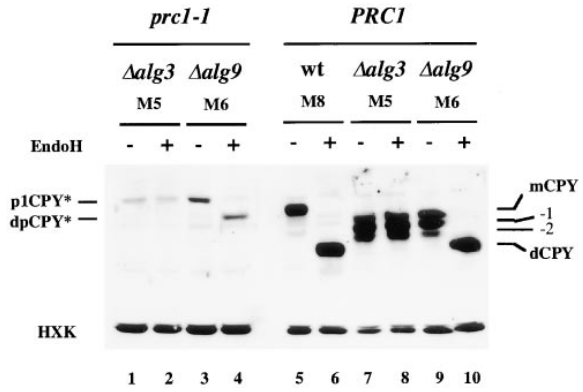

Figure 6. Processing of wild-type CPY occurs independent of NLO structure. Yeast cells were grown into stationary phase, equal cell numbers harvested and their proteins extracted. Extracts were analyzed before (−) or after endo H (+) treatment. The proteins were separated by SDS-PAGE, transferred to nitrocellulose and probed with antiserum to detect CPY. After exposure, membranes were stripped and reprobed with antiserum directed against hexokinase (*HXK*). *Δalg3* strains (lanes *1*, *2*, *7*, and *8*) and *Δalg9* strains (lanes *3*, *4*, *9*, and *10*) carrying a mutant *prc1-1* locus expressing CPY* (lanes *1–4*) or a wild-type *PRC1* locus (lanes *5–10*) were analyzed. The positions of the ER form p1CPY* and deglycosylated proCPY* (dpCPY*) are shown at the left (lanes *1–4*). Mature, wild-type CPY (mCPY), lacking one (−1) or two (−2) oligosaccharides and deglycosylated, mature CPY (dCPY) are indicated on the right side (lanes *5–10*). The following strains were used: YG797 (*Δalg3 prc1-1*), YG796 (*Δalg9 prc1-1*), SS328 (*PRC1*), YG228 (*Δalg3 PRC1*), and YG414 (*Δalg9 PRC1*).

whereas in *Δalg9* cells they were endo H sensitive (Fig. 6, lanes *8* and *10*).

In *Δalg3* or *Δalg9* cells, a much higher steady state level of CPY was observed compared with CPY* in various mutant cells (Fig. 6). This indicated that degradation of CPY* was not completely blocked by the altered oligosaccharide structure. However, our results demonstrated that oligosaccharide structures specifically affected the degradation of misfolded CPY accumulating in the ER, whereas processing and secretion of wild-type CPY was not altered.

Discussion

A Defined Oligosaccharide Structure Was Required for Efficient Degradation of Misfolded Glycoprotein

We observed a significant effect of the structure of N-linked oligosaccharides on degradation of misfolded CPY*, a model protein for degradation of glycoproteins retained in the ER (Wolf and Fink, 1975; Finger et al., 1993; Hiller et al., 1996; Knop et al., 1996). When expressed in *Δgls2* or *Δmns1* cells, we found a reduced degradation of CPY* (Fig. 5 *C*). The effect of the mannosidase I inactivation was significantly stronger than that of the glucosidase II deletion. A similar stabilization of CPY* as in *Δmns1* cells was observed in *Δalg9* and *Δalg12* cells, where incompletely mannosylated oligosaccharides ($Man_6GlcNAc_2$ and $Man_7GlcNAc_2$, respectively) were transferred to protein. In *Δalg3* cells, which contain protein-bound $Man_5GlcNAc_2$, degradation of CPY* was reduced, however, not to the same extent as in *Δalg9* or *Δalg12* cells. Interestingly, in *Δalg6* cells, where nonglucosylated oligosaccharide $Man_9GlcNAc_2$ was transferred to protein and trimmed to $Man_8GlcNAc_2$, no effect on the stabilization of CPY* was observed. We concluded, that the protein-bound $Man_8GlcNAc_2$ structure was an important recognition element in the degradation pathway of CPY*. Our results were best explained by a model (Fig. 7) where the $Man_8GlcNAc_2$ oligosaccharide on a misfolded glycoprotein acted as a positive signal for degradation. This oligosaccharide structure might be recognized by a lectin, since any changes in its structure reduced the efficiency of degradation. Such a carbohydrate-binding protein has also been postulated in the degradation of glycoproteins in mammalian cells (Yang et al., 1998) and might represent "the additional signal to direct them (soluble misfolded proteins) to the dislocation and the ubiquitation machinery" (Kopito, 1997). Our results showed that glucosylated oligosaccharides also reduced the degradation rate of CPY*, albeit to a lesser extent than the $Man_9GlcNAc_2$, $Man_7GlcNAc_2$ and $Man_6GlcNAc_2$ structures. We concluded that the α1,2-α1,2-dimannose branch of the $Man_8GlcNAc_2$ oligosaccharide was a less important structural element for oligosaccharide recognition than both the α1,6- and α1,3-branch affected by the *alg3, alg9* and *alg12* mutations (see Fig. 1). The observation that the $Man_5GlcNAc_2$-producing *Δalg3* mutation had a less severe effect on degradation was explained by the hypothesis that this oligosaccharide structure represents an intermediate

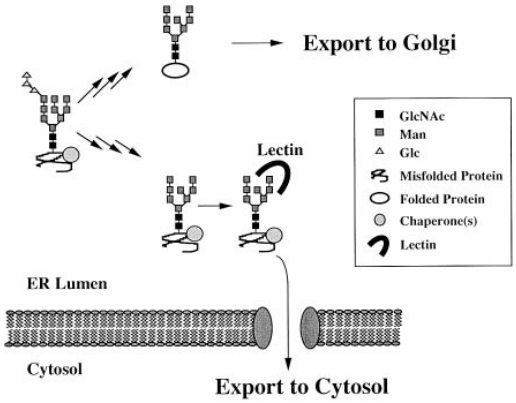

Figure 7. A role of N-linked oligosaccharides in the degradation of glycoproteins in yeast. A secreted glycoprotein folds in the lumen of the ER with the help of chaperone(s). The N-linked oligosaccharide of the glycoprotein is trimmed by glycosidases (indicated by the three arrows) to the $Man_8GlcNAc_2$ structure. The correctly folded protein is exported to the Golgi compartment. If folding of the glycoprotein is not completed within the time required for complete oligosaccharide trimming, the misfolded glycoprotein, bearing oligosaccharides of the $Man_8GlcNAc_2$ structure and associated with chaperone(s), is targeted for export to the cytosol, where degradation by the proteasome occurs. A lectin, recognizing specifically the $Man_8GlcNAc_2$ structure, is involved in the targeting of the malfolded protein to the degradation pathway.

in the degradation of glycoprotein as shown in higher eukaryotic cells (Villers et al., 1994; Ermonval et al., 1997).

Whether the postulated lectin additionally recognizes unfolded protein domains, as does UDP-glucose/glycoprotein glucosyltransferase, involved in the ER quality control pathway of higher eukaryotes (Sousa and Parodi, 1995), is not known. It is possible that the binding of both, the chaperones and the postulated lectin constitute a signal which targets the glycoprotein to the degradation pathway. Indeed Kar2p, the yeast homologue of BiP, transiently binds to wild-type CPY (te Heesen and Aebi, 1994; Simons et al., 1995). Furthermore, this Hsp70 protein was shown to be involved in the degradation of misfolded protein by the proteasome pathway (Plemper et al., 1997).

It has been proposed that the trimming of the protein-bound oligosaccharide in the endoplasmic reticulum represents a biological timer for the protein maturation in the ER of higher eukaryotes (Helenius et al., 1997). This timer function might be required to prevent permanent residence of misfolded glycoproteins in the ER due to the binding to calnexin and calreticulin in higher eukaryotes. Our results are fully compatible with this timer model: as in higher eukaryotic cells (Hubbard and Robbins, 1979), the protein bound oligosaccharide underwent a step-wise trimming process. Removal of the terminal α1,2-glucose by glucosidase I and the first α1,3-glucose by glucosidase II was a very rapid process, whereas the second α1,3-glucose was removed more slowly. The same difference in glucose hydrolysis was observed for glucosidase II of higher eukaryotic cells (Hubbard and Robbins, 1979) and it has been proposed that two different substrate binding sites of glucosidase II are responsible for this difference: the high affinity site would be responsible for the hydrolysis of the first glucose, the low affinity site for the hydrolysis of the second glucose (Alonso et al., 1993). Similar to the findings in higher eukaryotic cells, the mannose trimming was a slow process as compared with hydrolysis of the glucose residues of the protein-bound oligosaccharide. The removal of one specific α1,2-mannose residue by ER-α1,2-mannosidase might represent the time-point after which a misfolded protein is routed to the degradation pathway. We speculated that the processing by mannosidase I determined the time-scale in which a protein had to be correctly folded. If this was not achieved, the glycoprotein was degraded. Importantly, maturation and transport of correctly folded CPY is oligosaccharide-independent (Schwaiger et al., 1982; Winther et al., 1991) and was also not influenced by trimming or oligosaccharide structure (Figs. 3 and 6). It was also postulated that there is a selective export of proteins out of the ER in yeast (Kuehn and Schekman, 1997). Selective export of only correctly folded proteins in combination with degradation of misfolded proteins, timed by oligosaccharide trimming, might therefore represent an effective quality control system for glycoprotein folding in the ER of *S. cerevisiae*, where the calnexin/calreticulin cycle (reglucosylation of misfolded proteins) has not been found (Fernandez et al., 1994, Jakob et al., 1998).

In support of our model, we found that the degradation of a mutant form of the oligosaccharyltransferase component Stt3p, a glycoprotein with multiple transmembrane domains (Zufferey et al., 1995) was also controlled by oligosaccharide trimming (Bodmer, D., U. Spirig, and M. Aebi, manuscript in preparation), suggesting that resident ER glycoproteins are subject to the same degradation system as are glycoproteins that are exported from the ER.

Is there a similar role of the $Man_8GlcNAc_2$ oligosaccharide in the quality control process of glycoproteins in the ER of higher eukaryotic cells? In the trimming process of protein-bound oligosaccharides, removal of the glucose residues precedes the hydrolysis mannose trimming (Hubbard and Robbins, 1979). There is an α1,2-mannosidase activity that leads to the same $Man_8GlcNAc_2$ oligosaccharide as the yeast *MNS1* enzyme in the ER of higher eukaryotic cells (Bischoff and Kornfeld, 1983). However, there are additional ER mannosidase activities in the ER of higher eukaryotic cells and a different $Man_8GlcNAc_2$ oligosaccharide isomer, where the α1,2-mannose linked to the α1,6-mannose is removed, can be produced (Weng and Spiro, 1993, 1996; Moremen et al., 1994). The trimming process in higher eukaryotes is therefore more complex than in yeast. Nevertheless, our results obtained in yeast are compatible with reports on inhibition of α-mannosidase trimming by deoxymannojirimycin that stabilizes specific misfolded glycoproteins in the ER (Su et al., 1993; Daniel et al., 1994; Liu et al., 1997; Yang et al., 1998). On the other hand, degradation of some glycoproteins in the ER was not affected by mannosidase inhibition (Yang et al., 1998).

Alternative Pathways for ER Degradation?

Previous work has shown that CPY* remains in the ER, is ubiquitinated and then degraded in a proteasome-dependent pathway (Hiller et al., 1996). When we compared the level of wild-type CPY and mutant CPY* in both the *Δalg9* and *Δmns1* cells (Figs. 5 *A* and 6), it was apparent that more mature (vacuolar) CPY was present in wild-type cells than CPY* in the *prc1-1* cells. A major portion of CPY* was apparently degraded in these cells. Our results showed that alterations of the oligosaccharide structure did not completely block degradation of CPY*. Moreover, mutations reported to affect CPY* degradation do not completely block CPY* degradation either (Hiller et al., 1996; Knop et al., 1996; Plemper et al., 1997). Taken together, these results suggest an alternative, glycosylation-independent degradation pathway for misfolded glycoproteins in the ER of *S. cerevisiae*.

*Evidence for Posttranslocational N-Glycosylation of CPY**

N-linked oligosaccharides are added co- and posttranslocationally in yeast. For CPY, competition between glycosylation and folding has been reported (Holst et al., 1996). In cells containing *alg* mutations, incompletely assembled oligosaccharide is transferred to nascent protein (Stagljar et al., 1994), albeit at a reduced rate (Sharma et al., 1981). This is reflected by incomplete use of potential N-glycosylation sites. However, we noticed that in both *Δalg3* and *Δalg9* cells, only CPY* with all four potential N-glycosylation sites occupied accumulated, whereas in the *Δalg3* and *Δalg9* cells, wild-type CPY lacking one or two oligosaccharides were found (Fig. 6). Therefore, we postulated that the prolonged exposure of misfolded CPY* to the oli-

gosaccharyltransferase compensated for the reduced affinity of the oligosaccharyltransferase towards incompletely assembled oligosaccharide and resulted in fully glycosylated CPY* in both *Δalg3* and *Δalg9* cells.

The assembly of the lipid-linked oligosaccharide and its transfer to selected asparagine residues of polypeptides in the ER is a highly conserved process. The selective processing of the protein-bound oligosaccharide supports the idea that conservation of the transferred oligosaccharide structure is due to the function of specific trimming intermediates in glycoprotein maturation. Genetic tailoring of NLO structures will provide a useful tool to identify additional roles of the oligosaccharide in glycoprotein processing.

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