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 Man₉GlcNAc₂ oligosaccharide. Reduced rate of degradation was mainly observed for misfolded CPY bearing Man₉GlcNAc₂, Man-GlcNAc₂ and Man₈GlcNAc₂ oligosaccharides, whereas Man₈GlcNAc₂ and, to a lesser extent, Man₉GlcNAc₂ oligosaccharides supported degradation. These results suggest a role for the Man₈GlcNAc₂ oligosaccharide in the degradation process. They may indicate the presence of a Man₈GlcNAc₂-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), while present in the ER, is subject to trimming reactions (see Fig. 1) involving glucosidase I, glucosidase II, and mannosidase I (Herscovichs and Orlean, 1993; Moremen et al., 1994; Roth, 1995). In higher eukaryotes, a specific role of the trimming intermediate Glc₉Man₉GlcNAc₂ 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; Kopeito, 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 Ube6p 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.
Table I. Yeast Strains Used in This Study

| Strain | Genotype | Reference |
|--------|----------|-----------|
| SS328  | MATa ade2-101 ura3-52 his3Δ200 lys2-801 | Vijayraghavan et al. (1989) |
| YG268  | MATa ade2-101 ura3-52 his3Δ200 lys2-801 Δalg3::HIS3 | Aebi et al. (1996) |
| YG414  | MATa ade2-101 ura3-52 his3Δ200 lys2-801 Δalg9::KanMX | Burda et al. (1996) |
| YG427  | MATa ade2-101 ura3-52 his3Δ200 lys2-801 Δalg6::KanMX | Jakob et al. (1998) |
| YG590  | MATa ade2-101 ura3-52 his3Δ200 lys2-801 Δgls2::KanMX Δalg6::HIS3 | Jakob et al. (1998) |
| YG424  | MATa ade2-101 ura3-52 his3Δ200 lys2-801 Δalg8::HIS3 | Jakob et al. (1998) |
| YG491  | MATa ade2-101 ura3-52 his3Δ200 lys2-801 Δgls2::KanMX Δalg10::KanMX | Jakob et al. (1998) |
| YG746  | MATa ade2-101 ura3-52 his3Δ200 tyr1Δmns1::KanMX | This study |
| YG618  | MATa ade2-101 ura3-52 his3Δ200 lys2-801 pcr1-1 | This study |
| YG619  | MATa ade2-101 ura3-52 his3Δ200 lys2-801 Δgls2::KanMX pcr1-1 | This study |
| YG620  | MATa ade2-101 ura3-52 his3Δ200 lys2-801 Δalg6::HIS3 pcr1-1 | This study |
| YG623  | MATa ade2-101 ura3-52 his3Δ200 lys2-801 Δgls2::KanMX Δalg6::HIS3 pcr1-1 | This study |
| YG624  | MATa ade2-101 ura3-52 his3Δ200 lys2-801 Δalg8::HIS3 pcr1-1 | This study |
| YG696  | MATa ade2-101 ura3-52 his3Δ200 lys2-801 Δalg10::KanMX pcr1-1 | This study |
| YG796  | MATa ade2-101 ura3-52 lys2-801 his3Δ200 Δalg9::KanMX pcr1-1 | This study |
| YG797  | MATa ade2-101 ura3-52 lys2-801 his3Δ200 Δalg6::HIS3 pcr1-1 | This study |
| YG807  | MATa ade2-101 ura3-52 lys2-801 his3Δ200 Δalg12::KanMX pcr1-1 | This study |
| YG777  | MATa ade2-101 ura3-52 lys2-801 his3Δ200 Δmns1::KanMX pcr1-1 | This study |
| YG556  | MATa ade2-101 ura3-52 his3Δ200 lys2-801 leu2Δmhs1::KanMX pcr1-1 | This study |
| YG557  | MATa ade2-101 ura3-52 his3Δ200 lys2-801 leu2Δmhs1::KanMX sec18-50 | This study |
| YG821  | MATa ade2-101 ura3-52 his3Δ200 lys2-801 pcr1-1 YEp352 | This study |
| YG822  | MATa ade2-101 ura3-52 lys2-801 his3Δ200 Δalg9::KanMX pcr1-1 pALG9 | This study |
| YG823  | MATa ade2-101 ura3-52 lys2-801 his3Δ200 Δalg6::HIS3 pcr1-1 pALG3 | This study |
| YG824  | MATa ade2-101 ura3-52 lys2-801 his3Δ200 Δalg10::KanMX pcr1-1 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 ManαGlcNAc3 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 pcr1-1 mutation. Oligonucleotides (Microsynth, Balgach, Switzerland) used for gene deletion and screenings are listed in Table II. The integrative plasmid pRS316-PCR1-1 containing the mutated CPY gene was provided by Dr. D.H. Wolf (University of Stuttgart, Germany). The antisera 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                 | ggatcggctatccttg |
| MNS1revKan                 |                  |
| MNS1-431L                  |                  |
| ALG12forKan                |                  |
| ALG12revKan                |                  |
| ALG12forKan                |                  |
| KanMXu                     |                  |
| Primer for pcr1-1 screen   |                  |
| CPY462u                    |                  |
| CPY885L                    |                  |

* 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 + 451L 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 proce-
dure 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
(GG55R) of CPY (Wolf and Fink, 1975) was integrated into the PRC1 lo-
cus of various yeast strains, resulting in a duplication of the PRC1 locus.
Strains in which an excision of the duplication by homologous recombin-
a had occurred were selected on 5-FOA plates and the resulting colo-
nies 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 locus ORF YNR030w were identified by the resistance of the PCR fragment towards
the correct gene deletion (Table II).

Results

Trimming of Protein-bound Oligosaccharides In Vivo
To understand in more detail the role of NLO in glycopro-
tein 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 tempera-
ture-sensitive phenotype and prevents the fusion of ER-
derived vesicles with the Golgi apparatus at nonpermissive
temperature. No processing of protein-bound oligosaccha-
drives 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-
ing of NLO at the ER membrane. In each experiment the
terminal Galβ1,4Manβ1,2Fucα1,3Galβ1,4GlcNAc2 oligosaccharide
was used as a reference. On every time point 5·10^7
cells were resuspended in 250 µl YPD and 25 µl
35S-[3H]-methionine (50 µCi/ml; ICN Pharmaceuticals
Inc.) was added. After 10 min the cells were collected by
centrifugation, washed in minimal medium containing 0.1% glucose,
and resuspended in 450 µl of the same medium. The cells were
then incubated at 30°C for 20 min. After incubation the
Golgi apparatus was isolated by detergent solubilization.

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. 5·10^7 cells were harvested
and broken with glass beads in 50 mM Tris-HCl, pH 7.5, 1% SDS, 2 mM PMSEF
(Franzusoff et al., 1991; te Heesen et al., 1992). Protein extract equivalent to 7 · 10^7
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 con-
jugates on the nitrocellulose membranes were stripped by treatment in
0.1% SDS, 0.1% 2-Mercaptoethanol and 0.1% glycine. The stripped membranes
were blocked with 5% non-fat milk in PBS for 1 h and incubated with the
appropriate antibodies for 16 h. Bound antibodies were detected by an
enhanced chemiluminescence method (Amersham, England).

Analysis of Lipid-linked and Protein-linked Oligosaccharides

The analysis of lipid- and protein-linked oligosaccharides has been de-
scribed (Casan et al., 1993; Zutfen et al., 1995; Jakob et al., 1998). For
pulse–chase labeling of the oligosaccharides, typically 3 · 10^7 cells of a
logarithmically growing culture were pulsed, washed with YP0.1D (2% Yeast extract, 1% Bactopeptone, 0.1% glucose), and resuspended in 450 µl
YP0.1D containing 400 µCi 2-[3H]mannose (30 Ci/mmol; ICN Pharmaceuti-
cals). The oligosaccharides were labeled for 1 min at 26°C and the ra-
deoactivity was chased by adding nonradioactive (±)1D-mannose (111 mM
final concentration). At the given time points, 5 · 10^6 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 oligosaccha-
drides (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).
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 \( \text{Dgls2} \) mutation. Cells were labeled with \(^{3} \text{H}-\text{mannose} \) for 1 min at 26°C and the radioactivity was chased by adding an excess of nonradioactive \(^{6} \text{D}-\text{mannose} \). 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 \( \text{Dgls2} \) strain, only two trimming events occur in the ER: the removal of the terminal \( \alpha 1,2 \)-linked glucose residue by glucosidase I and the cleavage of an \( \alpha 1,2 \)-linked mannose residue by mannosidase I. As expected, a protein-bound oligosaccharide with the putative structure Glc-Man\(_{2}\)GlcNAc\(_{2}\) (G2M9)\(^{2} \) was found, which was slowly converted to Glc-Man\(_{2}\)GlcNAc\(_{2}\) (G2M8) with a half-life of \(~10\) min (Fig. 2, A and B, left). We were unable to detect protein-bound Glc-Man\(_{2}\)GlcNAc\(_{2}\).

We analyzed the structure of the different oligosaccharides in more detail. The endo H–released Glc-Man\(_{2}\)GlcNAc\(_{2}\) NLO comigrated with the Glc\(_{2}\)Man\(_{2}\)GlcNAc\(_{2}\) oligosaccharide. 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.

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\(^{2} \) 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.
Therefore, the two oligosaccharides found on protein in a linked glucose by glucosidase II was a rapid process. We A small amounts of diglucosylated oligosaccharides (Fig. 2, rapid process in vivo. Similarly, since we observed only protein-bound oligosaccharides by glucosidase I was a efficiency of the enzyme (Burda, P., unpublished data). Taken together, the results confirmed that release of the α,1,2-linked glucose from protein-bound oligosaccharide by glucosidase I was a rapid process in the Δgls2 strain. However, trimming of the oligosaccharide by endogenous α,1,2-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 Glc₂Man₉GlcNAc₂ 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 Glc₂Man₉GlcNAc₂ oligosaccharide but represented a minor fraction (<10%) of the total NLO. In contrast, significant amounts of Glc₁Man₉GlcNAc₂ 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-α,1,2-mannosidase (Fig. 2 C). This preparation contained small amounts of oligosaccharides comigrating with the Glc₁Man₉GlcNAc₂ standard and significant levels of oligosaccharides that migrated as expected for Man₉GlcNAc₂ and Man₈GlcNAc₂ oligosaccharide. Indeed, digestion by exo-α,1,2-mannosidase revealed that only the Glc₂Man₉GlcNAc₂ oligosaccharides contained a protective glucose residue and was converted to Glc₁Man₉GlcNAc₂, whereas the majority of the oligosaccharides was trimmed to Man₉GlcNAc₂. These results showed that the Glc₂Man₉GlcNAc₂ protein-bound oligosaccharide was converted primarily to Man₉GlcNAc₂ 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 α,1,2-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 α,1,3-linked glucose by glucosidase II was a rapid process. We concluded that under our experimental conditions, the monoglucosylated oligosaccharide Glc₁Man₉GlcNAc₂ was converted to the Man₉GlcNAc₂ 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 α,1,2-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 matures 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 α,1,2-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

![](image.png)

**Figure 3.** Maturation of CPY in wild-type and mannosidase-deficient mutant cells. Logarithmically growing cells were pulsed with [35S]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 |
|----------|--------------------|----------------------|-------------------|------------|
| Δalg2    | Glc₃Man₃GlcNAc₂     | Glc₃Man₃GlcNAc₂ (G2) | No                | This study |
| Δalg10Δalg2 | Glc₃Man₃GlcNAc₂ | Glc₃Man₃GlcNAc₂ (G2) | Yes               | Jakob et al. (1998) |
| Δalg8Δalg2 | Glc₃Man₃GlcNAc₂ | Glc₃Man₃GlcNAc₂ (G1) | Yes               | Jakob et al. (1998) |
| Δalg6Δalg2 | Man₅GlcNAc₂       | Man₅GlcNAc₂ (G0)    | Yes               | Jakob et al. (1998) |
| Δmns1    | Glc₃Man₃GlcNAc₂   | Man₅GlcNAc₂ (M9)    | No                | Puccia et al. (1993) |
| Wild-type | Glc₃Man₃GlcNAc₂   | Man₅GlcNAc₂ (M8)    | No                | Jakob et al. (1998), Verostek et al. (1991, 1993), Ziegler and Trimble (1991) |

*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.

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: Glc₃Man₃GlcNAc₂ (G2; Δalg10 Δalg2), Glc₃Man₃GlcNAc₂ (G1; Δalg8 Δalg2), Man₅GlcNAc₂ (G0; wild-type and Δalg6 Δalg2). It is important to note that 1,2-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 Δalg2) 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*, which was precipitated using CPY-specific antiserum. Precipitated CPY* was resolved by SDS-PAGE, autoradiography was performed using a PhosphorImager 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 Fig. 1 and above the different autoradiographs (B) were used in the analysis: wt; prcl-1, YG618; G0; Δalg6 Δalg2 prcl-1, YG623; G1; Δalg8 Δalg2 prcl-1, YG624; G2; Δalg10 Δalg2 prcl-1, YG625.

Figure 4. Degradation of misfolded CPY with glucosylated oligosaccharides. (A) Cells were labeled with a short pulse with 3S-labeled methionine, chased with an excess of unlabeled methionine, lysed 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 PhosphorImager 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 Fig. 1 and above the different autoradiographs (B) were used in the analysis: wt; prcl-1, YG618; G0; Δalg6 Δalg2 prcl-1, YG623; G1; Δalg8 Δalg2 prcl-1, YG624; G2; Δalg10 Δalg2 prcl-1, YG625.

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Efficient Degradation of Misfolded Glycoprotein

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 mammalian cells (Yang et al., 1998) and might represent “the additional signal to direct them (soluble misfolded proteins) to the dislocation and the ubiquitination machinery” (Kopito, 1997). Our results showed that glucosylated oligosaccharides also reduced the degradation rate of CPY*, albeit to a lesser extent than the ManαGlCNAC2, ManβGlCNAC2, and ManγGlCNAC2 structures. We concluded that the α1,2-dimannose branch of the Manα GlCNAC2 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 Manβ GlCNAC2-producing Δalg3 mutation had a less severe effect on degradation was explained by the hypothesis that this oligosaccharide structure represents an intermediate 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

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 prcl-1 locus expressing CPY* (lanes 1–4) or a wild-type PRC1 locus (lanes 5–10) were analyzed. The positions of the ER form (pCPY*) 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 prcl-1), YG796 (Δalg9 prcl-1), SS328 (PRC1), YG228 (Δalg3 PRC1), and YG414 (Δalg9 PRC1).

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 ManαGlCNAC2 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 ManαGlCNAC2 structure and associated with chaperone(s), is targeted for export to the cytosol, where degradation by the proteasome occurs. A lectin, recognizing specifically the ManαGlCNAC2 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 (Souza 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 Sst3p, 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 Man₄GlcNAc₂-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 Man₄GlcNAc₂-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 Man₄GlcNAc₂-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 pcr1-1 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 posttranslationally 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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