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Genetic tailoring of *N*-linked oligosaccharides: the role of glucose residues in glycoprotein processing of *Saccharomyces cerevisiae in vivo*

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In higher eukaryotes a quality control system monitoring the folding state of glycoproteins is located in the ER and is composed of the proteins calnexin, calreticulin, glucosidase II, and UDP-glucose: glycoprotein glucosyltransferase. It is believed that the innermost glucose residue of the *N*-linked oligosaccharide of a glycoprotein serves as a tag in this control system and therefore performs an important function in the protein folding pathway. To address this function, we constructed *Saccharomyces cerevisiae* strains which contain nonglucosylated (G0), monoglucosylated (G1), or diglucosylated (G2) glycoproteins in the ER and used these strains to study the role of glucose residues in the ER processing of glycoproteins. These alterations of the oligosaccharide structure did not result in a growth phenotype, but the induction of the unfolded protein response upon treatment with DTT was much higher in G0 and G2 strains as compared to wild-type and G1 strains. Our results provide *in vivo* evidence that the G1 oligosaccharide is an active oligosaccharide structure in the ER glycoprotein processing pathway of *S.cerevisiae*. Furthermore, by analyzing *N*-linked oligosaccharides of the constructed strains we can directly show that no general glycoprotein glucosyltransferase exists in *S.cerevisiae*.

Key words: protein folding/protein glycosylation/protein transport/quality control/yeast

Introduction

In higher eukaryotes, there is growing evidence of a quality control system monitoring the folding status of glycoproteins in the endoplasmic reticulum (ER). This system ensures that misfolded glycoproteins will be retained in the ER and either be subject to refolding or degradation. Correctly folded glycoproteins then exit the ER for further processing in the Golgi apparatus and are sorted to their final destinations. *N*-Linked glycoproteins are either co- or post-translationally translocated into the lumen of the ER whereby the oligosaccharyltransferase transfers a preassembled oligosaccharide $Glc_3Man_9GlcNAc_2$ (G3) from dolichyl-pyrophosphate to asparagine residues in the sequon Asn - X - Ser/Thr (Kornfeld and Kornfeld, 1985; Roth, 1995). Once transferred to protein the oligosaccharide is subject to trimming by the glucosidase I removing the terminal α1,2-linked glucose residue ($Glc_2Man_9GlcNAc_2$; G2), followed by the action of glucosidase II which cleaves sequentially the remaining α1,3-linked glucose residues ($Glc_1Man_9GlcNAc_2$, G1; $Man_9GlcNAc_2$, G0). Finally, in *S.cerevisiae*, α-mannosidase I selectively removes one α1,2-linked mannose residue ($Man_8GlcNAc_2$) before the glycoproteins exit the ER (Helenius, 1994; Moremen *et al.*, 1994). By using the glucosidase inhibitors castanospermine and 1-deoxynojirimycin, Hammond *et al.* (1994) and Hebert *et al.* (1995) demonstrated in mammalian cells the importance of monoglucosylated *N*-linked oligosaccharides for binding to calnexin. Moreover, UDP-glucose: glycoprotein glucosyltransferase, present in *S.pombe* (Fernandez *et al.*, 1994), *D.melanogaster* (Parker *et al.*, 1995) and rat (Trombetta and Parodi, 1992), is able to reglucosylate G0 oligosaccharides on *N*-linked glycoproteins which expose hydrophobic patches (Fernandez *et al.*, 1994). The misfolded reglucosylated glycoprotein can be recognized and bound by calnexin and thereby retained in the folding environment of the ER. The correctly folded glycoprotein would then become a substrate for glucosidase II and be able to exit the ER. This quality control model first postulated by Hammond and Helenius (1994) for mammalian cells defines a central role of monoglucosylated glycoproteins in the control of protein folding in the ER. To address the role of glucosylated glycoproteins *in vivo*, we used yeast genetic techniques and constructed strains which contain nonglucosylated or mono- or diglucosylated glycoproteins in the ER and studied possible effects on glycoprotein processing. Our data are compatible with the model that only the monoglucosylated and not the non- or diglucosylated *N*-linked oligosaccharides are the positive signal for protein folding in the ER *in vivo*.

Results

Sequence homologies of the ORF YBR229c to pig liver and human lymphocyte glucosidase II

Using the amino acid sequence derived from the pig liver glucosidase II (Flura *et al.*, 1997), we performed a database search in the nonredundant protein database and identified an open reading frame of *S.cerevisiae* with a sequence similarity of 34% (GenEMBL accession no. Z36098). The yeast amino acid sequence showed a sequence identity of 46% in the more conserved C-terminal regions (amino acids 234–954; data not shown). This ORF YBR229c, now termed *GLS2*, was replaced with the geneticin resistance gene by homologous recombination. In order to biochemically confirm that this locus encodes glucosidase II, membrane fractions of wild-type, *gls1–1* and Δ*gls2* cells were assayed for glucosidase II activity. The membrane-enriched protein preparation of the *GLS2*-deficient cells displayed a 5-fold reduced enzymatic activity as compared to both wild-type and *gls1–1* cells in two independent Δ*gls2* strains (Table II). We do not know the nature of the residual substrate-hydrolizing activity in the Δ*gls2* preparations since the substrate was stable in samples incubated without crude extract. Furthermore, the mobility of mature vacuolar CPY of the Δ*gls2* strains was slightly reduced due to the inability to trim the two glucose residues of each oligosaccharide (see below). These findings in addition to the amino acid sequence similarity confirmed that the locus ORF YBR229c encoded glucosidase II.

Table I. Yeast strains used in this study

Strain	Genotype	Reference
SS328	MATα ade2–101 ura3–52 his3Δ200 lys2–801	(Vijayraghavan *et al.*, 1989)
MLY1601	MATa leu2–3, 112 Δpep4::URA3 gls1–1	(Latterich and Schekman, 1994)
YG592	MATa ade2–101 ura3–52 his3Δ200 lys2–801 Δalg6::HIS3	(Reiss *et al.*, 1996)
YG126	MATα ade2–101 ura3–52 his3Δ200 lys2–801 Δalg8::HIS3	(Stagljar *et al.*, 1994)
YG428	MATα ade2–101 ura3–52 his3Δ200 tyr1 Δalg10::KanMX	This study
YG427	MATα ade2–101 ura3–52 his3Δ200 lys2–801 Δgls2::KanMX	This study
YG590	MATa ade2–101 ura3–52 his3Δ200 lys2–801 Δgls2::KanMX Δalg6::HIS3	This study
YG424	MATa ade2–101 ura3–52 his3Δ200 lys2–801 Δgls2::KanMX Δalg8::HIS3	This study
YG491	MATa ade2–101 ura3–52 his3Δ200 lys2–801 Δgls2::KanMX Δalg10::KanMX	This study
YG571	MATα ade2–101 ura3–52 his3Δ200 lys2–801 ura3–52::UPRE-lacZ-URA3	This study
YG572	MATα ade2–101 ura3–52 his3Δ200 lys2–801 Δgls2::KanMX ura3–52::UPRE-lacZ-URA3	This study
YG595	MATa ade2–101 ura3–52 his3Δ200 lys2–801 Δalg6::HIS3 ura3–52::UPRE-lacZ-URA3	This study
YG567	MATα ade2–101 ura3–52 his3Δ200 lys2–801 Δalg8::HIS3 ura3–52::UPRE-lacZ-URA3	This study
YG568	MATα ade2–101 ura3–52 his3Δ200 lys2–801 Δalg10::KanMX ura3–52::UPRE-lacZ-URA3	This study
YG596	MATa ade2–101 ura3–52 his3Δ200 lys2–801 Δgls2::KanMX Δalg6::HIS3 ura3–52::UPRE-lacZ- URA3	This study
YG569	MATa ade2–101 ura3–52 his3Δ200 lys2–801 Δgls2::KanMX Δalg8::HIS3 ura3–52::UPRE-lacZ-URA3	This study
YG570	MATa ade2–101 ura3–52 his3Δ200 lys2–801 Δgls2::KanMX Δalg10::KanMX ura3–52::UPRE-lacZ-URA3	This study
YG556	MATα ade2–101 ura3–52 his3Δ200 lys2–801 leu2 sec18–50	This study
YG603	MATα ade2–101 ura3–52 his3Δ200 lys2–801 leu2 Δgls2::KanMX Δalg6::HIS3 sec18–50	This study
YG562	MATα ade2–101 ura3–52 his3Δ200 lys2–801 leu2 Δgls2::KanMX Δalg8::HIS3 sec18–50	This study
YG563	MATa ade2–101 ura3–52 his3Δ200 lys2–801 leu2 Δgls2::KanMX Δalg10::KanMX sec18–50	This study

Table II. Glucosidase II activity of membrane-enriched protein preparations

Strain	Glucosidase II activity	Relative GLS2p activity
Wild-type	287 U mg protein^{-1}	1.0
gls1–1	270 U mg protein^{-1}	0.9
Δ*gls2* (clone 3)	57 U mg protein^{-1}	0.2
Δ*gls2* (clone 7)	50 U mg protein^{-1}	0.2

Logarithmically growing cells were dirupted in 0.25 M sucrose, 10 mM Tris–HCl (pH 7.5), 1.5 mM AEBSF using glass beads. The membranes were enriched by the addition of 1% triton-X-100 and centrifugation. The glucosidase II activity was assayed according to Brada and Dubach (1984) using 4-methyl-umbelliferyl-α-D-glucopyranosid as substrate. The specific enzyme activity was calculated from the range of maximal velocity (incubation < 5 min). One unit enzyme activity was defined as the release of 1 μmol 4-methyl-umbelliferon per minute. The protein concentrations were determined by using the Bio-Rad protein assay. The following strains were used: wild-type, SS328; *gls1–1*, MLY1601; Δ*gls2* (clone 7), YG427.

Genetic tailoring of oligosaccharide structures

In order to obtain *N*-linked oligosaccharides of defined structure on glycoproteins in the ER of *S.cerevisiae*, the Δ*gls2* mutation was crossed with several yeast strains harboring deletions in the lipid-linked oligosaccharide biosynthetic pathway. Incomplete lipid-linked oligosaccharide can be transferred to protein, albeit with a reduced efficiency (Stagljar *et al.*, 1994). In Δ*alg6* cells nonglucosylated oligosaccharide (G0; Reiss *et al.*, 1996) is transferred, in Δ*alg8* strains monoglucosylated (G1; Stagljar *et al.*, 1994) and in Δ*alg10* strains diglucosylated oligosaccharide (G2; this study and Burda *et al.*, unpublished observations) as depicted in Figure 1. In the absence of glucosidase II activity, glucose residues should remain on the protein-bound oligosaccharides. Therefore, the combination of specific *alg* deletions with the Δ*gls2* mutation should make it possible to genetically tailor the structure of the oligosaccharide on glycoproteins in the ER (Figure 1, Table III). To test this hypothesis, appropriate Δ*algX*Δ*gls2* strains were constructed and characterized. The growth rates of all strains were equal to wild-type cells and no temperature-dependent growth phenotype was observed (data not shown). Nonlethal mutations in the *alg* genes lead to an incomplete assembly of the dolichol-linked oligosaccharide and thereby form a suboptimal substrate for the oligosaccharyltransferase (Sharma *et al.*, 1981). The effect of this suboptimal substrate *in vivo* is apparent by the occurrence of underglycosylated glycoproteins such as CPY and Wbp1p. Protein extracts of exponential growing yeast strains were subjected to Western blot analysis using anti-CPY and anti-Wbp1p sera. As expected the Δ*gls2* mutant showed no underglycosylation of both glycoproteins CPY and Wbp1p (Figure 2, lane 2), because the completely assembled dolichol-linked oligosaccharide is transferred to the nascent polypeptide chains. Furthermore, the combination of Δ*gls2* with deletions in *alg* genes did not modulate the extent of underglycosylation of CPY and Wbp1p (Figure 2, lanes 3–8): the pattern of underglycosylation of both, CPY and Wbp1p, was not altered in the Δ*alg* cells by addition of the Δ*gls2* mutation. However, we observed a different molecular weight of the resulting glycoproteins due to the altered biosynthesis and trimming of the oligosaccharides. This alteration is visualized best in the case of the fully glycosylated Wbp1p which showed the slowest mobility in Δ*gls2* and Δ*alg10*Δ*gls2* cells (Figure 2B, lanes 2 and 8), and an intermediate mobility in Δ*alg8*Δ*gls2* cells (Figure 2B, lane 7). In Δ*alg6*Δ*gls2* cells, the fully glycosylated form of Wbp1p had the same molecular weight as the wild-type cells (Figure 2B, lanes 6 and 1, respectively). A similar change in mobility was observed for the different CPY glycoforms. These altered mobilities showed that in the absence of glucosidase II,

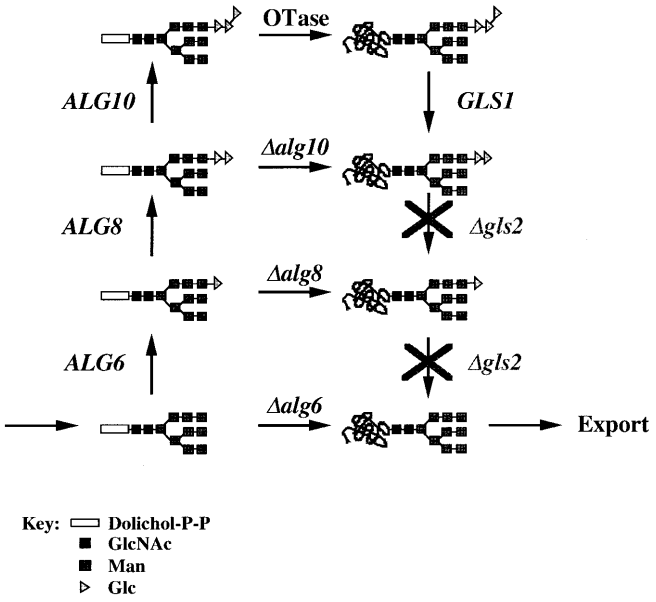

Fig. 1. Principle of genetic tailoring of oligosaccharides. By the introduction of Δ*gls2* deletion alone and by combination of the deletions Δ*alg10*Δ*gls2*, it was possible to maintain the structure of *N*-linked oligosaccharides after transfer to a protein in the diglucosylated form, in Δ*alg8*Δ*gls2* in the monoglucosylated form and in Δ*alg6* or Δ*alg6*Δ*gls2* in the unglucosylated form. In the Δ*gls2* strains the *N*-linked oligosaccharides remain unmodified with respect to the α1,3-linked glucose residues due to the inactivation of the glucosidase II whereas in *alg* deletion strains the oligosaccharide is sequentially trimmed by the glucosidase II. OTase, oligosaccharyltransferase.

α1,3-linked glucose residues were not removed from glycoproteins and were still found on vacuolar proteins (e.g., mature CPY). Most significantly, these glucose residues were also retained on the ER-resident protein Wbp1p. This data shows by differences in electrophoretic mobility that the combination of *alg* and *gls2* mutations makes it possible to tailor the glucosylation state of glycoproteins *in vivo*.

N-Linked oligosaccharide composition of glycoproteins of the tailored strains

In order to verify the differences in protein-linked oligosaccharide composition as observed by SDS–PAGE (Figure 2), lipid-linked oligosaccharides (LLO) and *N*-linked oligosaccharides (NLO) were labeled with [^{3}H]mannose, extracted, released with EndoH, and analyzed by HPLC. To prevent modification by Golgi mannosyltransferases the temperature-conditional *sec18–50* mutation (Esmon *et al.*, 1981; Novick *et al.*, 1981; Verostek *et al.*, 1991, 1993) was introduced into all analyzed strains. The cells were shifted to nonpermissive temperature for 30 min prior to labeling to block the fusion of ER vesicles to the Golgi. As expected, the Δ*alg10*Δ*gls2* mutation lead to the accumulation of the incomplete LLO $Glc_2Man_9GlcNAc_2$ (G2M9), the Δ*alg8*Δ*gls2* mutations to the $Glc_1Man_9GlcNAc_2$ (G1M9) and the Δ*alg6*Δ*gls2* mutations to the $Man_9GlcNAc_2$ (M9) LLO (Figure 3; LLO). The identity of the EndoH-released NLO signals (Figure 3; NLO) was determined by mixing appropriate LLO and NLO extracts and HPLC analysis (Figure 3; LLO+NLO). In the wild-type strain, the NLO consisted of $Man_9GlcNAc_2$ and $Man_8GlcNAc_2$ oligosaccharides. In the Δ*alg6*Δ*gls2* mutant strain the same oligosaccharide composition as in the wild-type strain was found ($Man_9GlcNAc_2$ and $Man_8GlcNAc_2$). The $Man_9GlcNAc_2$ and $Man_8GlcNAc_2$ signals completely disappeared upon digestion with α-mannosidase (data not shown). In the Δ*alg8*Δ*gls2* double mutant the $Glc_1Man_9GlcNAc_2$ was identified by mixing experiments, the other occurring peak appears to be $Glc_1Man_8GlcNAc_2$ (G1M8, see *Discussion*) since only one oligosaccharide species ($Glc_1Man_4GlcNAc_2$) was detectable after α-mannosidase treatment (data not shown). Likewise, in the case of the Δ*alg10*Δ*gls2* double mutant the NLO structure $Glc_2Man_9GlcNAc_2$ was confirmed and presumably the $Glc_2Man_8GlcNAc_2$ (G2M8, see discussion) also exists. Upon α-mannosidase digestion of these NLO only one single oligosaccharide ($Glc_2Man_4GlcNAc_2$) was detectable (data not shown). Hence, by HPLC analysis of the *N*-linked oligosaccharides, we could directly show that the oligosaccharide structures as predicted by genetics occurred in our strains *in vivo*.

Absence of a general glycoprotein glucosyltransferase activity

Since the Δ*alg6*Δ*gls2* strain is glucosidase II deficient, glucose residues transferred to protein-bound oligosaccharides by a general glycoprotein glucosyltransferase ought to be visible by analyzing the NLO profile of the Δ*alg6*Δ*gls2* strain. The LLO of the Δ*alg6*Δ*gls2* mutant strain consisted mainly of $Man_9GlcNAc_2$ (Figure 3, LLO). No glucose-containing LLO were detected. Likewise, no NLO of a larger size than $Man_9GlcNAc_2$ of the same strain were observed (Figure 3, NLO) confirming the absence of a general glycoprotein glucosyltransferase in *S.cerevisiae* (Fernandez *et al.*, 1994).

Table III. Structure of oligosaccharides of N-linked glycoproteins in mutant yeast strains

Genotype	LLO structure[a]	Final NLO structure[2]	Underglycosylation
Wild-type	$Glc_3Man_9GlcNAc_2$	$Man_9GlcNAc_2$	No
Δalg6	$Man_9GlcNAc_2$	$Man_9GlcNAc_2$	Yes
Δalg8	$Glc_1Man_9GlcNAc_2$	$Man_9GlcNAc_2$	Yes
Δalg10	$Glc_2Man_9GlcNAc_2$	$Man_9GlcNAc_2$	Yes
Δgls2	$Glc_3Man_9GlcNAc_2$	$Glc_2Man_9GlcNAc_2$ (G2)	No
Δalg6 Δgls2	$Man_9GlcNAc_2$	$Man_9GlcNAc_2$ (G0)	Yes
Δalg8 Δgls2	$Glc_1Man_9GlcNAc_2$	$Glc_1Man_9GlcNAc_2$ (G1)	Yes
Δalg10 Δgls2	$Glc_2Man_9GlcNAc_2$	$Glc_2Man_9GlcNAc_2$ (G2)	Yes

[a]Structure of dolichol-linked oligosaccharides (LLO) and oligosaccharides on glycoproteins before trimming.
[b]Oligosaccharide structure on glycoproteins (NLO) after endogenous glucosidase trimming.

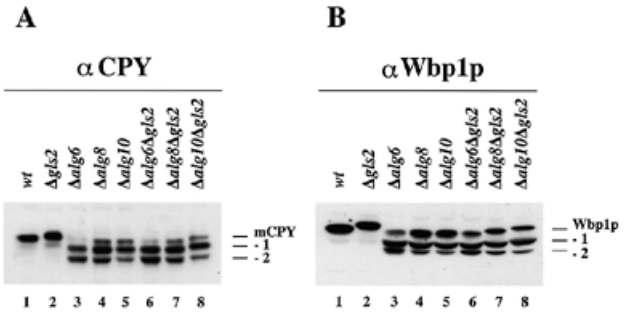

Fig. 2. Glycosylation patterns of CPY and Wbp1p in various yeast strains. Logarithmically growing cells were pelleted and broken with glass beads in the presence of 2 mM PMSF. The protein extract of 0.375 OD equivalents was separated by SDS–PAGE (7.5% acrylamide) and transferred to nitrocellulose. CPY (A) and Wbp1p (B) was visualized by Western blotting by using chemiluminescence. The positions of mature proteins (mCPY, mWbp1p) and the glycoforms lacking one (-1) or two (-2) oligosaccharides are indicated. The introduction of the Δ*gls2* mutation did not modulate the extent of CPY or Wbp1p glycosylation. The following strains were used: SS328 (1); YG427 (2); YG592 (3); YG126 (4); YG428 (5); YG590 (6); YG424 (7); YG491 (8). The relevant genotype is indicated above each lane.

Disturbing the protein folding and transport of CPY by DTT

The absence of any detectable growth phenotype in Δ*algX*Δ*gls2* strains demonstrate that a potential quality control/folding system requiring correctly glucosylated glycoproteins was not limiting for growth in these strains. In order to assay for the role of glucose residues on glycoprotein folding, we have chosen two experimental approaches: first, reducing the efficiency of protein folding by the addition of DTT (Simons *et al.*, 1995), and second, determining the degree of induction of the unfolded protein response (UPR; Mori *et al.*, 1992) as a measure for the extent of misfolded proteins *in vivo*. Reducing agents such as DTT modulate the redox potential in the ER. In yeast, it has been shown that proteins containing disulfides are retained in the ER, whereas the transport of proteins devoid of disulfide bonds such as α-factor is not affected (Jamsa *et al.*, 1994; Simons *et al.*, 1995). We examined the concentration-dependent interference of DTT on the transport of CPY by monitoring the proteolytic digestion of the protein during its maturation. Concentrations of higher than 0.5 mM DTT reversibly blocked the export of CPY out of the ER irrespective of the oligosaccharide structure (Figure 4). Furthermore, an underglycosylated form of the ER-resident p1CPY (Figure 4A–C, p1CPY*) occurred. Both, the p1CPY and the underglycosylated p1CPY* displayed identical molecular weight upon treatment with endoglycosidase H (Figure 4B). Upon diluting DTT to 0.5 mM, the transport and maturation of CPY reoccurred demonstrating the reversibility of the DTT-induced transport block. Moreover, upon reinitiating the protein transport, the p1CPY* then disappeared, either due to glycosylation and export or degradation (Figure 4C). In a pulse chase experiment, we were able to demonstrate that CPY, trapped in the ER by DTT, maturated normally and was targeted to the vacuole upon dilution

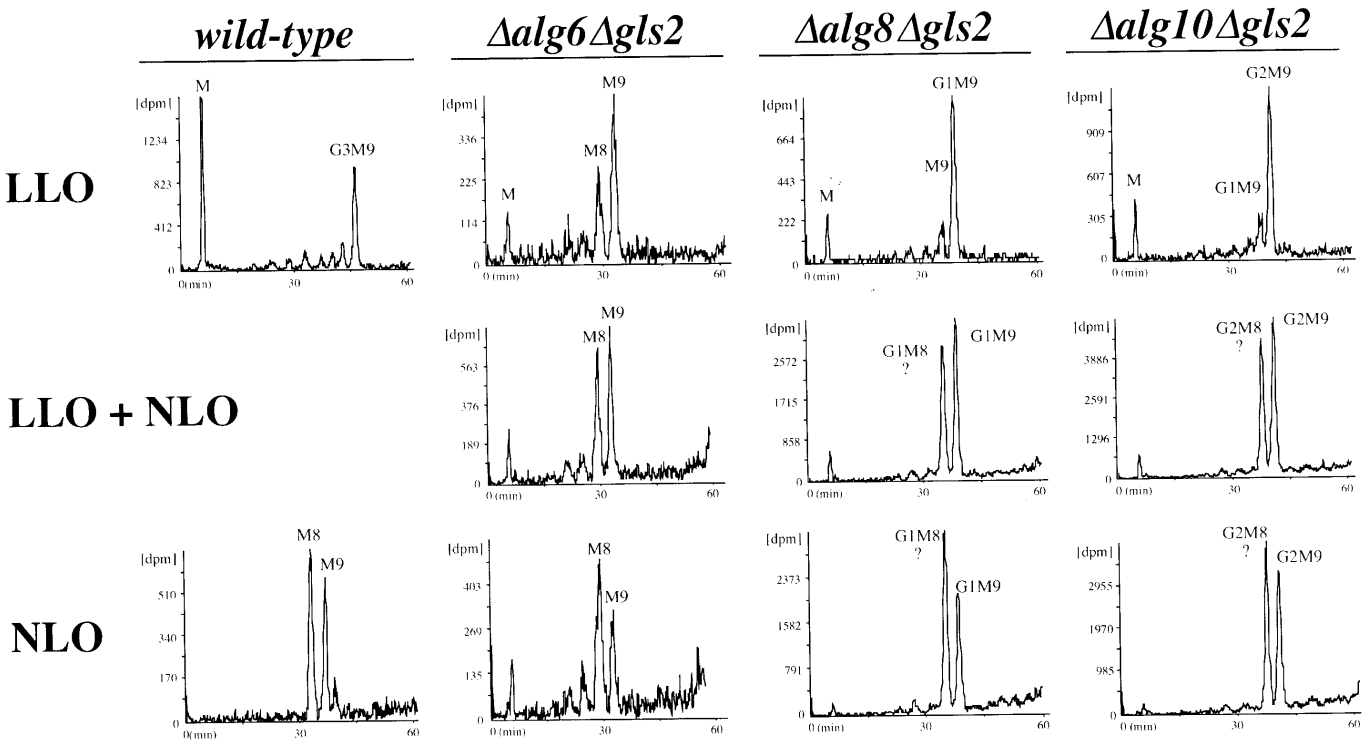

Fig. 3. Analysis of oligosaccharide composition of LLO and NLO by HPLC. The LLO were labeled for 30 min, extracted and hydrolized according to Zufferey *et al.* (1995). The hydrolized LLO were then trimmed with EndoH and desalted by ion-exchanger chromatography. The NLO were obtained after extraction of the LLO. The glycoproteins were digested with trypsin and the NLO released by EndoH treatment. The samples were desalted by ion-exchanger chromatography. The LLO, NLO, and LLO/NLO mixing samples were separated and analyzed by HPLC as described previously (Cacan *et al.*, 1993; Zufferey *et al.*, 1995). The lipid-linked (LLO) or *N*-linked (NLO) origin of the oligosaccharides is indicated. The identity of the oligosaccharide structures are given: M, free Man; M8, $Man_8GlcNAc_2$; M9, $Man_9GlcNAc_2$; G1M8, $Glc_1Man_8GlcNAc_2$; G1M9, $Glc_1Man_9GlcNAc_2$; G2M8, $Glc_2Man_8GlcNAc_2$; G2M9, $Glc_2Man_9GlcNAc_2$; G3M9, $Glc_3Man_9GlcNAc_2$. Question marks indicate the proposed structures. The following strains were used in this figure: wild-type (YG556); Δ*alg6*Δ*gls2* (YG603); Δ*alg8*Δ*gls2* (YG562); Δ*alg10*Δ*gls2* (YG563).

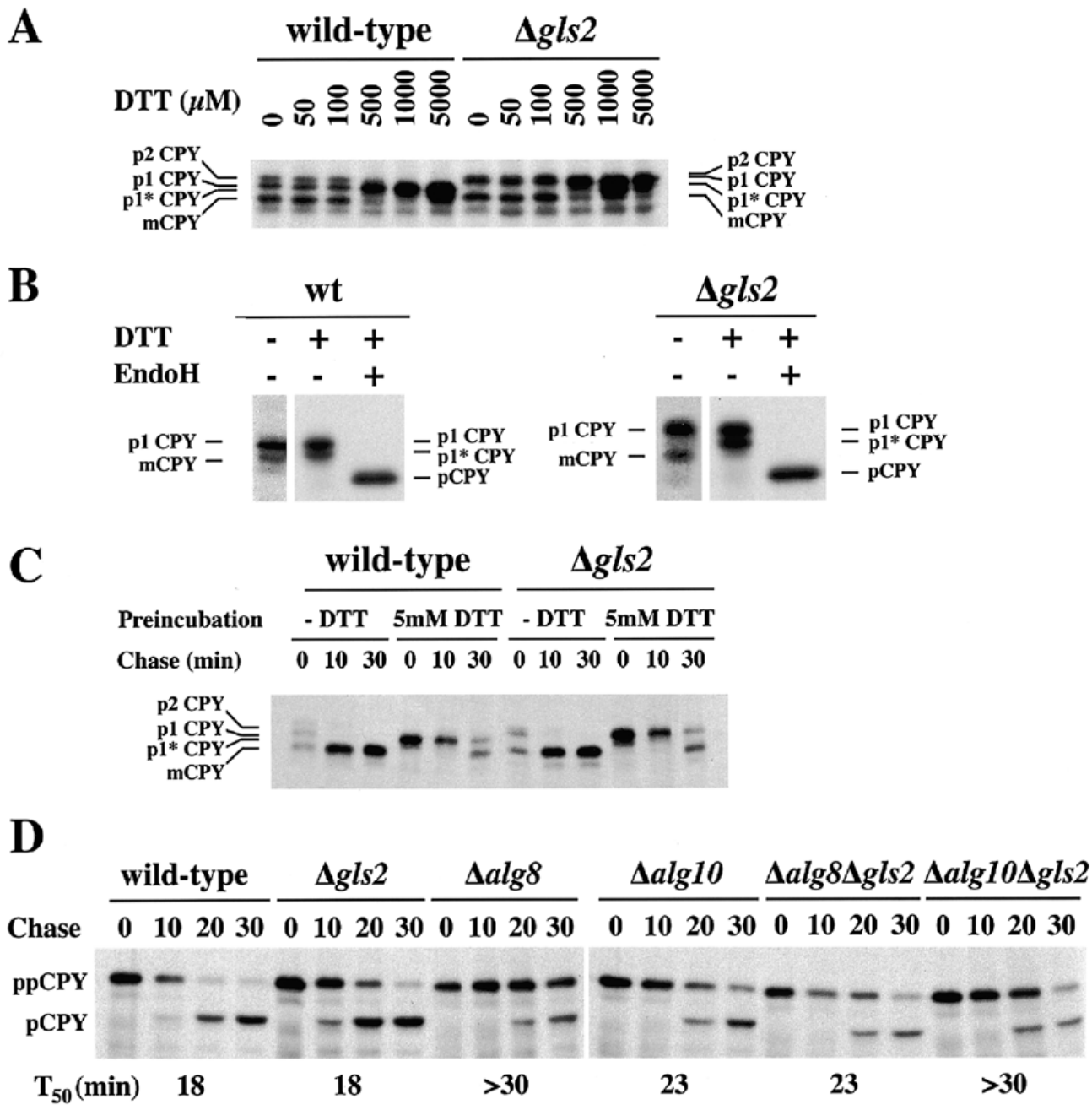

Fig. 4. Influence of DTT on transport and glycosylation of CPY. (**A**) Two OD units of each yeast strain per DTT concentration were labeled with [35-S]methionine at 37°C for 10 min. The cells were broken in the presence of 2 mM PMSF, CPY was immunoprecipitated and subjected to SDS–PAGE. The labeled proteins were visualized by salicylate-enhanced autoradiography. Irrespective of the genotypes, CPY was retained in the ER-resident p1 form at concentrations higher than 500 μM DTT. In addition, an underglycosylated form of CPY (p1CPY*) appeared. (**B**) Two OD units of cells were preincubated with 5 mM DTT at 37°C for 10 min before adding the radioactive label. The difference in molecular weight disappeared upon treatment with EndoH. (**C**) Two OD units of cells per time point were preincubated with 5 mM DTT at 37°C for 10 min before adding the radioactive label. The pulse was for 10 min. The chase was initiated (time = 0 min) by a 10-fold dilution of DTT and an excess of unlabeled methionine. The chase reaction was terminated by adding 20 mM azide at the given time points (chase time is given in minutes). Upon dilution of the DTT, the p1CPY and the underglycosylated p1*CPY were normally processed and transported to Golgi and the vacuole. (**D**) After preincubation in 5 mM DTT for 10 min and labeling with [^{35}S]methionine for 10 min the chase was initiated by addition of an excess of unlabeled methionine and cysteine and the DTT was diluted to 0.5 mM. The chase reaction was terminated by adding 20 mM azide at given time points. The cells were broken in the presence of 2 mM PMSF and CPY was immunoprecipitated, EndoH-treated and then subjected to SDS–PAGE. The labeled proteins were visualized by salicylate-enhanced autoradiography. The intensity of the bands were quantified by using a PhosphoImager and the transport rate determined as T_{50} indicating the time point when half of the labeled protein was present in the vacuolar form. The following strains were used: wt, SS328; Δ*gls2*, YG427; Δ*alg6*, YG526; Δ*alg8*, YG126; Δ*alg10*, YG428; Δ*alg6*Δ*gls2*, YG590; Δ*alg8*Δ*gls2*, YG424; Δ*alg10*Δ*gls2*, YG491. The relevant genotype is indicated. Abbreviations: p1CPY, ER proCPY; p2CPY, Golgi-modified proCPY; mCPY, mature vacuolar CPY; ppCPY, EndoH-deglycosylated p1CPY and p2CPY, respectively; pCPY, EndoH-deglycosylated mCPY.

of the reducing agent (Figure 4D). Furthermore, the transport of CPY was independent of the glucose composition (wild-type, G1 or G2) of the *N*-linked oligosaccharides (Figure 4D). We determined the transport rate of CPY by quantifying the bands of the proCPY (ER and Golgi form) and the CPY (vacuolar form) using a Phosphoimager. The transport rate of CPY of the wild-type and Δ*gls2* strains were identical (T_{50} = 18 min). In the other strains, CPY was processed at a slightly slower rate (T_{50} = 23 and >30 min, respectively), however, no glucose-dependent transport rate was observed. CPY bearing G1 glycoproteins was transported from ER to vacuole at the same rate as CPY exhibiting the G2 oligosaccharide (Figure 4D). Likewise, the CPY transport from ER to Golgi was not influenced by the glucose composition of the oligosaccharide (data not shown). A reduced transport rate of underglycosylated CPY has been reported previously (Winther *et al.*, 1991; te Heesen and Aebi, 1994).

Induction of unfolded protein response

In yeast, the presence of misfolded proteins in the ER is transmitted from the ER lumen into the nucleus via a signal transduction pathway requiring the *IRE1* protein kinase (Cox *et al.*, 1993; Mori *et al.*, 1993; Nikawa *et al.*, 1993). Specific transcription factors such as Hac1p (Cox and Walter, 1996) bind to specific promoter segments, the unfolded protein response elements (UPRE), present and highly conserved in the promoters of *KAR2*/BiP, *PDI1*, *EUG1*, and *FKB2* (Shamu *et al.*, 1994) and activate the appropriate genes. Such a regulatory system allows for a direct measurement of the unfolded protein content in the ER *in vivo* (Mori *et al.*, 1992). We used a reporter system based on the *E.coli* ß-galactosidase *(LacZ)* gene under the control of the UPRE of the yeast *KAR2*/BiP promoter. We ligated the UPRE upstream of the ß-galactosidase gene according to Mori *et al.* (1992) and integrated it as a single copy into the *ura3–52* locus. This single copy reporter construct, placed at the identical location in all strains tested, allowed a direct comparison of the UPR in the different strains used. By the sensitive ß-galactosidase read-out, we quantified the extent of misfolded proteins in the ER of the various yeast strains with genetically tailored *N*-linked oligosaccharides. Tunicamycin is known to strongly induce the unfolded protein response (Mori *et al.*, 1992). Upon tunicamycin treatment, the ß-galactosidase activity was strongly stimulated in all strains as compared to untreated cells (40- to 50-fold induction in single experiment, data not shown). When incubating the cells with 0.5 mM DTT, a concentration interfering with protein folding but not abolishing the ER to Golgi transport (Figure 4A,D), we observed a differential response. Wild-type cells did not induce ß-galactosidase activity upon DTT treatment, whereas Δ*gls2* cells did (Figure 5). The deletion of *GLS2* leads to conservation of the *N*-linked oligosaccharides in the diglucosylated G2 form (Table III). The Δ*alg6* mutation prevents the addition of the innermost α1,3-linked glucose residue and thereby renders the *N*-linked oligosaccharide structure in the unglucosylated G0 form. In Δ*alg6* cells, the UPR was also stimulated upon treatment with DTT. Furthermore, the additional loss of glucosidase II activity in Δ*alg6*Δ*gls2* cells did not change this response to DTT (Figure 5). The Δ*alg8* mutation leads to the transfer of monoglucosylated oligosaccharides (G1) and in combination with the Δ*gls2* deletion the single glucose remains on the oligosaccharide of glycoproteins. In contrast to Δ*alg6*, Δ*alg6*Δ*gls2*, and Δ*gls2* cells, the UPR was not induced neither in Δ*alg8* nor in Δ*alg8*Δ*gls2* cells. Alg10p transfers the outermost

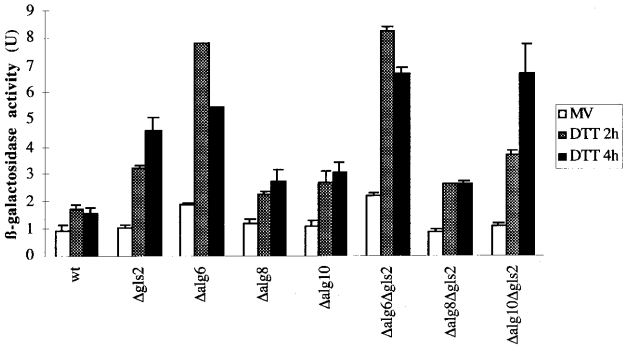

Fig. 5. Induction of β-galactosidase by unfolded protein response. The modified UPRE-*lacZ* plasmid (Mori *et al.*, 1992) was integrated into the genome as a single copy into yeast strains with genetically tailored oligosaccharides. The yeast strains were incubated in minimal medium containing the appropriate amino acids and 0.5 mM DTT for 2 or 4 h. One OD unit of cells were pelleted and subjected to the β-galactosidase assay according to Guarente (1983) using o-nitrophenyl-ß-d-galactopyranosid as a substrate. The galactosidase activity was corrected for cell numbers and reaction time. The enzyme activity was assayed in duplicates in two separate experiments. The following strains were used in this figure: wt, wild-type (YG571); Δ*gls2* (YG572); Δ*alg6* (YG595); Δ*alg8* (YG567); Δ*alg10* (YG568); Δ*alg6*Δ*gls2* (YG596); Δ*alg8*Δ*gls2* (YG569); Δ*alg10*Δ*gls2* (YG570). ±SD. MV, minimal medium.

glucose residue to complete the synthesis of the dolichol-linked oligosaccharide precursor. Upon inactivation of this locus (Δ*alg10*) the lipid-linked oligosaccharide is transferred in the diglucosylated form (G2) to glycoproteins. The trimming of the glucose residues was not impaired. In the Δ*alg10* strain the UPR was the same as in the wild-type, Δ*alg8* and Δ*alg8*Δ*gls2* strains. However, upon combination of Δ*alg10* with Δ*gls2* and thereby preserving the *N*-linked oligosaccharide structure in the G2 form, the unfolded protein response was induced by DTT. We conclude that with respect to the induction of the UPR by DTT the Δ*alg8* mutation was epistatic over Δ*gls2*, but Δ*alg10* was not.

Discussion

Using the combined approach of the complete yeast genome information and yeast genetic techniques, we were able to obtain yeast strains with tailored oligosaccharides not only on lipid-linked precursors but also linked to proteins. By HPLC analysis we confirmed the structures of the oligosaccharides suggested by genetics. Interestingly, the NLO in the wild-type and in the Δ*alg6*Δ*gls2* strains consisted of two different oligosaccharides ($Man_9GlcNAc_2$ and $Man_8GlcNAc_2$). In the Δ*alg8*Δ*gls2* and Δ*alg10*Δ*gls2* strains one signal did not coelute with marker oligosaccharides. The NLO $Man_9GlcNAc_2$ is converted to $Man_8GlcNAc_2$ by the action of the ER mannosidase I (Grondin and Herscovics, 1992; Lipari and Herscovics, 1994). In analogy to the mannosidase I-trimmed $Man_9GlcNAc_2$ structures in the wild-type and in the Δ*alg6*Δ*gls2* strains, we suppose that the second oligosaccharide species represents the corresponding mannosidase I-trimmed products (Δ*alg8*Δ*gls2*, $Glc_1Man_8GlcNAc_2$; Δ*alg10*Δ*gls2*, $Glc_2Man_8GlcNAc_2$). The two oligosaccharide species can be converted to only one by digesting with α-mannosidase confirming the identity of $Glc_1Man_9GlcNAc_2$/$Glc_1Man_8GlcNAc_2$ and $Glc_2Man_8GlcNAc_2$/$Glc_2Man_8GlcNAc_2$ in the Δ*alg8*Δ*gls2* and Δ*alg10*Δ*gls2* strains, respectively. The presence of these two oligosaccharides suggests that glucose trimming is not a prerequisite for mannosidase

I activity. Interestingly, trimming of the lipid-linked oligosaccharide by mannosidase I seems not to occur.

These strains with tailored NLO provided the tools to investigate the involvement of the innermost α1,3-linked glucose residue of *N*-linked oligosaccharides of *S.cerevisiae* in coping cellular stress and its involvement in protein folding *in vivo*. In higher eukaryotic cells the innermost glucose residue of *N*-linked oligosaccharides is involved in binding to calnexin and calreticulin as shown by experiments using the glucosidase inhibitors castanospermine and 1-deoxynojirimycin (Hammond *et al.*, 1994; Hebert *et al.*, 1996), glucosidase II-deficient mouse lymphoma cells (Ora and Helenius, 1995) and *in vitro* reglucosylated ribonuclease B (Rodan *et al.*, 1996; Zapun *et al.*, 1997). According to the model of Hammond and Helenius (1994) monoglucosylated oligosaccharides of *N*-linked glycoproteins play a central role in the protein refolding process. However, the *in vivo* importance of this glucose residue for the overall folding process of proteins in the ER has not been demonstrated directly in any organism. In *S.cerevisiae*, using a reporter construct under the control of the UPRE of *KAR2*/BiP (Mori *et al.*, 1992), we were able to demonstrate the involvement of the monoglucosylated *N*-linked oligosaccharides in glycoprotein folding. In our experiments we sought for mild nonlethal stress conditions interfering with protein folding. Since we wanted to study the influence of *N*-linked oligosaccharides it was not feasible to employ tunicamycin, a drug blocking *N*-linked glycosylation and known to strongly induce the unfolded protein response (UPR). We used a low concentration of DTT instead shown to still allow protein transport albeit at a reduced rate. Glycoproteins with fixed nonglucosylated and diglucosylated *N*-linked oligosaccharides induced the UPR under these mild reducing conditions whereas the response of the strain with monoglucosylated oligosaccharides was the same as in wild-type cells. We postulate that under these conditions of stress the monoglucosylated oligosaccharide-dependent folding process may be required in wild-type cells. Inactivation of this system leads to a higher degree of misfolded proteins in the ER and a subsequent induction of the UPR. Apparently, the G0 and the G2 oligosaccharides failed to interact with elements of the folding system, e.g., Cne1p, the calnexin analogue (de Virgilio *et al.*, 1993; Parlati *et al.*, 1995) and hence, the cells have a reduced ability to handle misfolded proteins. However, in *S.cerevisiae*, a direct involvement of Cne1p in the refolding network has never been demonstrated. In higher eukaryotes though, Ware *et al.* (1995) and Spiro *et al.* (1996) showed that calnexin and calreticulin, respectively, bound to oligosaccharides of the structure $Glc_1Man_{5-9}GlcNAc_2$. More recently, Rodan *et al.* (1996) and Zapun *et al.* (1997) were able to show that the single monoglucosylated oligosaccharide of ribonuclease B was responsible for the binding to dog pancreas calnexin and calreticulin.

We note that the unfolded protein response of Δ*gls2* strain (G2) was lower than the response of the Δ*alg10*Δ*gls2* (G2), the Δ*alg6* (G0) or the Δ*alg6*Δ*gls2* (G0) strains. We propose that this difference is due to the underglycosylation of glycoproteins in the Δ*alg* cells (Huffaker and Robbins, 1981, 1983; Stagljar *et al.*, 1994; te Heesen *et al.*, 1994; Burda *et al.*, 1996). Underglycosylation may lead to a reduced folding of proteins in the ER (Hickman *et al.*, 1977; Copeland *et al.*, 1988; Marquardt and Helenius, 1992; Imperiali and Rickert, 1995). The underglycosylation of glycoproteins and the impaired glucose trimming add up to the cumulative effect in stimulating the UPR in the Δ*alg6* (G0), Δ*alg6*Δ*gls2* (G0), and Δ*alg10*Δ*gls2* (G2).

In eukaryotes, the UDP-glucose: glycoprotein glucosyltransferase reglucosylates proteins displaying hydrophobic patches on their surface (Fernandez *et al.*, 1994). In *S.cerevisiae*, *KRE5*, a glucosyltransferase probably involved in cell wall biosynthesis (Meaden *et al.*, 1990) is the only locus displaying significant sequence homology to the UDP-glucose: glycoprotein glucosyltransferase protein. However, no glycoprotein glucosyltransferase activity has been determined in *S.cerevisiae*. Our NLO analysis data and the UPRE-*LacZ* results support the view that a general glycoprotein glucosyltransferase is absent in *S.cerevisiae* (Fernandez *et al.*, 1994): the Δ*alg6* single and Δ*alg6*Δ*gls2* double mutant cells contain no glucose residues on their lipid-linked oligosaccharides and the Δ*gls2* mutation would prevent the trimming of putatively reglucosylated *N*-linked glycoproteins. We analyzed both, the LLO and NLO of the Δ*alg6*Δ*gls2* strain. As expected the LLO consisted mainly of $Man_9GlcNAc_2$. The NLO profile showed the presence of $Man_9GlcNAc_2$ and $Man_8GlcNAc_2$ structures, but there was no evidence for glucose-containing oligosaccharides in this strain. However, a protein-specific glucosyltransferase reglucosylating *N*-linked high-mannose oligosaccharides of only a minor portion of glycoproteins cannot completely be excluded by our HPLC experiments since such an oligosaccharide would be below the limit of detection. Furthermore, using the UPRE-*lacZ* reporter under mild reducing conditions, both, the Δ*alg6* and the Δ*alg6*Δ*gls2* strains, induced the UPR to a similar extent, indicating that such a general glycoprotein glucosyltransferase in *S.cerevisiae* is very unlikely to exist. The lack of this glucosyltransferase makes a quality control system apparently leaky. The question remains how *S.cerevisiae* copes with such an open system. There are several possibilities to be considered. Recently, Zapun *et al.* (1997) have shown that the calnexin-oligosaccharide interaction prevented the glucose cleavage by glucosidase II. By such means, a G1 oligosaccharide-binding protein in the ER of *S.cerevisiae* (e.g., Cne1p) may reduce the cleavage rate of glucosidase II by monitoring the folding state of a bound glycoprotein. Further, a chaperone-like function of glucosidase II may also be possible. Here, glucosidase II would discriminate between folded and misfolded glycoproteins. The trimming of the innermost glucose residue would only occur if a protein has attained its correct three-dimensional structure. However, such a property needs to be demonstrated.

Our experimental system made it possible to assign a specific function of the monoglucosylated oligosaccharide in the processing of glycoproteins in the ER. However, this function is not essential, because a Δ*gls2* strains has no detectable growth phenotype. For specific glycoproteins, e.g., CPY, folding, export, and correct targeting is not dependent on the oligosaccharide (Schwaiger *et al.*, 1982; Winther *et al.*, 1991) suggesting back-up systems in the process of protein maturation. However, the degradation of incorrectly folded CPY molecules in the ER requires the oligosaccharide structures (Knop *et al.*, 1996). The genetic tailoring of these structures makes it possible to address their function in this process.

Materials and methods

Yeast strains and manipulations

Yeast strains used are listed in Table I. Standard protocols (Guthrie and Fink, 1991) were followed for growth of yeast, mating, sporulation, and ascus dissection. If not otherwise stated, the cells were grown at 30°C in either YPD medium (2%

Bacto-Peptone, 1% yeast extract, 2% glucose) or for metabolic labeling experiments overnight in MV medium (0.67% yeast nitrogen base, 2% glucose supplemented with the appropriate amino acids).

Construction of strains

Disruption of the GLS2 locus. The *GLS2* locus (GenEMBL accession no. Z36098, ORF YBR229c) showed high sequence similarities to the peptide sequence of pig liver glucosidase II (Flura *et al.*, 1997). A large portion of the N-terminal sequence of this ORF was removed by homologous recombination using a PCR product containing the complete kanamycin resistance (Wach *et al.*, 1994) gene flanked by *GLS2*-specific regions. The sequence of the kanamycin resistance gene was amplified by using the pFA6a-KanMX4 plasmid (Wach *et al.*, 1994) and by using the primers 5′-**ctgcgtatcttaaaatagcggtctcgaatcaaccgtatgcaac**tcgatgaattcgagctc and 5′-**tctaaaaaatgggtaccataagcccgcttggtaccaacgtgc**cgtacgctgcaggtcgac (boldface letters represent *GLS2* sequence). The resulting PCR fragment was used to transform the haploid yeast strain SS328 selecting for resistance to G418 (200 µg/ml) (Wach *et al.*, 1994). Transformants were analyzed for correct integration by whole cell PCR (Sathe *et al.*, 1991) using KanMX- (5′-gtattgatgttggacgag-3’) and *GLS2*-specific primers (5′-gagagctataactcaatg-3′, 5′-gaattgtggaatactggt-3′).

Disruption of the ALG10 locus. The identification of the *ALG10* locus (GenEMBL accession no. X87941, ORF YGR227w) will be described elsewhere (P. Burda, unpublished observations). A large portion of this ORF was removed by homologous recombination using a PCR product containing the complete kanamycin resistance gene flanked by *ALG10*-specific regions (Wach *et al.*, 1994). The sequence of the kanamycin resistance gene was amplified by PCR using the primers 5′-**atttatagaacgtctttttgcaactataatcaattctgttca**cgtacgctgcaggtcgac-3′ and 5′-**caatatcccaggtacaccgagcccagtcacatcgaacttata**tcgatgaattcgagctcg-3′ (boldface letters represent *ALG10* sequence), the resulting DNA transformed into strain SS328 and the cells were selected on G418 plates as described above. Transformants were analyzed for correct integration as described above using KanMX- (5′-gtattgatgttggacgag-3′) and *ALG10*-specific primers (5‘-cccagcaacgttagatga-3′).

Metabolic labeling and immunoprecipitations

The metabolic labeling was performed as described previously (Simons *et al.*, 1995). After labeling the cells were resuspended in 50 mM Tris–HCl, pH 7.5, 1% SDS, 2 mM PMSF, disrupted by vortexing with glass beads and boiled for 5 min. The immunoprecipitation of carboxypeptidase Y (CPY) was performed as described previously (Franzusoff *et al.*, 1991; te Heesen *et al.*, 1992). One unit of endoglycosidase H was added where indicated and incubated at 37°C for 3 h before SDS–PAGE.

Glucosidase II assay

Logarithmically growing cells were disrupted in 0.25 M sucrose, 10 mM Tris–HCl (pH 7.5), 1.5 mM AEBSF (Calbiochem, San Diego, CA) using glass beads. The membranes were enriched by subsequent centrifugation steps discarding the supernatant (600 × *g*, 10 min; 8500 × *g*, 10 min; 100,000 × *g*, 1 h). The pellet was resuspended in 50 mM sodium phosphate (pH 7.0) and homogenized by passing through a G24 needle. Finally, Triton X-100 was added to 1% final concentration and the solution was subjected to ultracentrifugation 100,000 × *g*, 1 h). The glucosidase II activity was assayed from the resulting supernatant according to Brada and Dubach (1984) using 4-methyl-umbelliferyl-α-D-glucopyranosid as artificial substrate. The specific enzyme activity was calculated from the range of maximal velocity (incubation < 5 min). One unit enzyme activity was defined as the release of 1 µmol 4-methyl-umbelliferon per min. The protein concentrations were determined by the Bio-Rad protein assay.

Labeling, extraction and analysis of LLO and NLO

The labeling and extraction of the LLO was performed according to Zufferey *et al.* (1995). However, the cells were preincubated at 37°C for 30 min prior to the labeling. The labeling reaction was terminated after 30 min. The hydrolyzed LLO were digested with EndoH (2 U; Boehringer Mannheim, Germany) in 25 mM sodium citrate, pH 5.5 at 37°C for 3 h and then desalted by applying onto spin columns (Bio-Rad, Bio-Spin; Hercules, CA) filled with each 0.5 ml equilibrated Serdolit Red and Blue (Serva, Heidelberg, Germany) ion-exchanger matrix. The run-through was filtered through a 0.45 µm filter (Millipore UFC3OHV00) and stored frozen at -20°C. The HPLC analysis was performed as described previously (Cacan *et al.*, 1993; Zufferey *et al.*, 1995). For the extraction of the NLO, the protein pellet obtained from the LLO extraction was digested with trypsin (TPCK-treated; 1 mg/ml; Sigma, St. Louis, MO) in 0.1 M ammonium bicarbonate, pH 7.5, over night at 37°C. The digest was boiled for 10 min to destroy the trypsin. The labeled *N*-linked oligosaccharides were released by EndoH cleavage and processed as described above. To identify the nature of the oligosaccharide species, NLO were incubated with each 50 mU of α-mannosidase (*Canavalia ensiformis*, Boehringer Mannheim, Germany) in 25 mM sodium citrate (pH 5.5) at ambient temperature for 2 h. The digestion was terminated by extracting the oligosaccharides once with chloroform/methanol (5:1, v/v). The aqueous phase was dried, resuspended, filtered, and subjected to HPLC analysis.

Integration of the UPRE-LacZ reporter construct

From the plasmid pLG-Δ178 UPRE-Y containing the *E.coli LacZ* gene under the control of the unfolded protein response element of yeast *KAR2*/BiP (Mori *et al.*, 1992) the 2µ element was excised using *Hind*III, the plasmid religated and then linearized with the *Stu*I restriction endonuclease before integrating the plasmid into the *ura3–52* locus of various strains. The correct integration of the reporter gene was tested by incubating the cells with 5 µg/ml tunicamycin for 2 h and then performing the ß-galactosidase assay (see below).

Beta-galactosidase assay

The ß-galactosidase assays were performed according to Guarente (1983) using o-nitrophenyl-ß-D-galactopyranoside (Sigma Chemicals, St. Louis, MO) as a substrate. The assays were performed in duplicates in two separate experiments.

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Abbreviations

CPY, carboxypeptidase Y; EndoH, endoglycosidase H; ER, endoplasmic reticulum; LLO, lipid-linked oligosaccharide; NLO, protein *N*-linked oligosaccharide.

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