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Ordered assembly of the asymmetrically branched lipid-linked oligosaccharide in the endoplasmic reticulum is ensured by the substrate specificity of the individual glycosyltransferases

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The assembly of the lipid-linked core oligosaccharide Glc₃Man₉GlcNAc₂, the substrate for N-linked glycosylation of proteins in the endoplasmic reticulum (ER), is catalyzed by different glycosyltransferases located at the membrane of the ER. We report on the identification and characterization of the *ALG12* locus encoding a novel mannosyltransferase responsible for the addition of the α -1,6 mannose to dolichol-linked Man₇GlcNAc₂. The biosynthesis of the highly branched oligosaccharide follows an ordered pathway which ensures that only completely assembled oligosaccharide is transferred from the lipid anchor to proteins. Using the combination of mutant strains affected in the assembly pathway of lipid-linked oligosaccharides and overexpression of distinct glycosyltransferases, we were able to define the substrate specificities of the transferases that are critical for branching. Our results demonstrate that branched oligosaccharide structures can be specifically recognized by the ER glycosyltransferases. This substrate specificity of the different transferases explains the ordered assembly of the complex structure of lipid-linked Glc₃Man₉GlcNAc₂ in the endoplasmic reticulum.

Key words: protein glycosylation/lipid-linked oligosaccharide/glycosyltransferases/endoplasmic reticulum/*Saccharomyces cerevisiae*

Introduction

Catalyzed by specific glycosyltransferases, the lipid-linked core oligosaccharide Glc₃Man₉GlcNAc₂ is formed by the sequential addition of sugars from their activated derivatives to the lipid carrier dolichyl-pyrophosphate (Kornfeld and Kornfeld, 1985; Tanner and Lehle, 1987; Herscovics and Orlean, 1993). The “one-linkage-one glycosyltransferase” hypothesis suggests that there is one distinct glycosyltransferase for every specific glycosidic linkage (Schachter, 1995). In the yeast *Saccharomyces cerevisiae alg* mutant strains (defective in asparagine-linked glycosylation), which are affected in different glycosyltransferases involved in the assembly of the lipid-linked core oligosaccharide, were

identified (Herscovics and Orlean, 1993; Orlean, 1997; Burda and Aebi, 1999). A common characteristic of the different *alg* mutant strains is the accumulation of a biosynthetic oligosaccharide intermediate specific for the defective *ALG* locus. Based on the assumption that the accumulating intermediate is the acceptor oligosaccharide of the reaction affected, defined glycosyltransferase activities could be assigned to the different *ALG* loci (Burda and Aebi, 1999). In addition, mutations in *ALG* loci lead to underglycosylation of secreted proteins *in vivo*. The reason for this underglycosylation appears to be the decreased affinity of the oligosaccharyltransferase toward incompletely assembled oligosaccharides; however, these oligosaccharides are still transferred to protein, albeit with a strongly reduced efficiency.

The synthesis of a highly branched and complex oligosaccharide requires a well organized assembly pathway and the notion that “the complex type of biosynthesis of the carbohydrate component of glycoproteins is truly amazing, and it is very difficult to see at present how this sequence of enzymatic reactions involving controlled addition and deletion of sugars to and from the glycopeptide is regulated” (Neuenberger, 1995) also applies to the lipid-linked oligosaccharide (Figure 1). Biosynthesis of such an asymmetric oligosaccharide structure requires highly specific enzymes working in an orchestrated fashion on the growing oligosaccharide chain. Here we report on the characterization of the *ALG12* locus encoding a dolichyl-phosphomannose dependent α -1,6 mannosyltransferase. We provide evidence that the branched mannose structure observed in the core oligosaccharide and its highly ordered assembly is due to the exact substrate specificity of glycosyltransferases involved in the assembly of lipid-linked Glc₃Man₉GlcNAc₂.

Results

Mild hypoglycosylation of CPY protein and altered CPY glycoforms in Δ alg12 strains

Previously we reported on the identification and characterization of the *ALG9* locus encoding an α -1,2 mannosyltransferase. It was proposed that Alg9p adds a mannose residue to the α -1,3-linked mannose (Burda *et al.*, 1996)(Figure 1). When searching the databases for sequences similar to the *ALG9* protein, we detected a family of yeast proteins which also share a common sequence motif with the PIG-B protein (for sequence alignments, see Canivence-Gansel *et al.*, 1998), a human Dol-P-Man-dependent mannosyltransferase required for GPI assembly (Takahashi *et al.*, 1996). Besides Alg9p the yeast protein family contained Gpi10p (Canivence-Gansel *et al.*, 1998; Sütterlin, C. *et al.*, 1998) and Smp3p (Irie *et al.*, 1991; H. Riezmann, personal communication), both essential α -1,2 mannosyltransferases required for GPI anchor biosynthesis. A fourth yeast protein of this family is encoded by ORF *YNR030w*. Mutants in this ORF were identified in a screen directed toward the isolation of mutant strains with

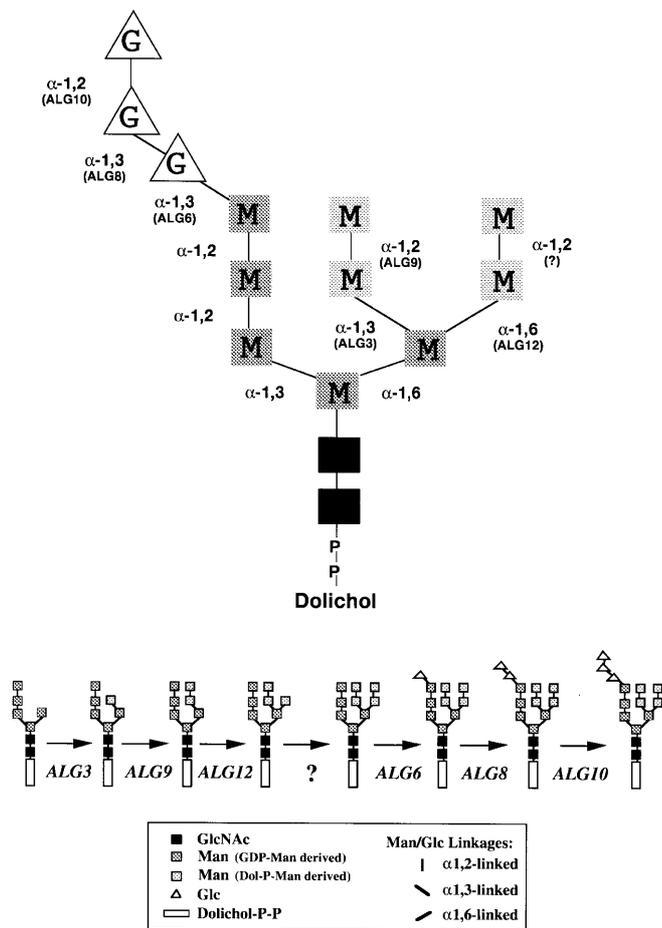


Fig. 1. Structure and assembly pathway of the dolicholpyrophosphate-linked oligosaccharide $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$. The stepwise synthesis occurs at the membrane of the ER catalyzed by a series of highly specific glycosyltransferases (encoded by *ALG* loci). The portion of the assembly pathway occurring at the luminal side of the ER is shown. The linkage of each individual glycosyl residue and the loci coding for the corresponding glycosyltransferases are indicated.

altered cell wall biogenesis (Lussier *et al.*, 1997). The locus was named *EMC39*. Due to our detailed analysis of the primary function of this locus (see below), we named it *ALG12*. In addition, Alg9p and Alg12p share a very similar, highly hydrophobic protein structure (data not shown). We disrupted the *ALG12* ORF in the wild-type strain SS328 by the integration of a kanamycin cassette and the haploid deletion strain proved to be fully viable. Next we asked whether the resulting $\Delta\text{alg12}::\text{kanMX4}$ strain (YG839), which showed no apparent growth phenotype, was affected in the N-linked protein glycosylation process.

Known *alg* mutant strains that are unable to complete the synthesis of the lipid-linked oligosaccharide at the luminal side of the ER lead to underglycosylation of secreted proteins because of the decreased affinity of the oligosaccharyltransferase (OTase) towards truncated lipid-linked oligosaccharides. In combination with a reduced OTase activity (*wbp1* mutation) (Stagljar *et al.*, 1994; Zufferey *et al.*, 1995) these *alg* mutants showed a synthetic growth defect at 30°C due to a severe glycosylation deficiency. We therefore crossed the Δalg12 strain with a *wbp1-2* mutant strain and a tetraploid tetrad was analyzed for growth and for

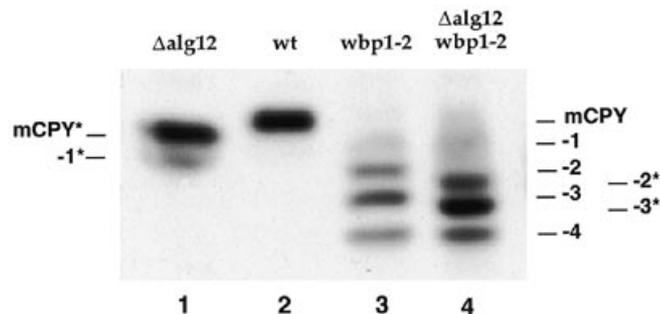


Fig. 2. The *alg12* mutation alters glycosylation of CPY *in vivo*. Four strains derived from a tetraploid tetrad of a cross $\Delta\text{alg12}\times\text{wbp1-2}$ were used for CPY-specific immunoblotting. The relevant genotype of the strains is indicated above the lanes. The position of mature CPY (mCPY) and the different glycoforms lacking up to four N-linked oligosaccharides (-1 to -4) are given. The position of mature CPY derived from strains containing the *Δalg12* mutation (mCPY*) and the different glycoforms also are indicated (-1* to -3*). Strains: YG840 (Δalg12 , lane 1), YG841 (wt, lane 2), YG842 (*wbp1-2*, lane 3), YG843 ($\Delta\text{alg12 wbp1-2}$, lane 4).

N-glycosylation of secreted proteins. In contrast to previously analyzed *alg wbp1* mutant strains, the resulting double mutant strain $\Delta\text{alg12 wbp1-2}$ (YG843) was able to grow at 30°C (data not shown). When we examined the N-glycosylation of carboxypeptidase Y (CPY) by Western blot analysis (Figure 2), we noticed a very weak hypoglycosylation of CPY in the Δalg12 strain (Figure 2, lane 1). In combination with the *wbp1-2* mutation, a severe hypoglycosylation was observed, but the same extent of glycosylation deficiency also was observed in the *wbp1-2* single mutant strain (Figure 2, lanes 3 and 4). However, we detected an effect of the *Δalg12* mutation on the mobility of the different CPY glycoforms: mature CPY in the Δalg12 strain as well as the different glycoforms in the $\Delta\text{alg12 wbp1-2}$ double mutant strain (Figure 2, lanes 1 and 4) migrated faster in SDS-PAGE than the corresponding molecules in either the wild-type (Figure 2, lane 2) or the *wbp1-2* strain (Figure 2, lane 3). Nonglycosylated CPY protein in both the *wbp1-2* and the $\Delta\text{alg12 wbp1-2}$ strain had the same mobility. This mobility shift of the glycoforms can be attributed to the transfer of incomplete assembled oligosaccharide to protein in the ER and also was observed in other mutant strains affected in the biosynthesis of the lipid-linked oligosaccharide (Burda *et al.*, 1996; Jakob *et al.*, 1998).

Δalg12 mutant strains accumulate lipid-linked Man₇GlcNAc₂ and low levels of lipid-linked Glc₃Man₇GlcNAc₂

We analyzed the dolichol-linked oligosaccharides that accumulate in a Δalg12 strain. For that purpose, we labeled Δalg12 cells *in vivo* with [³H]mannose and isolated the radiolabeled lipid-linked oligosaccharide. After the release of the oligosaccharide from the lipid-carrier dolichol by acidic hydrolysis we separated the oligosaccharides by HPLC. Oligosaccharides of known structure were used as standards. Indeed, an altered biosynthesis of lipid-linked oligosaccharides was observed in the Δalg12 strain (Figure 3): this mutant strain was not able to synthesize lipid-linked $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$. A major oligosaccharide intermediate (eluting after 42 min in this experiment) and a minor peak (at 57 min) were detected (Figure 3B). Comparison to the oligosaccharide profile observed in the wild-type strain (Figure 3A) suggested that the major peak represents a $\text{Hex}_7\text{GlcNAc}_2$

oligosaccharide, whereas the minor peak had the retention time expected for Hex₁₀GlcNAc₂ (Hex = mannose or glucose). The amount of the minor peak was found to be dependent on the genetic background of the $\Delta alg12$ strains. A deletion of the *ALG12* locus in the SS328 wild-type background resulted in a decrease of this Hex₁₀GlcNAc₂ peak (Figure 4C). We first analyzed the structure of the putative Man₇GlcNAc₂ oligosaccharide in more detail. Previously we reported on the isolation of the *ALG9* locus and showed that $\Delta alg9$ cells accumulate lipid-linked Man₆GlcNAc₂. Detailed analysis by Trimble and co-workers using ¹H-NMR technique revealed that the Man₆GlcNAc₂ oligosaccharide accumulating in $\Delta alg9$ cells contained an additional α -1,3 mannose (Trimble, personal communication) linked to the Man₅GlcNAc₂ oligosaccharide found in $\Delta alg3$ strains (Verostek *et al.*, 1993a; Figure 1). Digestion of this Man₆GlcNAc₂ oligosaccharide by an α -1,2 mannosidase resulted in a Man₄GlcNAc₂ structure (Figure 4A,B). The same oligosaccharide was observed after α -1,2 mannosidase treatment of the Man₇GlcNAc₂ accumulating in $\Delta alg12$ cells (Figure 4C,D). Knowing that the $\Delta alg9$ strain accumulates the same oligosaccharide intermediate as the $\Delta alg9$ $\Delta alg12$ double mutant strain ($\Delta alg9$ mutation is epistatic over $\Delta alg12$; data not shown), we conclude that lipid-linked Man₇GlcNAc₂ oligosaccharides in $\Delta alg12$ cells contain the α -1,3- α -1,2 di-mannose branch of the lipid-linked oligosaccharide. Therefore, the *ALG12* locus most likely encodes the α -1,6 mannosyltransferase required for the synthesis of Man₈GlcNAc₂-PP-Dol.

Earlier studies in yeast cells indicated that the oligosaccharide intermediate Man₅GlcNAc₂-PP-Dol can be glucosylated yielding Glc₃Man₅GlcNAc₂-PP-Dol, albeit at a low level (Verostek *et al.*, 1993a). Thus, we speculated that in $\Delta alg12$ strains the accumulating Man₇GlcNAc₂ might be a (suboptimal) substrate for the Alg6p glucosyltransferase (Runge *et al.*, 1984; Reiss *et al.*, 1996) and subsequent glucosylation by Alg8p (Stagljar, I. *et al.*, 1994) and Alg10p (Burda and Aebi, 1998) transferases might result in the formation of Glc₃Man₇GlcNAc₂-PP-Dol, the minor peak observed in $\Delta alg12$ strains (Figure 3 B). To test this hypothesis we transformed a $\Delta alg12$ mutant strain with a high copy number plasmid overexpressing Alg6p glucosyltransferase (Reiss *et al.*, 1996) and asked, whether biosynthesis of the oligosaccharide could be shifted towards the Hex₁₀GlcNAc₂-PP-Dol. This was indeed the case. Almost equal amounts of Man₇GlcNAc₂ and Hex₁₀GlcNAc₂ oligosaccharide were observed in the Alg6p overexpressing strain (Figure 5B). To show that the postulated Glc₃Man₇GlcNAc₂ peak contained glucose residues, we constructed a $\Delta alg12$ $\Delta alg5$ double mutant strain. A disruption of the *ALG5* locus causes loss of Dol-P-Glc synthase activity (Runge *et al.*, 1984; te Heesen *et al.*, 1994); thus, cells are devoid of Dol-P-Glc, the donor for the glucosylation reactions in the biosynthesis of the putative Glc₃Man₇GlcNAc₂-PP-Dol. We transformed this $\Delta alg12$ $\Delta alg5$ double mutant strain with the Alg6p overexpressing plasmid. In contrast to the $\Delta alg12$ single mutant overexpressing Alg6p (Figure 5B), the Alg6p overexpressing $\Delta alg12$ $\Delta alg5$ double mutant strain (YG846) accumulated only one oligosaccharide intermediate which comigrated with the Man₇GlcNAc₂ oligosaccharide (Figure 5B,C). This result confirms the presence of glucose residues in the minor Glc₃Man₇GlcNAc₂ peak observed in the $\Delta alg12$ strain. Taken together, our data show that the *ALG12* locus is required for the assembly of the lipid-linked core oligosaccharide Glc₃Man₉GlcNAc₂. $\Delta alg12$ mutant strains accumulate lipid-linked

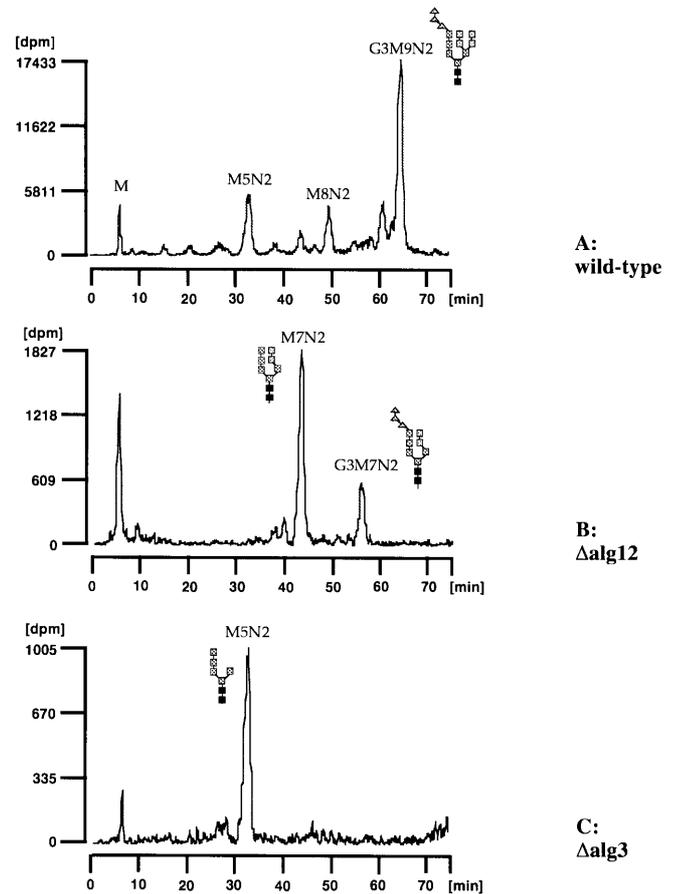


Fig. 3. Analysis of lipid-linked oligosaccharides of the $\Delta alg12$ mutant strain FHEN005-02C(A). Cells were labeled with [³H] mannose. Lipid-linked oligosaccharides were extracted and hydrolyzed, and the liberated oligosaccharides were analyzed by HPLC. Oligosaccharides isolated from strain SS328 (wild-type) and YG248 ($\Delta alg3$) served as standards. The position of mannose (M), Man_{5,7,8}GlcNAc₂ (M5,7,8N2) and Glc₃Man₉GlcNAc₂ (G3M9N2) are indicated. The structure of the corresponding oligosaccharides is illustrated. (A) Wild-type strain SS328. (B) $\Delta alg12$ mutant strain FHEN005-02C(A). (C) $\Delta alg3$ mutant strain YG248.

Man₇GlcNAc₂; however, Man₇GlcNAc₂-PP-Dol can be glucosylated in these cells resulting in Glc₃Man₇GlcNAc₂-PP-Dol.

Glucosylation of intermediates of the LLO assembly pathway

The observation that the α -1,2- α -1,2-di-mannose branch in $\Delta alg12$ strains can be glucosylated before completion of the Man₉GlcNAc₂ core prompted us to test whether glucosylation of oligosaccharides lacking the two Dol-P-Man derived di-mannose arms is also possible. For that purpose the Alg6p glucosyltransferase was overexpressed in both $\Delta alg3$ and $\Delta alg9$ mutant strains, the LLOs accumulating in these strains were analyzed by HPLC and further characterized by α -1,2 mannosidase digestion (Figure 6). In the $\Delta alg3$ strain overexpressing Alg6p we observed two oligosaccharide species (Figure 6A). The oligosaccharide eluting at 37 min comigrates with the Man₅GlcNAc₂ oligosaccharide (data not shown), whereas the minor oligosaccharide eluting at 52 min was not observed in $\Delta alg3$ cells (Aebi *et al.*, 1996) (data not shown). As expected, α -1,2 mannosidase

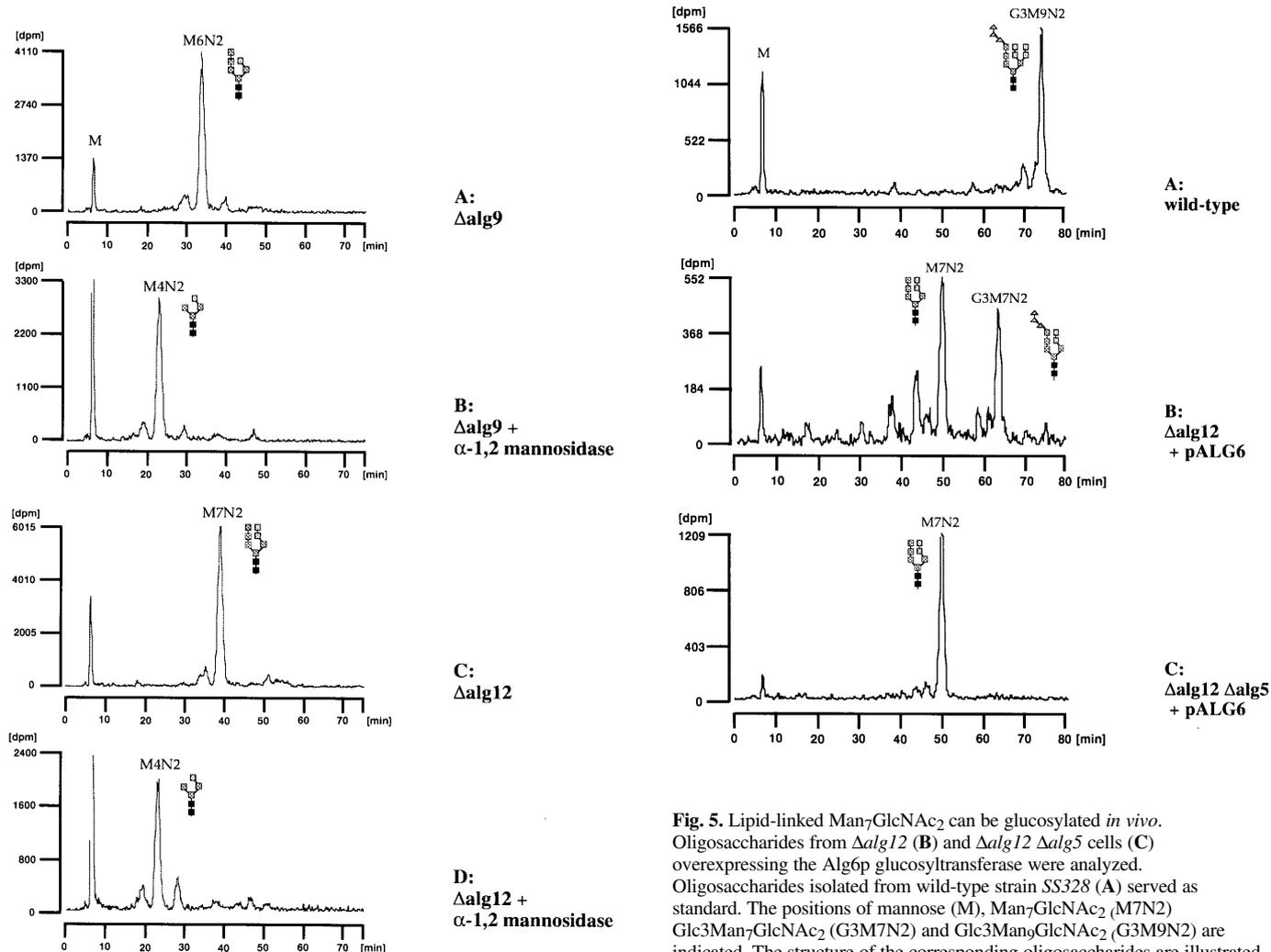


Fig. 4. HPLC analysis of exo- α -1,2 mannosidase digestion products of $\Delta alg9$ and $\Delta alg12$ derived oligosaccharides. The radiolabeled oligosaccharides were prepared and digested with *A.saitoi* α -1,2 mannosidase. The digested oligosaccharide products were analyzed by HPLC. The positions of mannose (M), $Man_4GlcNAc_2$ (M4N2), $Man_6GlcNAc_2$ (M6N2) and $Man_7GlcNAc_2$ (M7N2) and the corresponding oligosaccharide structures are shown. (A) Oligosaccharide derived from $\Delta alg9$ strain YG414. (B) Oligosaccharide from $\Delta alg9$ strain (A) treated with exo- α -1,2 mannosidase. (C) Oligosaccharide derived from $\Delta alg12$ strain YG839. (D) Oligosaccharide from $\Delta alg12$ strain (C) treated with exo- α -1,2 mannosidase.

digestion resulted in a shift of the $Man_5GlcNAc_2$ peak due to the removal of the two α -1,2-linked mannose residues yielding $Man_3GlcNAc_2$ (Figure 6B). However, the peak at 53 min was not affected by the mannosidase treatment, compatible with the hypothesis that the addition of glucose residues is protecting the two α -1,2-linked mannoses from hydrolysis by the exo- α -1,2 mannosidase. The same effect of Alg6p overexpression was observed in $\Delta alg9$ strains. A novel oligosaccharide species eluting at a position compatible with $Glc_3Man_6GlcNAc_2$ was found to be resistant toward α -1,2 mannosidase, whereas the $Man_6GlcNAc_2$ oligosaccharide was cleaved to yield $Man_4GlcNAc_2$ (Figure 6C,D). When we compared the amount of these glucosylated oligosaccharide intermediates to those synthesized in $\Delta alg12$ cells overexpressing Alg6p (Figure 5B), we

Fig. 5. Lipid-linked $Man_7GlcNAc_2$ can be glucosylated *in vivo*. Oligosaccharides from $\Delta alg12$ (B) and $\Delta alg12 \Delta alg5$ cells (C) overexpressing the Alg6p glucosyltransferase were analyzed. Oligosaccharides isolated from wild-type strain SS328 (A) served as standard. The positions of mannose (M), $Man_7GlcNAc_2$ (M7N2), $Glc_3Man_7GlcNAc_2$ (G3M7N2) and $Glc_3Man_9GlcNAc_2$ (G3M9N2) are indicated. The structure of the corresponding oligosaccharides are illustrated. (A) Wild-type strain SS328. (B) $\Delta alg12$ mutant strain transformed with the high copy number plasmid pALG6 (YG845, SS328 background). (C) $\Delta alg12 \Delta alg5$ mutant strain transformed with the high copy number plasmid pALG6 (YG846).

observed that $Man_7GlcNAc_2$ oligosaccharide is glucosylated more efficiently as compared to $Man_5GlcNAc_2$ and $Man_6GlcNAc_2$, respectively. The dependence of the glucosylated oligosaccharide species on the glucose-donor Dol-P-Glc was proven by the result that these species were missing when either $\Delta alg3 \Delta alg5$ or $\Delta alg9 \Delta alg5$ double mutant strains were used for Alg6p overexpression (data not shown). Furthermore, overexpression of the Alg8p glucosyltransferase (Stagljar et al., 1994) catalyzing the addition of the second α -1,3-linked oligosaccharide (Figure 1) did not result in glucosylation of the $Man_5GlcNAc_2$ oligosaccharide in $\Delta alg3$ cells (data not shown). In conclusion, our data suggest that the α -1,2-linked mannose of the α -1,3- α -1,2 di-mannose branch (missing in both $\Delta alg3$ and $\Delta alg9$ mutant strains) is an important determinant of Alg6p substrate specificity and that synthesis of the $Man_9GlcNAc_2$ core is a prerequisite for efficient Alg6p-dependent glucosylation of the lipid-linked oligosaccharide.

Glucosylated lipid-linked oligosaccharide intermediates are transferred more efficiently to protein in vivo

It has been demonstrated both *in vitro* (Murphy and Spiro, 1981) and *in vivo* (Burda and Aebi, 1998) that glucosylation of the

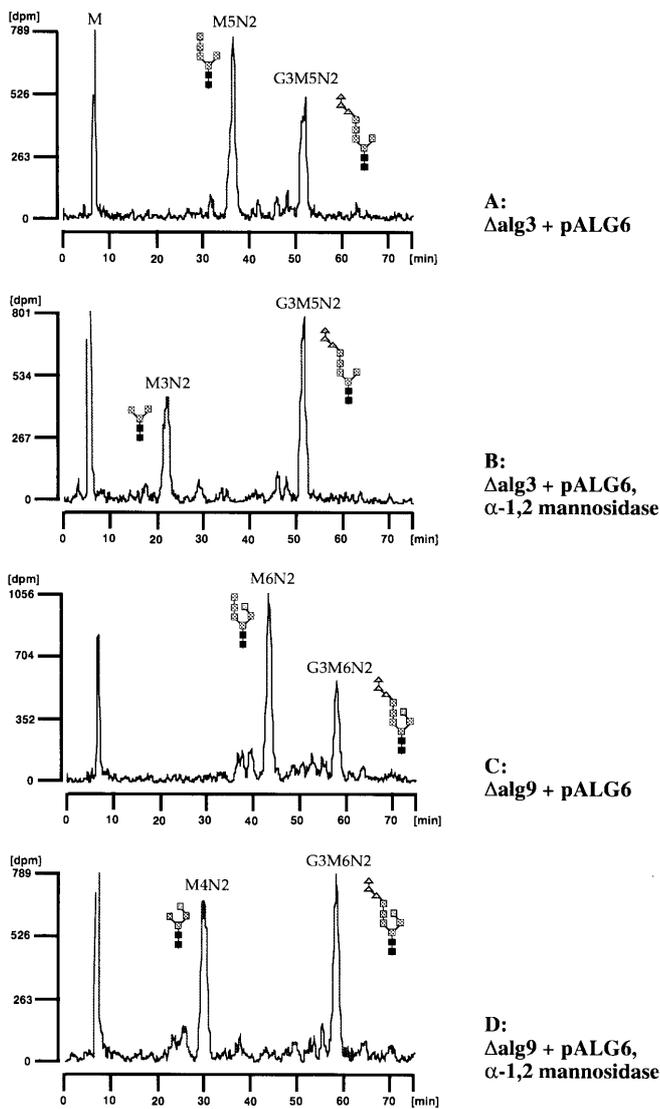


Fig. 6. Glycosylation of incompletely mannosylated lipid-linked oligosaccharides. The Alg6p glucosyltransferase was overexpressed both in *Δalg3* and *Δalg9* mutant cells. The oligosaccharides from these strains were characterized by exo- α -1,2 mannosidase digestion followed by HPLC analysis. The position of mannose (M), Man₃GlcNAc₂ (M3N2), Man₄GlcNAc₂ (M4N2), Man₅GlcNAc₂ (M5N2), Man₆GlcNAc₂ (M6N2), Glc3Man₅GlcNAc₂ (G3M5N2), and Glc3Man₆GlcNAc₂ (G3M6N2) and the corresponding oligosaccharide structures are given. (A) Oligosaccharides isolated from *Δalg3* strain overexpressing Alg6p glucosyltransferase (YG859). (B) Oligosaccharides isolated from *Δalg3* strain overexpressing Alg6p glucosyltransferase digested with exo- α -1,2 mannosidase. (C) Oligosaccharides isolated from *Δalg9* strain overexpressing Alg6p glucosyltransferase (YG849). (D) Oligosaccharides from *Δalg9* strain overexpressing Alg6p glucosyltransferase digested with exo- α -1,2 mannosidase.

lipid-linked oligosaccharide, in particular the presence of the terminal α -1,2-linked glucose, is necessary for efficient transfer of the core oligosaccharide to protein. To test a fully glucosylated, but partially mannosylated lipid-linked oligosaccharide as a substrate for the oligosaccharyltransferase *in vivo*, we analyzed the glycosylation of the model protein CPY in different *Δalg3* mutant strains (Figure 7). Glycosylation of CPY was improved due to Alg6p overexpression both in a *Δalg3* and a *Δalg3 wbp1-2*

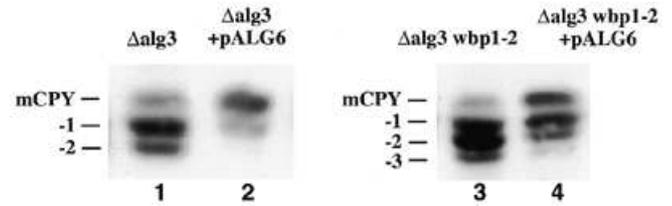


Fig. 7. N-linked glycosylation of CPY in *Δalg3* strains overexpressing Alg6p glucosyltransferase. *Δalg3* single mutant and *Δalg3 wbp1-2* double mutant strain, respectively, were transformed with the high copy number plasmid pALG6. Glycosylation of CPY was analyzed by CPY-specific immunoblotting. CPY isolated from the pALG6 transformed mutant cells was compared to CPY molecules of untransformed mutant strains. The relevant genotype of the strains is indicated above the lanes. The position of mature CPY (mCPY) and the different glycoforms lacking up to three N-linked oligosaccharides (-1 to -3) are indicated. Strains: YG248 (*Δalg3*, lane 1), YG859 (*Δalg3* + pALG6, lane 2), YG857 (*Δalg3 wbp1-2*, lane 3), YG858 (*Δalg3 wbp1-2* + pALG6, lane 4).

strain. The same observations upon CPY analysis were made in either a *Δalg9* strain or a *Δalg9 wbp1-2* strain (data not shown). In addition, overexpression of Alg6p in both *Δalg3 wbp1-2* and *Δalg9 wbp1-2* double mutant strains restored the viability at 30°C (data not shown). This supports the hypothesis that complete glucosylation of the lipid-linked oligosaccharide is a central factor in the recognition of the oligosaccharide substrate by the oligosaccharyltransferase complex.

Acceptor specificity of Alg12p mannosyltransferase

As shown above, efficient glucosylation of the lipid-linked oligosaccharide requires the completion of the Man₅GlcNAc₂ structure. Nevertheless, addition of glucose residues occurred on an earlier intermediate in the biosynthesis, however with reduced efficiency. Likewise, in principle it is possible to add either α -1,3- or α -1,6 mannose to the α -1,6 mannose of the lipid-linked Man₅GlcNAc₂ which is (according to the currently accepted topological model of LLO biosynthesis) translocated into the lumen of the ER. However, the accumulation of Man₅GlcNAc₂-PP-Dol in a *Δalg3* strain and of Man₆GlcNAc₂-PP-Dol in a *Δalg9* strain, respectively, suggests that the order of addition is determined by the specificity of the α -1,6 mannosyltransferase Alg12p: no oligosaccharide containing the α -1,6-linked mannose was detected in an *Δalg3* strain (Verostek *et al.*, 1993b). We therefore asked whether overexpression of the Alg12p activity in a *Δalg3* or in a *alg9* mutant strain may result in oligosaccharide intermediates containing the α -1,6 mannose. For that purpose *Δalg3* and *Δalg9* cells were transformed with a high copy number vector carrying the *ALG12* locus, the resulting strains were labeled with [³H]-mannose and the radiolabeled oligosaccharides isolated from these strains analyzed by HPLC. The Man₅GlcNAc₂ oligosaccharide was found in *Δalg3* cells overexpressing Alg12p (Figure 8A). It comigrated with the oligosaccharide accumulating in *Δalg3* strains (data not shown) and was reduced to Man₃GlcNAc₂ when treated with exo- α -1,2 mannosidase (Figure 8B). No additional oligosaccharide due to Alg12p overexpression was observed. However, when we analyzed the oligosaccharides deriving from *Δalg9* cells overexpressing *ALG12*, we detected an additional oligosaccharide intermediate (Figure 8C) that was not present in the *Δalg9* mutant strain (Figure 4A). According to the retention time we postulated that this additional oligosaccharide is likely Man₇GlcNAc₂, since it comigrated with the Man₇GlcNAc₂ intermediate observed in *Δalg12* cells (data not shown). When we analyzed these

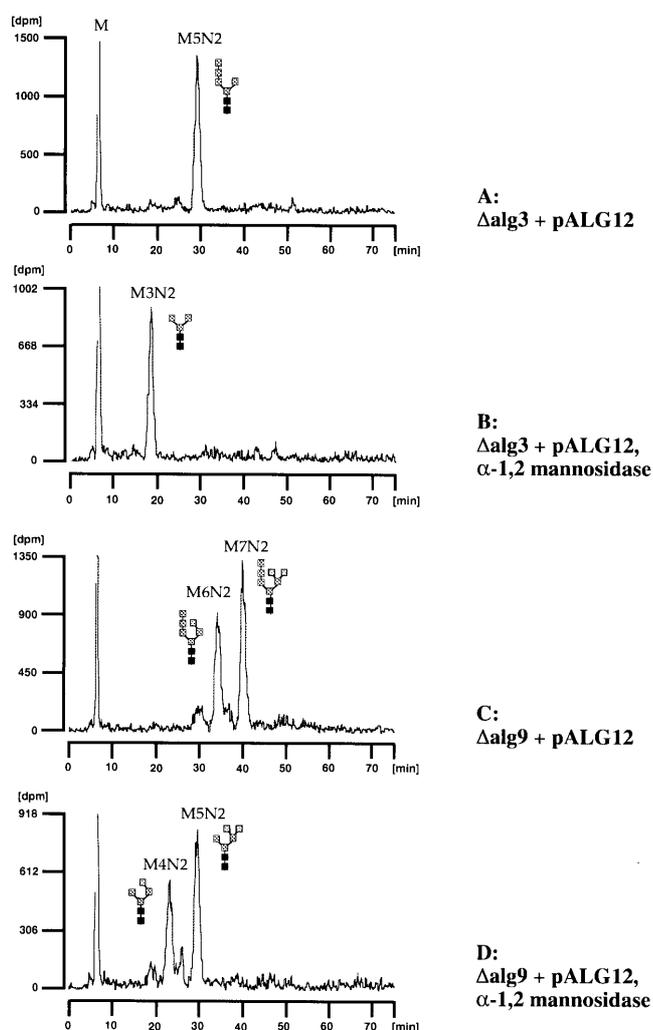


Fig. 8. Analysis of lipid-linked oligosaccharides in *alg* mutant strains overexpressing Alg12p. Lipid-linked oligosaccharides accumulating in $\Delta alg9$ and $\Delta alg3$ cells overexpressing Alg12p mannosyltransferase were isolated and analyzed using HPLC. The structure of the oligosaccharides was investigated by exo- α -1,2 mannosidase digestion. The position of mannose (M), $\text{Man}_3\text{GlcNAc}_2$ (M3N2), $\text{Man}_4\text{GlcNAc}_2$ (M4N2), $\text{Man}_5\text{GlcNAc}_2$ (M5N2), $\text{Man}_6\text{GlcNAc}_2$ (M6N2), and $\text{Man}_7\text{GlcNAc}_2$ (M7N2) is shown and the corresponding oligosaccharide structures are indicated. (A) Oligosaccharides isolated from $\Delta alg3$ strain transformed with pALG12 (YG855) (A) and treated with α -1,2 mannosidase (B). Oligosaccharides isolated from $\Delta alg9$ strain transformed with pALG12 (YG848) (C) and treated with α -1,2 mannosidase (D).

oligosaccharides by α -1,2 mannosidase digestion, we noticed two peaks representing $\text{Man}_4\text{GlcNAc}_2$ and $\text{Man}_5\text{GlcNAc}_2$. The same ratio of the $\text{Man}_4\text{GlcNAc}_2$ to $\text{Man}_5\text{GlcNAc}_2$ oligosaccharide was observed after α -1,2 mannosidase digestion as for $\text{Man}_6\text{GlcNAc}_2$ to $\text{Man}_7\text{GlcNAc}_2$, and therefore we conclude that the $\text{Man}_4\text{GlcNAc}_2$ oligosaccharide derives from digestion of $\text{Man}_6\text{GlcNAc}_2$ and the $\text{Man}_5\text{GlcNAc}_2$ oligosaccharide from $\text{Man}_7\text{GlcNAc}_2$. The structural analysis by α -1,2 mannosidase shows that in contrast to the $\text{Man}_7\text{GlcNAc}_2$ intermediate from $\Delta alg12$ cells, the $\text{Man}_7\text{GlcNAc}_2$ species from $\Delta alg9$ cells overexpressing Alg12p has only two cleavable α -1,2 linked mannose residues. Thus, it contains an additional mannose residue which is added by the overexpressed *ALG12* protein. We propose that the additional residue is the α -1,6-linked mannose.

Our results demonstrate that the Alg3p and Alg12p mannosyltransferase clearly differ in their acceptor oligosaccharide specificity, because the addition of the α -1,6-linked mannose by Alg12p requires a minimal structure including the α -1,3-linked mannose residue added by Alg3p. Moreover, these experiments provide further evidence that the *ALG12* locus indeed encodes a mannosyltransferase, because overexpression of this protein in a $\Delta alg9$ strain results in a novel lipid-linked oligosaccharide intermediate.

Discussion

The assembly of lipid-linked $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ takes place at the membrane of the endoplasmic reticulum (ER). Current topological models suggest that the first part of lipid-linked oligosaccharide biosynthesis takes place at the cytoplasmic side of the ER membrane, whereas the synthesis continues in the lumen after flipping of the lipid-linked $\text{Man}_5\text{GlcNAc}_2$ intermediate across the ER membrane. In this discussion, we will focus on the assembly pathway of the oligosaccharide after the translocation of $\text{Man}_5\text{GlcNAc}_2\text{-PP-Dol}$ to the lumen of the ER. Four mannose and three glucose residues are added by specific glycosyltransferases using as substrates dolichylphosphate-activated mannose and glucose, respectively. In recent years, different yeast loci have been identified which are supposed to encode such specific mannosyl- or glucosyltransferases (Orlean, 1997; Burda and Aebi, 1999). In this report, we describe the *ALG12* locus encoding a novel α -1,6 mannosyltransferase involved in the biosynthesis of the lipid-linked oligosaccharide. *ALG12* deletion strains are not able to synthesize the complete lipid-linked core oligosaccharide $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$, but accumulate as a major product $\text{Man}_7\text{GlcNAc}_2\text{-PP-Dol}$. Our observation that overexpression of the *ALG12* locus in a $\Delta alg9$ strain results in a novel oligosaccharide species containing one additional mannose residue which is normally not present in $\Delta alg9$ cells, strongly suggests that Alg12p is indeed the Dol-P-Man dependent α -1,6 mannosyltransferase. The altered mobility of glycoforms of the marker protein CPY in $\Delta alg12$ cells as compared to wild-type cells shows that incompletely assembled oligosaccharide is transferred to protein. However, we detected glucosylated oligosaccharide intermediates ($\text{Glc}_3\text{Man}_7\text{GlcNAc}_2$) in $\Delta alg12$ mutant strains. This observation might explain the mild hypoglycosylation phenotype noticed in these cells, because the presence of the terminal α -1,2 linked glucose on the glucosylated oligosaccharide intermediate makes it a good substrate of the oligosaccharyltransferase (OTase) complex. Consistent with this observation, the $\Delta alg12$ mutation does not synthetically interact with the OTase mutation *wbp1-2* and was therefore not identified in a screen directed towards mutants with altered biosynthesis of lipid-linked oligosaccharides (Zufferey *et al.*, 1995). The preference for glucosylated lipid-linked oligosaccharides also has been observed in higher eukaryotes. A CHO mutant cell line unable to synthesize Dol-P-Man accumulated $\text{Man}_5\text{GlcNAc}_2\text{-PP-Dol}$; however, a minor proportion of lipid-linked oligosaccharide was glucosylated yielding $\text{Glc}_3\text{Man}_5\text{GlcNAc}_2$. Analysis of the protein-bound oligosaccharides revealed that, nevertheless, the glucosylated species were preferentially transferred to the nascent polypeptide chain (Stoll *et al.*, 1992).

alg12 mutant strains have been found previously in a screen for mutants with altered cell wall biogenesis (Lussier *et al.*, 1997). Due to the essential role of mannoproteins in yeast cell wall biogenesis (Klis, 1994) the incomplete oligosaccharide structure

which is transferred to protein in *Δalg12* cells might be the cause of this cell wall phenotype.

We have identified the *ALG12* locus by searching the available databases for sequences similar to the *ALG9* protein. As all other ER glycosyltransferases using dolichylphosphate-activated hexose, Alg12p is a highly hydrophobic protein. In addition, Alg12p possesses a putative N-terminal signal sequence (von Heijne, 1986) directing the import of the protein into the ER membrane. The database search revealed a family of yeast proteins with a common sequence motif (Canivence-Gansel *et al.*, 1998). The homologous proteins Gpi10p and Smp3p are essential for viability and both encode Dol-P-Man-dependent mannosyltransferases that are located in the ER and involved in the biosynthesis of GPI anchors (Benghezal *et al.*, 1995; Canivence-Gansel *et al.*, 1998; Sütterlin *et al.*, 1998; Riezman, personal communication). The sequence motif shared by this mannosyltransferase family might represent a recognition sequence for the common substrate Dol-P-Man. However, we did not detect this motif in the sequence of the Dol-P-Man-dependent *ALG3* mannosyltransferase.

As soon as the core oligosaccharide is transferred to protein, trimming of the protein-linked Glc₃Man₉GlcNAc₂ occurs in the ER by the enzymes glucosidase I, glucosidase II, and mannosidase I (for a review, see Moremen *et al.*, 1994). In recent years, it has become more and more evident that individual sugar residues of the protein-bound oligosaccharide are required for specific functions in the endoplasmic reticulum (Helenius *et al.*, 1997; Jakob *et al.*, 1998). In particular, the Glc₁Man₉GlcNAc₂ structure is specifically recognized by calnexin and calreticulin, a function required in the quality control of glycoprotein folding. *In vivo* studies by Verostek and co-workers (Verostek *et al.*, 1993b) showed that the complete Man₉GlcNAc₂ core might be important for efficient glucose trimming of protein-bound oligosaccharides. Other studies on the yeast ER processing of oligosaccharides revealed that mannosidase I activity was dependent upon the terminal α-1,2-linked mannose of the α-1,6-α-1,2 di-mannose branch of protein-linked oligosaccharide (Ziegler and Trimble, 1991). In addition, recognition and degradation of malformed glycoproteins in the ER in yeast seems to be dependent on the correctly processed Man₈GlcNAc₂ oligosaccharide structure (Knop *et al.*, 1996). Therefore, the transfer of fully assembled Glc₃Man₉GlcNAc₂ oligosaccharide to protein has to be ensured.

The selective transfer of only complete assembled oligosaccharide is guaranteed by the substrate specificity of the oligosaccharyltransferase (Silberstein and Gilmore, 1996). However, reduced transfer rate of lipid-linked biosynthetic oligosaccharide intermediates to protein has been observed both *in vitro* and *in vivo*. Small oligosaccharides such as chitobiose (GlcNAc₂; Sharma *et al.*, 1981) and different oligosaccharide intermediates synthesized by a series of *alg* mutant strains (Burda and Aebi, 1999) are transferred to protein, albeit at a reduced level. For efficient transfer, the terminal α-1,2-linked glucose residue of the lipid-linked oligosaccharide is required, and from our *in vivo* data we estimate that oligosaccharides lacking this terminal glucose are transferred to protein with a 10-fold reduced efficiency (Burda and Aebi, 1998). Based on these data, we speculate that the OTase recognizes the oligosaccharide substrate via two structurally distant motifs, the chitobiose stem and the terminal α-1,2 glucose residue. Our observation that N-linked protein glycosylation is an efficient process in *Δalg12* cells (which accumulate Glc₃Man₇GlcNAc₂) also suggests the di-mannose side branches contribute little to the substrate recognition by the OTase. It is therefore essential that the biosynthesis of the lipid-linked

oligosaccharide follows a highly defined pathway which terminates in the addition of the α-1,2-linked glucose residue by the *ALG10* glucosyltransferase.

This precision in the assembly of lipid-linked Glc₃Man₉GlcNAc₂ is achieved by the high specificity of the ER glycosyltransferases towards their lipid-linked oligosaccharide substrates. Our *in vivo* data obtained in yeast demonstrate that the α-1,3-α-1,2 di-mannose arm is assembled prior to the α-1,6-α-1,2 di-mannose antenna resulting in lipid-linked Man₉GlcNAc₂ (Figure 1), an assembly order also found in higher eukaryotes (Rearick *et al.*, 1981). The defined assembly of the Man₉GlcNAc₂ core is the consequence of the acceptor oligosaccharide specificity of the branching mannosyltransferases Alg3p and Alg12p, respectively. The addition of the α-1,6-linked mannose by Alg12p requires the presence of the α-1,3 mannose added by the *ALG3* mannosyltransferase. In contrast, Alg3p recognizes efficiently lipid-linked Man₅GlcNAc₂ and catalyzes the addition of the α-1,3-linked mannose (Aebi *et al.*, 1996; Kuster and te Heesen, unpublished observations). The fact that the *Δalg9* mutation is epistatic over *Δalg12* suggests that the presence of the complete α-1,3-α-1,2 di-mannose antenna is a prerequisite for Alg12p activity. However, absence of the α-1,2 mannose can be overcome by overexpression of Alg12p.

The fact that incomplete mannosylated oligosaccharide intermediates can be glucosylated raises an interesting aspect of the substrate specificity of the Alg6p glucosyltransferase that adds the first glucose residue to lipid-linked Man₉GlcNAc₂ (Runge *et al.*, 1984; Reiss *et al.*, 1996). The significant amount of Glc₃Man₇GlcNAc₂ observed in *Δalg12* mutant cells and the absence of detectable amounts of glucosylated LLO in *Δalg3*, *Δalg9*, and *Δalg9* mutant strain overexpressing Alg12p suggests that the α-1,3-α-1,2 di-mannose antenna of the oligosaccharide is an important determinant of Alg6p substrate specificity. However, the α-1,6-α-1,2 di-mannose branch must be recognized as well, because pronounced glucosylation of Man₇GlcNAc₂ is detected only under specific conditions. This specificity of Alg6p ensures that in wild-type cells the first glucose is predominantly attached to the fully assembled Man₉GlcNAc₂ core. The Alg6p acceptor specificity therefore represents a checkpoint for complete mannosylated oligosaccharide intermediates. Furthermore, Alg6p also has to recognize the outer α-1,3-α-1,2-α-1,2 tri-mannose arm in order to avoid glucose addition to the other terminal α-1,2 mannose residues. This suggests that several of the mannose residues of the Man₉GlcNAc₂ oligosaccharide determine the substrate specificity for the Alg6p mediated glucosylation reaction. How such a complex substrate recognition by the highly hydrophobic *ALG6* protein is achieved requires further investigation. The addition of the last two glucoses catalyzed by Alg8p and Alg10p, respectively (Stagljar *et al.*, 1994; Burda and Aebi, 1998), seems to be independent of the mannosylation state of the oligosaccharide, because we did not observe mono-or-di-glucosylated Man₅₋₇GlcNAc₂-PP-Dol. This suggests that the presence of the first α-1,2-linked glucose residue is sufficient for Alg8p acceptor specificity. Even though Alg8p shares significant sequence homology to Alg6p, the activity is highly specific for the addition of the second α-1,3 glucose (Reiss *et al.*, 1996). Also the Alg10p glucosyltransferase adding the terminal α-1,2 glucose to the lipid-linked oligosaccharide shows a very stringent acceptor specificity towards its acceptor Glc₂Man₉GlcNAc₂-PP-Dol (Burda and Aebi, 1998). Taken together, the highly ordered LLO assembly pathway and the specific substrate recognition by the OTase ensures that only completely assembled and correctly branched oligosaccharide is transferred to protein.

Table I. Yeast strains used in this study

Strain	Genotype	Reference
SS328	Mat α ade2-101 ura3-52 his3 Δ 200 lys2-801	Vijayraghavan <i>et al.</i> , 1989
FHEN005-02C(A)	Mata ura3-52 trp1-63 Δ alg12::kanMX4-loxP	This study
YG839	Mata ade2-101 ura3-52 his3 Δ 200 tyr1 Δ alg12::kanMX4	This study
YG840	Mata ade2-101 ura3-52 his3 Δ 200 lys2-801 Δ alg12::kanMX4	This study
YG841	Mat α ade2-101 ade3 ura3-52 his3 Δ 200	This study
YG842	Mata ade2-101 ura3-52 his3 Δ 200 leu2 lys2-801 wbp1-2	This study
YG843	Mat α ade2-101 ade3 ura3-52 his3 Δ 200 leu2 Δ alg12::kanMX4 wbp1-2	This study
YG844	Mat α ade2-101 ura3-52 his3 Δ 200 tyr1 Δ alg12::kanMX4 pYEp352	This study
YG845	Mat α ade2-101 ura3-52 his3 Δ 200 lys2-801 Δ alg12::kanMX4 p[ALG6]	This study
YG846	Mat α ade2-101 ura3-52 his3 Δ 200 Δ alg12::kanMX4 Δ alg5::HIS3 p[ALG6]	This study
YG414	Mat α ade2-101 ura3-52 his3 Δ 200 lys2-801 Δ alg9::kanMX4	Burda <i>et al.</i> , 1996
YG847	Mat α ade2-101 ura3-52 his3 Δ 200 lys2-801 Δ alg9::kanMX4 pYEp352	This study
YG848	Mat α ade2-101 ura3-52 his3 Δ 200 lys2-801 Δ alg9::kanMX4 p[ALG12]	This study
YG849	Mat α ade2-101 ura3-52 his3 Δ 200 lys2-801 Δ alg9::kanMX4 p[ALG6]	This study
YG850	Mata ade2-101 ura3-52 his3 Δ 200 lys2 Δ alg9::kanMX4 Δ alg5::HIS3 p[ALG6]	This study
YG851	Mat α ade2-101 ura3-52 his3 Δ 200 Δ alg9::kanMX4 Δ alg12::kanMX4	This study
YG852	Mat α ade2-101 ade3 ura3-52 his3 Δ 200 lys2 Δ alg9::kanMX4 wbp1-2 pYEp352	This study
YG853	Mat α ade2-101 ade3 ura3-52 his3 Δ 200 lys2 Δ alg9::kanMX4 wbp1-2 p[ALG6]	This study
YG248	Mata ade2-101 ura3-52 his3 Δ 200 lys2 Δ alg3::HIS3	This study
YG854	Mata ade2-101 ura3-52 his3 Δ 200 lys2 Δ alg3::HIS3 p[ALG8]	This study
YG855	Mata ade2-101 ura3-52 his3 Δ 200 lys2 Δ alg3::HIS3 p[ALG12]	This study
YG856	Mata ade2-101 ura3-52 his3 Δ 200 lys2 Δ alg3::HIS3 Δ alg5::HIS3 p[ALG6]	This study
YG857	Mat α ade2-101 ura3-52 his3 Δ 200 lys2 Δ alg3::HIS3 wbp1-2 pYEp352	This study
YG858	Mat α ade2-101 ura3-52 his3 Δ 200 lys2 Δ alg3::HIS3 wbp1-2 p[ALG6]	This study
YG859	Mata ade2-101 ura3-52 his3 Δ 200 lys2 Δ alg3::HIS3 p[ALG6]	This study

The high conservation of the lipid-linked oligosaccharide structure required for N-linked protein glycosylation suggests that individual sugar residues fulfill specific functions in glycoprotein processing. Using the combination of *alg* mutant strains and overexpression of glycosyltransferases, we are now in a position to genetically tailor the structure of both lipid-linked and protein-bound oligosaccharides in yeast. This will make it possible to address the functions of the individual sugar residues *in vivo*.

Materials and methods

Yeast strains and media

The strains of *Saccharomyces cerevisiae* used in this study are listed in Table I. The pALG6 plasmid has been described (Reiss *et al.*, 1996). Standard yeast media and genetic techniques were applied (Guthrie and Fink, 1991).

Isolation and disruption of the *ALG12* locus

The *ALG12* gene (GenBank Accession No. 1302525, ORF YNR030w) was isolated using the gap repair strategy resulting in plasmid pYCG_NR030w, which includes a 2.3 kb *NotI* fragment (bp 10–2312) containing the complete *ALG12* ORF (bp 405–2060). The disruption of the *ALG12* gene in strain SS328 was performed according to the PCR-based gene disruption using the KanMX4-module (Wach *et al.*, 1994). A 1.77-kb-long PCR fragment was amplified using pFA6a-KanMX4 as template and two primers (primer 1: 5'-AAAAGAGTTGAATAAAGCCATTA-

AACAACGATTCAGTTGACATCGATGAATTCGAGCTC-3'; primer 2: 5'-GCTCGCTATATATTTTATTGGAATTGACGTTA-GCTATTATCACGTACGCTGCAGGTCGAC-3') (bold sequences: homologous to pFA6a-KanMX4; other sequences: homologous either to the region directly upstream of the startcodon (primer 1) or to the region directly downstream to the stop codon (primer 2) of the *ALG12* ORF.

Construction of a high copy number plasmid overexpressing *Alg12p*

Plasmid pYCG_NR030w (see above) was cut with *NotI* generating a 2.3 kb fragment that contained the complete *ALG12* ORF. This fragment was cloned into the vector pRS306. The resulting plasmid was cut with *KpnI* and *SacI* (flanking the *NotI* sites) generating a 2.3 kb fragment which was cloned into the high copy number vector YEp352 resulting in plasmid pALG12.

Immunological techniques

Western-blot analysis was performed as described previously (Burda *et al.*, 1996) using anti-CPY specific antibodies.

Extraction and analysis of lipid-linked oligosaccharides of various yeast strains

In vivo metabolic labeling with [³H]mannose (20 Ci/mmol; ICN Pharmaceuticals), extraction of lipid-linked oligosaccharides, and HPLC analysis of the radiolabeled oligosaccharides were performed as described previously (Burda and Aebi, 1998).

Oligosaccharide cleavage with *exo- α -1,2 mannosidase*

Digestion of radiolabeled oligosaccharides with *exo- α -1,2 mannosidase* (from *Aspergillus saitoi*; Oxford GlycoSciences) was performed with 2.5 μ U of enzyme in 60 μ l 100 mM sodium acetate pH 5.0 for 24 h at 37°C. To inactivate the enzyme the samples were heated for 5 min at 95°C and filtered through a 0.45 μ m filter (Millipore UFC30HV00) prior to analysis of the digested oligosaccharides by HPLC.

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Abbreviations

CPY, carboxypeptidase Y; Dol, dolichol; Dol-P-Glc, dolichyl-phosphoglucose; Dol-P-Man, dolichyl-phosphomannose; ER, endoplasmic reticulum; LLO, lipid-linked oligosaccharide; -PP-Dol, dolichyl-pyrophosphate-linked; OTase, oligosaccharyltransferase.

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