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

Patricie Burda, Claude A. Jakob1, Jens Beinhauer2, Johannes H. Hegemann3 and Markus Aebi3

Mikrobiologisches Institut, ETH Zürich, CH-8092 Zürich, Switzerland, 1Division of Cell and Molecular Pathology, Department of Pathology, University of Zurich, CH-8091 Zurich, Switzerland and 2Henrich-Henze-Universität Düsseldorf, Institut für Mikrobiologie, D-40225 Düsseldorf, Germany

Received on September 15, 1998; revised on October 16, 1998; accepted on October 27, 1998

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 Man9GlcNAc2. 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-phosphosphate (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 α-Nparasparaginyl-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 oligosaccharide transferase 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 Δalg12 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
growth defect at 30 °C due to a severe glycosylation deficiency. 

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 (Δalg12 wbp1-2, lane 4).

Fig. 1. Structure and assembly pathway of the dolichylpyrophosphate-linked oligosaccharide Glc3Man9GlcNAc2. 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 lumenal side of the ER is shown. The linkage of each individual glycosyl residue and the loci coding for the corresponding glycosyltransferases are indicated.

![Dolichol](image)

Fig. 2. The Δalg12 mutation alters glycosylation of CPY in vivo. Four strains derived from a tetrapole tetrad of a cross Δalg12×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 (Δalg12 wbp1-2, lane 4).

N-glycosylation of secreted proteins. In contrast to previously analyzed alg wbp1 mutant strains, the resulting double mutant strain Δalg12 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 Δalg12 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 Δalg12 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 ManyGlcNAc2 and low levels of lipid-linked Glc3ManGlcNAc2

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 Glc3ManGlcNAc2. 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 Hex7GlcNAc2.

![Image](image)
oligosaccharide, whereas the minor peak had the retention time expected for Hex$_{10}$GlcNAc$_2$ (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 Hex$_{10}$GlcNAc$_2$ peak (Figure 4C). We first analyzed the structure of the putative Man$_7$GlcNAc$_2$ oligosaccharide in more detail. Previously we reported on the isolation of the ALG9 locus and showed that $\Delta$alg9 cells accumulate lipid-linked Man$_9$GlcNAc$_2$. Detailed analysis by Trimble and co-workers using $^3$H-NMR technique revealed that the Man$_9$GlcNAc$_2$ oligosaccharide accumulating in $\Delta$alg9 cells contained an additional α-1,3 mannose (Trimble, personal communication) linked to the Man$_5$GlcNAc$_2$ oligosaccharide found in $\Delta$alg3 strains (Verostek et al., 1993a; Figure 1). Digestion of this Man$_9$GlcNAc$_2$ oligosaccharide by an exo-α-1,2 mannosidase resulted in a Man$_5$GlcNAc$_2$ structure (Figure 4A,B). The same oligosaccharide was observed after α-1,2 mannosidase treatment of the Man$_7$GlcNAc$_2$ accumulating in $\Delta$alg12 cells (Figure 4C,D). Knowing that the $\Delta$alg9 strain accumulates the same oligosaccharide 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 Man$_9$GlcNAc$_2$ 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 Man$_9$GlcNAc$_2$-PP-Dol.

Earlier studies in yeast cells indicated that the oligosaccharide intermediate Man$_3$GlcNAc$_2$-PP-Dol can be glucosylated yielding Glc$_3$Man$_3$GlcNAc$_2$-PP-Dol, albeit at a low level (Verostek et al., 1993a). Thus, we speculated that in $\Delta$alg12 strains the accumulating Man$_9$GlcNAc$_2$ might be a (suboptimal) substrate for the Alg6p glucosyltransferase (Runge et al., 1984; Reiss et al., 1996) and subsequent glucosylation by Alg8p (Stagljær, I. et al., 1994) and Alg10p (Burda and Aebi, 1998) transferases might result in the formation of Glc$_3$Man$_3$GlcNAc$_2$-PP-Dol, the minor peak observed in $\Delta$alg12 strains (Figure 3B). 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 Hex$_{10}$GlcNAc$_2$-PP-Dol. This was indeed the case. Almost equal amounts of Man$_9$GlcNAc$_2$ and Hex$_{10}$GlcNAc$_2$ oligosaccharide were observed in the Alg6p overexpressing strain (Figure 5B). To show that the postulated Glc$_3$Man$_3$GlcNAc$_2$ 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 Glc$_3$Man$_3$GlcNAc$_2$-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 Man$_9$GlcNAc$_2$ oligosaccharide (Figure 5B,C). This result confirms the presence of glucose residues in the minor Glc$_3$Man$_3$GlcNAc$_2$ peak observed in the $\Delta$alg12 strain. Taken together, our data show that the $\Delta$alg12 locus is required for the assembly of the lipid-linked core oligosaccharide Glc$_3$Man$_3$GlcNAc$_2$. $\Delta$alg12 mutant strains accumulate lipid-linked Man$_9$GlcNAc$_2$; however, Man$_9$GlcNAc$_2$-PP-Dol can be glucosylated in these cells resulting in Glc$_3$Man$_3$GlcNAc$_2$-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 Man$_9$GlcNAc$_2$ 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 exo-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 Man$_3$GlcNAc$_2$ 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, exo-α-1,2 mannosidase...
Fig. 4. HPLC analysis of exo-α-1,2 mannosidase digestion products of Δalg9 and Δalg12 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), Man4GlcNAc2 (M4N2) and Man6GlcNAc2 (M6N2) and the corresponding oligosaccharide structures are shown. (A) Oligosaccharide derived from Δalg9 strain YG414. (B) Oligosaccharide from Δalg9 strain (A) treated with exo-α-1,2 mannosidase. (C) Oligosaccharide derived from Δalg12 strain YG839. (D) Oligosaccharide from Δalg12 strain (C) treated with exo-α-1,2 mannosidase.

digestion resulted in a shift of the Man5GlcNAc2 peak due to the removal of the two α-1,2-linked mannose residues yielding Man4GlcNAc2 (M4N2) and Man3GlcNAc2 (M3N2) and the corresponding oligosaccharide structures are shown. (A) Oligosaccharide derived from Δalg9 strain YG414. (B) Oligosaccharide from Δalg9 strain (A) treated with exo-α-1,2 mannosidase. (C) Oligosaccharide derived from Δalg12 strain YG839. (D) Oligosaccharide from Δalg12 strain (C) treated with exo-α-1,2 mannosidase.

observed that Man5GlcNAc2 oligosaccharide is glucosylated more efficiently as compared to Man3GlcNAc2 and Man4GlcNAc2, 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 Δalg3 Δalg5 or Δalg9 Δalg5 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 Man5GlcNAc2 oligosaccharide in Δalg3 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 Δalg3 and Δalg9 mutant strains) is an important determinant of Alg6p substrate specificity and that synthesis of the Man5GlcNAc2 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
Δ due to Alg6p overexpression both in a mutant strains (Figure 7). Glycosylation of CPY was improved lipid-linked oligosaccharide, in particular the presence of the core oligosaccharide to protein. To test a fully glucosylated, overexpressing Alg6p glucosyltransferase digested with exo-α-mannosidase. Oligosaccharides isolated from B (YG859). (C) Oligosaccharides isolated from Δalg9 strain overexpressing Alg6p glucosyltransferase digested with exo-α-1,2 mannosidase. (D) Oligosaccharides isolated from Δalg9 strain overexpressing Alg6p glucosyltransferase (YG849). 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.

Accepter specificity of Alg12p mannosyltransferase

As shown above, efficient glucosylation of the lipid-linked oligosaccharide requires the completion of the Man9GlcNAc2 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 mannoside to the α-1,6 mannoside of the lipid-linked Man9GlcNAc2 which is (according to the currently accepted topological model of LLO biosynthesis) translocated into the lumen of the ER. However, the accumulation of Man9GlcNAc2-PP-Dol in a Δalg3 strain and of Man9GlcNAc2-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 Δalg9 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 Man9GlcNAc2 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 Man7GlcNAc2 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
propose that the additional residue is the residue which is added by the overexpressed mannose residues. Thus, it contains an additional mannose-1,2 linked overexpressing Alg12p has only two cleavable α ratio of the Man 4 GlcNAc 2 to Man 5 GlcNAc 2 oligosaccharide was investigated by exo-α-1,2 mannosidase digestion as for Man 6 GlcNAc 2 and the Man 5 GlcNAc 2 oligosaccharide from Δalg9 cells transformed with pALG12. 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 Δalg9 strain results in a novel lipid-linked oligosaccharide intermediate.

Discussion

The assembly of lipid-linked Glc3ManαGlcNAc2 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 ManαGlcNAc2 intermediate across the ER membrane. In this discussion, we will focus on the assembly pathway of the oligosaccharide after the translocation of ManαGlcNAc2-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 Glc3ManαGlcNAc2, but accumulate as a major product ManγGlcNAc2-PP-Dol. Our observation that overexpression of the ALG12 locus in a Δalg9 strain results in a novel oligosaccharide species containing one additional mannose residue which is normally not present in Δalg9 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 Δalg12 cells as compared to wild-type cells shows that incompletely assembled oligosaccharide is transferred to protein. However, we detected glucosylated oligosaccharide intermediates (Glc3ManαGlcNAc2) in Δalg12 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 oligosaccharidyltransferase (OTase) complex. Consistent with this observation, the Δalg12 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 ManβGlcNAc2-PP-Dol; however, a minor proportion of lipid-linked oligosaccharide was glucosylated yielding Glc3ManαGlcNAc2. 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 mannanproteins 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 Glc2Man9GlcNAc2 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 Glc2Man9GlcNAc2 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 mannoside 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 malfolded glycoproteins in the ER in yeast seems to be dependent on the correctly processed Man9GlcNAc2 oligosaccharide structure (Knap et al., 1996). Therefore, the transfer of fully assembled Glc2Man9GlcNAc2 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 Glc2Man9GlcNAc2) 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 Glc2Man9GlcNAc2 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 (Reaick 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 mannoside by Alg12p requires the presence of the α-1,3 mannoside added by the ALG3 mannosyltransferase. In contrast, Alg3p recognizes efficiently lipid-linked Man9GlcNAc2 and catalyzes the addition of the α-1,3-linked mannoside (Aebi et al., 1996; Kuster and te Heesen, unpublished observations). The fact that the Alg9 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 mannoside 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 Glc2Man9GlcNAc2 observed in Δalg12 mutant cells and the absence of detectable amounts of glucosylated LLO in Δalg5, Δalg9, and Δalg7 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 Man9GlcNAc2 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 tri-mannose arm in order to avoid glucose addition to the other terminal α-1,2 mannoside residues. This suggests that several of the mannoside 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 glucose 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.
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′-AAAGAGATTGAAATACAGCCATTACAGGATTCAGTTGACATCGATGAAATCGAGCTC-3′; primer 2: 5′-GCTCGCTA TA TA TTTTA TTGGAA TTGACGTTA-CTATTATCACTACGTCGAGGCAGTCAGC-3′) (bold sequences: homologous to pFA6a-KAnMX4; other sequences: homologous either to the region directly upstream of the start codon (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/mmole; ICN Pharmaceuticals), extraction of lipid-linked oligosaccharides, and HPLC analysis of the radiolabeled oligosaccharides were performed as described previously (Burda and Aebi, 1998).

---

Table I. Yeast strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS328</td>
<td>Matot ade2-101 ura3-52 his3A200 lys2-801</td>
<td>Vijayaraghavan et al., 1989</td>
</tr>
<tr>
<td>FYEN005–02(t)</td>
<td>Mata ura3-52 trpl-63 Δalg12::kanMX4-1oxP</td>
<td>This study</td>
</tr>
<tr>
<td>YG839</td>
<td>Mata ade2-101 ura3-52 his3Δ200 tyr1 Δalg12::kanMX4</td>
<td>This study</td>
</tr>
<tr>
<td>YG840</td>
<td>Mata ade2-101 ura3-52 his3Δ200 lys2-801 Δalg12::kanMX4</td>
<td>This study</td>
</tr>
<tr>
<td>YG841</td>
<td>Mata ade2-101 ade3 ura3-52 his3Δ200</td>
<td>This study</td>
</tr>
<tr>
<td>YG842</td>
<td>Mata ade2-101 ura3-52 his3Δ200 leu2 lys2-801 wbp1-2</td>
<td>This study</td>
</tr>
<tr>
<td>YG843</td>
<td>Mata ade2-101 ade3 ura3-52 his3Δ200 leu2 Δalg12::kanMX4 wbp1-2</td>
<td>This study</td>
</tr>
<tr>
<td>YG844</td>
<td>Mata ade2-101 ura3-52 his3Δ200 tyr1 Δalg12::kanMX4 YEpt352</td>
<td>This study</td>
</tr>
<tr>
<td>YG845</td>
<td>Mata ade2-101 ura3-52 his3Δ200 lys2-801 Δalg12::kanMX4 p[ALG6]</td>
<td>This study</td>
</tr>
<tr>
<td>YG846</td>
<td>Mata ade2-101 ura3-52 his3Δ200 Δalg12::kanMX4 Δalg5::HIS3 p[ALG6]</td>
<td>This study</td>
</tr>
<tr>
<td>YG847</td>
<td>Mata ade2-101 ura3-52 his3Δ200 lys2-801 Δalg9::kanMX4 YEpt352</td>
<td>This study</td>
</tr>
<tr>
<td>YG848</td>
<td>Mata ade2-101 ura3-52 his3Δ200 lys2-801 Δalg9::kanMX4 p[ALG12]</td>
<td>This study</td>
</tr>
<tr>
<td>YG849</td>
<td>Mata ade2-101 ura3-52 his3Δ200 lys2-801 Δalg9::kanMX4 p[ALG6]</td>
<td>This study</td>
</tr>
<tr>
<td>YG850</td>
<td>Mata ade2-101 ura3-52 his3Δ200 lys2 Δalg9::kanMX4 Δalg5::HIS3 p[ALG6]</td>
<td>This study</td>
</tr>
<tr>
<td>YG851</td>
<td>Mata ade2-101 ura3-52 his3Δ200 Δalg9::kanMX4 Δalg12::kanMX4</td>
<td>This study</td>
</tr>
<tr>
<td>YG852</td>
<td>Mata ade2-101 ade3 ura3-52 his3Δ200 lys2 Δalg9::kanMX4 wbp1-2 pYEpt352</td>
<td>This study</td>
</tr>
<tr>
<td>YG853</td>
<td>Mata ade2-101 ade3 ura3-52 his3Δ200 lys2 Δalg9::kanMX4 wbp1-2 p[ALG6]</td>
<td>This study</td>
</tr>
<tr>
<td>YG248</td>
<td>Mata ade2-101 ura3-52 his3Δ200 lys2 Δalg5::HIS3</td>
<td>This study</td>
</tr>
<tr>
<td>YG854</td>
<td>Mata ade2-101 ura3-52 his3Δ200 lys2 Δalg5::HIS3 p[ALG8]</td>
<td>This study</td>
</tr>
<tr>
<td>YG855</td>
<td>Mata ade2-101 ura3-52 his3Δ200 lys2 Δalg5::HIS3 p[ALG12]</td>
<td>This study</td>
</tr>
<tr>
<td>YG856</td>
<td>Mata ade2-101 ura3-52 his3Δ200 lys2 Δalg5::HIS3 Δalg5::HIS3 p[ALG6]</td>
<td>This study</td>
</tr>
<tr>
<td>YG857</td>
<td>Mata ade2-101 ura3-52 his3Δ200 lys2 Δalg3::HIS3 wbp1-2 pYEpt352</td>
<td>This study</td>
</tr>
<tr>
<td>YG858</td>
<td>Mata ade2-101 ura3-52 his3Δ200 lys2 Δalg3::HIS3 wbp1-2 p[ALG6]</td>
<td>This study</td>
</tr>
<tr>
<td>YG859</td>
<td>Mata ade2-101 ura3-52 his3Δ200 lys2 Δalg3::HIS3 p[ALG6]</td>
<td>This study</td>
</tr>
</tbody>
</table>

---

References:

- Vijayaraghavan et al., 1989
- Guthrie and Fink, 1991
- Wach et al., 1994
- Burda et al., 1996
- Reiss et al., 1996
- Burda et al., 1996
- 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 µM of enzyme in 60 µl 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 µm filter (Millipore UFC09HV00) prior to analysis of the digested oligosaccharides by HPLC.

Acknowledgments

We thank Prof. Howard Riezman for bringing the ORF YNR030w to our attention. We thank Prof. Howard Riezman, Prof. Andreas Conzelmann and Prof. Rob Trimble for sharing results prior to publication and Dr. Rob Bouliaune for critically reading the manuscript. This work was supported by the Swiss National Science Foundation to M.A. (Grant 3100-040350.94).

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.

References

Aebi, M., Gassenhuber, J., Domdey, H. and te Heesen, S. (1996) Cloning and 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 µM of enzyme in 60 µl 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 µm filter (Millipore UFC09HV00) prior to analysis of the digested oligosaccharides by HPLC.

Aebi, M., Gassenhuber, J., Domdey, H. and te Heesen, S. (1996) Cloning and 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 µM of enzyme in 60 µl 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 µm filter (Millipore UFC09HV00) prior to analysis of the digested oligosaccharides by HPLC.

Aebi, M., Gassenhuber, J., Domdey, H. and te Heesen, S. (1996) Cloning and 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 µM of enzyme in 60 µl 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 µm filter (Millipore UFC09HV00) prior to analysis of the digested oligosaccharides by HPLC.

Aebi, M., Gassenhuber, J., Domdey, H. and te Heesen, S. (1996) Cloning and 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 µM of enzyme in 60 µl 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 µm filter (Millipore UFC09HV00) prior to analysis of the digested oligosaccharides by HPLC.

Aebi, M., Gassenhuber, J., Domdey, H. and te Heesen, S. (1996) Cloning and 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 µM of enzyme in 60 µl 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 µm filter (Millipore UFC09HV00) prior to analysis of the digested oligosaccharides by HPLC.

Aebi, M., Gassenhuber, J., Domdey, H. and te Heesen, S. (1996) Cloning and 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 µM of enzyme in 60 µl 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 µm filter (Millipore UFC09HV00) prior to analysis of the digested oligosaccharides by HPLC.

Aebi, M., Gassenhuber, J., Domdey, H. and te Heesen, S. (1996) Cloning and 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 µM of enzyme in 60 µl 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 µm filter (Millipore UFC09HV00) prior to analysis of the digested oligosaccharides by HPLC.

Aebi, M., Gassenhuber, J., Domdey, H. and te Heesen, S. (1996) Cloning and 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 µM of enzyme in 60 µl 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 µm filter (Millipore UFC09HV00) prior to analysis of the digested oligosaccharides by HPLC.

Aebi, M., Gassenhuber, J., Domdey, H. and te Heesen, S. (1996) Cloning and 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 µM of enzyme in 60 µl 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 µm filter (Millipore UFC09HV00) prior to analysis of the digested oligosaccharides by HPLC.

Aebi, M., Gassenhuber, J., Domdey, H. and te Heesen, S. (1996) Cloning and 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 µM of enzyme in 60 µl 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 µm filter (Millipore UFC09HV00) prior to analysis of the digested oligosaccharides by HPLC.

Aebi, M., Gassenhuber, J., Domdey, H. and te Heesen, S. (1996) Cloning and 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 µM of enzyme in 60 µl 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 µm filter (Millipore UFC09HV00) prior to analysis of the digested oligosaccharides by HPLC.

Aebi, M., Gassenhuber, J., Domdey, H. and te Heesen, S. (1996) Cloning and 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 µM of enzyme in 60 µl 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 µm filter (Millipore UFC09HV00) prior to analysis of the digested oligosaccharides by HPLC.