

## ***ALG9* mannosyltransferase is involved in two different steps of lipid-linked oligosaccharide biosynthesis**

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**N-linked protein glycosylation follows a conserved pathway in eukaryotic cells. The assembly of the lipid-linked core oligosaccharide  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ , the substrate for the oligosaccharyltransferase (OST), is catalyzed by different glycosyltransferases located at the membrane of the endoplasmic reticulum (ER). The substrate specificity of the different glycosyltransferase guarantees the ordered assembly of the branched oligosaccharide and ensures that only completely assembled oligosaccharide is transferred to protein. The glycosyltransferases involved in this pathway are highly specific, catalyzing the addition of one single hexose unit to the lipid-linked oligosaccharide (LLO). Here, we show that the dolichylphosphomannose-dependent *ALG9* mannosyltransferase is the exception from this rule and is required for the addition of two different  $\alpha$ -1,2-linked mannose residues to the LLO. This report completes the list of lumen-oriented glycosyltransferases required for the assembly of the LLO.**

**Key words:** endoplasmic reticulum/dolichol/*Saccharomyces cerevisiae*

### **Introduction**

N-linked glycosylation in eukaryotic cells is an essential post-translational modification of proteins in the secretory pathway (Kornfeld and Kornfeld, 1985). A key feature is the transfer of the pre-assembled branched oligosaccharide  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ . The assembly of  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$  on the lipid-carrier dolichylpyrophosphate (PP-Dol) at the membrane of the endoplasmic reticulum (ER) and the initial trimming reactions of the oligosaccharide after transfer to protein are highly conserved processes in eukaryotes (Herscovics and Orlean, 1993; Burda and Aebi, 1999). The N-linked oligosaccharide fulfills different functions in the maturation of the glycoprotein, such as glycoprotein quality control or lectin-mediated sorting in the secretory pathway (Helenius, 2001; Trombetta, 2003; Helenius and Aebi, 2004). Therefore, it is of central importance that only the complete  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$  oligosaccharide is transferred

to protein, not biosynthetic intermediates or incorrectly assembled structures. This is accomplished primarily by the substrate specificity of the oligosaccharyltransferase (OST) that transfers the oligosaccharide to protein and the individual glycosyltransferases involved in the substrate assembly (Murphy and Spiro, 1981; Burda *et al.*, 1999; Cipollo and Trimble, 2000; Karaoglu *et al.*, 2001).

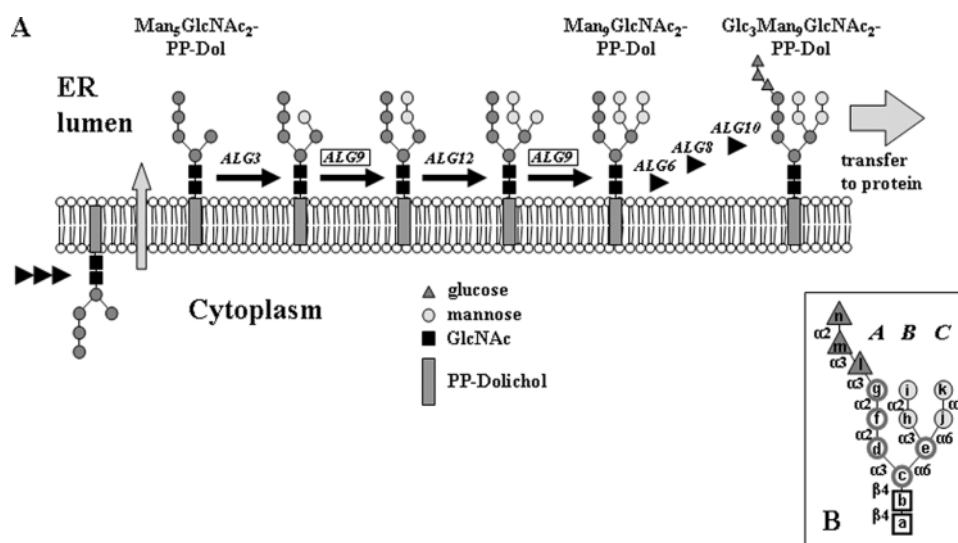
Lipid-linked oligosaccharide (LLO) biosynthesis starts on the cytoplasmic side of the ER membrane by transfer of GlcNAc-P to dolichylphosphate (P-Dol), followed by the addition of GlcNAc and five mannose residue-yielding  $\text{Man}_5\text{GlcNAc}_2\text{-PP-Dol}$  (Figure 1). Nucleotide-activated sugars serve as substrates for these reactions. After Rft1p-dependent translocation of the heptasaccharide moiety into the lumen of the ER (Helenius and Aebi, 2002), four more mannoses are added from the lipid-linked donor Man-P-Dol, and the 14mer oligosaccharide is completed by transfer of three glucoses from Glc-P-Dol. Finally, the multi-subunit complex OST recognizes the full-length substrate and transfers it to asparagine residues in the context Asn-X amino acid-Ser/Thr, where X amino acid can be any amino acid except proline (Gavel and Von Heijne, 1990; Knauer and Lehle, 1999; Yan and Lennarz, 1999; Dempski and Imperiali, 2002).

Yeast *alg* (asparagine-linked glycosylation) (Huffaker and Robbins, 1982) mutants have been pivotal for elucidation of the pathway components, as all 10 known genes encoding glycosyltransferases involved in LLO biosynthesis were initially cloned by complementation of corresponding mutations (Burda *et al.*, 1999). *alg* mutant strains are characterized by accumulation of the LLO substrate of the affected glycosyltransferases, suggesting the absence of redundant transferases. However, because of the branched structure of the oligosaccharide, LLO biosynthesis can proceed in some mutant strains, resulting in aberrant oligosaccharide structures that are then transferred to protein (Cipollo *et al.*, 2001; Helenius and Aebi, 2002).

The high degree of conservation of this pathway in eukaryotes has enabled rapid elucidation of the molecular basis of several human diseases caused by deficiencies in the ER glycosylation pathway, known as congenital disorders of glycosylation (CDG) (Aebi and Hennet, 2001; Marquardt and Denecke, 2003). As mentioned above, the understanding of the LLO biosynthetic pathway is not complete. In particular, the mannosyltransferase responsible for the addition of the terminal  $\alpha$ -1,2-linked mannose of the C-antenna (residue k in Figure 1B) has not been identified (Burda *et al.*, 1999). On the basis of the current models (Figure 1), it is proposed that Man-P-Dol serves as a donor in this reaction. The known Man-P-Dol-dependent mannosyltransferases of the pathway, *Alg3p*, *Alg9p*, and *Alg12p*, are hydrophobic proteins with multiple predicted transmembrane

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**Fig. 1.** Scheme of lipid-linked oligosaccharide (LLO) biosynthesis at the luminal side of the endoplasmic reticulum (ER) membrane. Four mannoses and three glucoses are added in a stepwise manner from Man-P-Dol and Glc-P-Dol, respectively. Genes involved in the respective glycosyl-transfer step are indicated atop the arrow. Darker circles indicate mannoses added from GDP-Man on the cytoplasmic side, lighter circles indicate mannoses added on the luminal side from Man-P-Dol. Inset: linkage map of the Man<sub>9</sub>GlcNAc<sub>2</sub> oligosaccharide. Monosaccharides are labeled with letters in alphabetic order of addition. The A-, B-, and C-arm are indicated by italic letters. Abbreviations used are  $\alpha 2$  ( $\alpha$ -1,2),  $\alpha 3$  ( $\alpha$ -1,3),  $\alpha 6$  ( $\alpha$ -1,6), and  $\beta 4$  ( $\beta$ -1,4).

domains. They belong to a group of related transferases which includes also enzymes involved in GPI-anchor formation (Oriol *et al.*, 2002). Members of this family without assigned function would be prime candidates for the missing transferase. Yet in *Saccharomyces cerevisiae*, all family members have already a suggested function. Interestingly, no yeast mutant strain accumulating Man<sub>8</sub>GlcNAc<sub>2</sub>-PP-Dol (residues a–j in Figure 1) has been isolated so far, despite several genetic screens that have been conducted to identify mutants in this pathway (Huffaker and Robbins, 1982; Roos *et al.*, 1994; Burda *et al.*, 1996, 1999; Burda and Aebi, 1998).

The failure to isolate a Man<sub>8</sub>GlcNAc<sub>2</sub>-PP-Dol-accumulating mutant could be explained by a model in which a mannosyltransferase acting earlier in the pathway also catalyzes addition of the mannose in question.

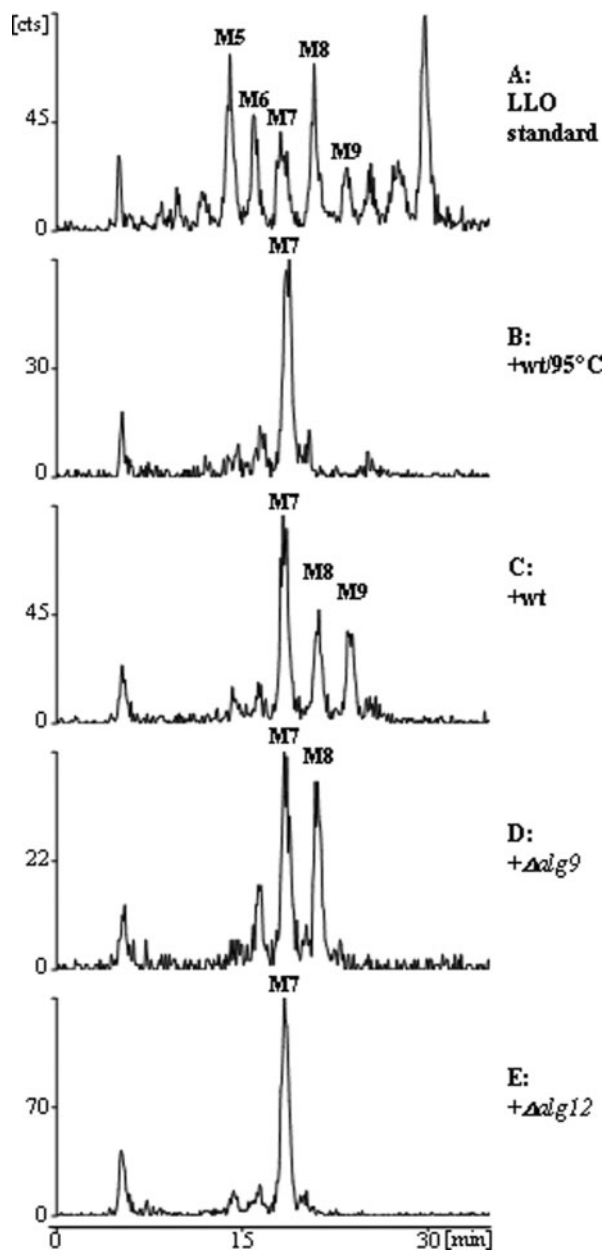
On the basis of the assumption that there is a high linkage specificity of the glycosyltransferases involved, the Man-P-Dol-dependent mannosyltransferases Alg3p and Alg12p can be ruled out as possible dual-function candidates, as these transferases add an  $\alpha$ -1,3- or  $\alpha$ -1,6-linked mannose, respectively. In contrast, the ALG9 mannosyltransferase has previously been shown to be required for the addition of the  $\alpha$ -1,2-linked seventh mannose which terminates the B-arm (residue i in Figure 1) (Burda *et al.*, 1996; Cipollo and Trimble, 2000). Cells lacking Alg9p accumulate Man<sub>6</sub>GlcNAc<sub>2</sub>-PP-Dol, but an abnormal Man<sub>7</sub>GlcNAc<sub>2</sub> LLO with the acceptor residue (j in Figure 1) for mannose k can be obtained by overexpression of ALG12 in a  $\Delta alg9$  strain (Burda *et al.*, 1999). Interestingly, this Man<sub>7</sub>GlcNAc<sub>2</sub> LLO lacks both terminal  $\alpha$ -1,2-linked mannose residues despite the absence of one single transferase, Alg9p. This implies that either the responsible mannosyltransferase has a strict requirement for presence of the terminal  $\alpha$ -1,2 mannose on the B-arm or that this transferase is Alg9p.

We have investigated the hypothesis that mannose k is added by Alg9p. Using an *in vitro* assay for elongation of LLO and by analysis of the effects of Alg9p overexpression, we provided evidence that Alg9p is involved in addition of both the seventh and the ninth mannose to the LLO of N-glycan biosynthesis. It is thus the first example of a transferase in this conserved pathway found to add two saccharides.

## Results

First, we followed a biochemical approach to test whether Alg9p is required for addition of the ninth mannose to Man<sub>8</sub>GlcNAc<sub>2</sub>-PP-Dol. This employed an *in vitro* assay for elongation of Man<sub>7</sub>GlcNAc<sub>2</sub>-PP-Dol to Man<sub>9</sub>GlcNAc<sub>2</sub>-PP-Dol. We used [<sup>3</sup>H]mannose-labeled Man<sub>7</sub>GlcNAc<sub>2</sub>-PP-Dol (residues a–i) isolated from a strain lacking Alg12p (Burda *et al.*, 1999) as the acceptor LLO substrate. Crude membrane extracts isolated from wildtype  $\Delta alg9$  or  $\Delta alg12$  strains were mixed with LLO substrate in the presence of detergent. Addition of GDP mannose was required to allow formation of Man-P-Dol by dolichol-phosphate-mannose synthase *in vitro*, while UDP-Glc was left out to avoid formation of glucosylated LLOs. Alterations of the radioactive oligosaccharide were then analyzed by HPLC.

Wildtype membranes elongated the input substrate (Figure 2B) by one or two hexose units to species co-migrating with Man<sub>8</sub>GlcNAc<sub>2</sub> and Man<sub>9</sub>GlcNAc<sub>2</sub>, respectively (Figure 2C). In contrast, membranes from  $\Delta alg9$  cells elongated Man<sub>7</sub>GlcNAc<sub>2</sub>-PP-Dol only by one hexose unit (Figure 2D). No elongation of Man<sub>7</sub>GlcNAc<sub>2</sub>-PP-Dol was observed with  $\Delta alg12$  membranes (Figure 2E). This demonstrates that the first elongation was Alg12p dependent and that the second elongation from Man<sub>8</sub>GlcNAc<sub>2</sub>-PP-Dol to Man<sub>9</sub>GlcNAc<sub>2</sub>-PP-Dol depended on Alg9p. Like *in vivo*,



**Fig. 2.** *In vitro* assay for elongation of Man<sub>7</sub>GlcNAc<sub>2</sub>-PP-Dol. HPLC analysis of [<sup>3</sup>H]mannose-labeled lipid-linked oligosaccharide (LLO) substrate from *Δalg12* yeast cells after incubation with membranes isolated from different yeast strains. (A) Oligosaccharide standard mix from [<sup>3</sup>H]mannose-labeled yeast LLO. (B) LLO substrate incubated with heat-inactivated (5 min/95°C) wildtype membranes. (C) LLO substrate incubated with wildtype membranes. (D) LLO substrate incubated with *Δalg9* membranes. (E) LLO substrate incubated with *Δalg12* membranes. Peaks co-migrating with standard oligosaccharides and several selected standard oligosaccharides are indicated. M5–M9 refers to Man<sub>5</sub>GlcNAc<sub>2</sub>–Man<sub>9</sub>GlcNAc<sub>2</sub>.

also in the *in vitro* assay addition of the  $\alpha$ -1,6-linked mannose on the C-arm (residue j) by Alg12p preceded addition of the terminal  $\alpha$ -1,2-linked mannose.

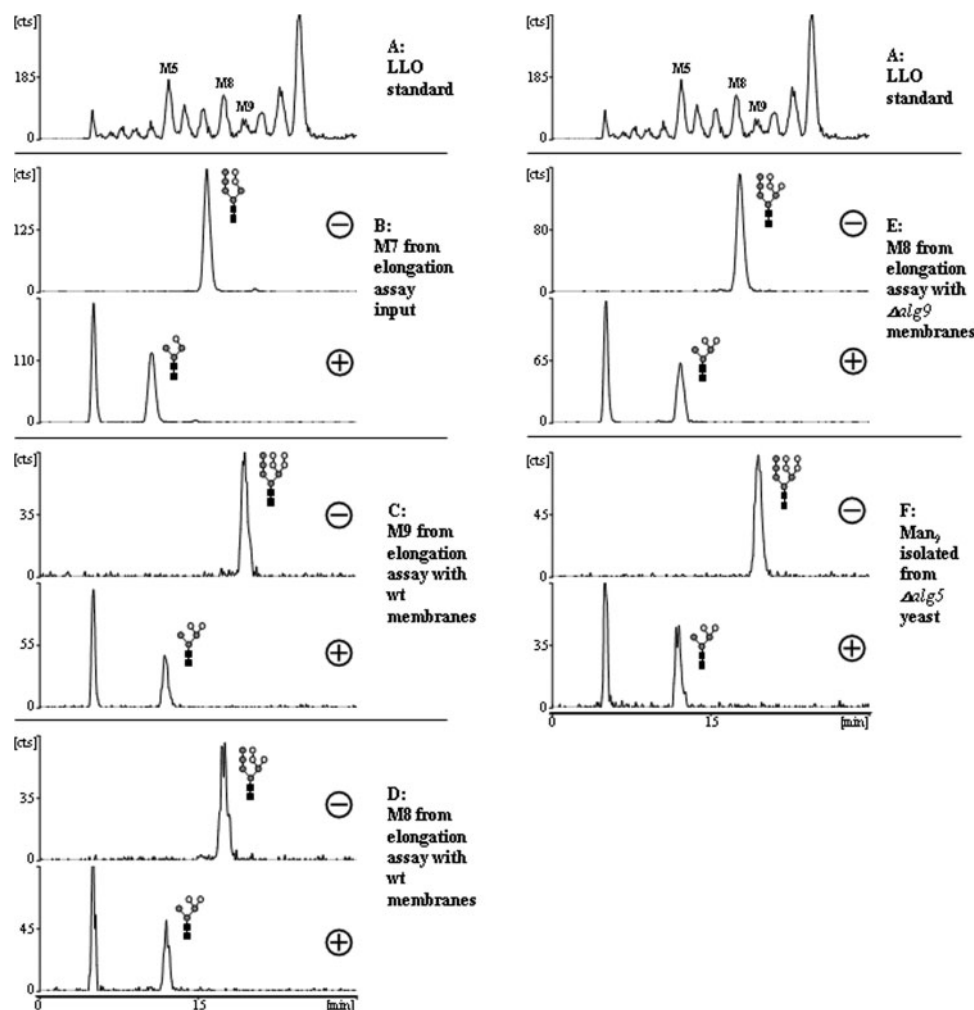
To exclude that deletion of *ALG9* results in an inhibiting activity which would mask the  $\alpha$ -1,2 mannosyltransferase activity, we mixed membranes from wildtype and *Δalg9*

cells and looked for substrate elongation. A peak at position of Man<sub>9</sub>GlcNAc<sub>2</sub> could still be observed (data not shown), which rules out the presence of an inhibiting activity.

To verify that the oligosaccharides generated in the assay had the presumed Man<sub>8</sub>GlcNAc<sub>2</sub> or Man<sub>9</sub>GlcNAc<sub>2</sub> structures, we collected individual oligosaccharide species, digested them with an  $\alpha$ -1,2 specific exo-mannosidase and analyzed them by HPLC (Figure 3). The material from elongation assays with wildtype membranes showed the expected behavior (Figure 3B–D): Man<sub>7</sub>GlcNAc<sub>2</sub> shifted upon treatment to a Man<sub>4</sub>GlcNAc<sub>2</sub> isomer, while Man<sub>8</sub>GlcNAc<sub>2</sub> and Man<sub>9</sub>GlcNAc<sub>2</sub> both shifted to the same Man<sub>5</sub>GlcNAc<sub>2</sub> isomer, demonstrating that they only differed by a terminal  $\alpha$ -1,2-linked mannose.

Digestion of the Man<sub>8</sub>GlcNAc<sub>2</sub> peak generated with *Δalg9* membranes produced Man<sub>5</sub>GlcNAc<sub>2</sub>, demonstrating that only the Alg12p-dependent elongation occurred and not addition of an  $\alpha$ -1,2-linked mannose. To confirm that the Man<sub>9</sub>GlcNAc<sub>2</sub> species produced in the assay by wildtype membranes was identical to the naturally occurring Man<sub>9</sub>GlcNAc<sub>2</sub>, we compared it with a Man<sub>9</sub>GlcNAc<sub>2</sub> reference oligosaccharide isolated from a *Δalg5* strain. Cells lacking *ALG5* cannot produce Glc-P-Dol and therefore accumulate Man<sub>9</sub>GlcNAc<sub>2</sub>-PP-Dol (te Heesen *et al.*, 1994). The Man<sub>5</sub>GlcNAc<sub>2</sub> isomer of the digested assay products co-migrated with the Man<sub>5</sub>GlcNAc<sub>2</sub> isomer obtained after exo-mannosidase digestion of Man<sub>9</sub>GlcNAc<sub>2</sub> from *Δalg5*.

To confirm our results by a genetic approach, we overexpressed *ALG9* in wildtype yeast and analyzed resulting changes in the LLO profile. Cells were transformed with a high-copy plasmid containing the *ALG9* gene under control of the endogenous promoter. We then analyzed the pulse-labeling LLO profile by HPLC (Figure 4) and compared it with LLO profiles of control cells bearing an empty plasmid. Three prominent glycan peaks are usually detected by the HPLC analysis if LLOs of our wildtype cells are labeled by a [<sup>3</sup>H]mannose pulse: [<sup>3</sup>H]Man<sub>5</sub>GlcNAc<sub>2</sub>, [<sup>3</sup>H]Man<sub>8</sub>GlcNAc<sub>2</sub> and the full-length oligosaccharide [<sup>3</sup>H]Glc<sub>3</sub>Man<sub>9</sub>GlcNAc<sub>2</sub>. Accumulation of Man<sub>5</sub>GlcNAc<sub>2</sub>-PP-Dol can be explained by the potentially rate-limiting translocation step that precedes elongation, by the Alg3p mannosyltransferase. The full-length precursor likely accumulates because of lack of acceptor peptides or limiting OST activity. We envisioned that Man<sub>8</sub>GlcNAc<sub>2</sub>-PP-Dol accumulates due to limited mannosyltransferase activity for elongation. In support of this hypothesis, we found that *ALG9* overexpression significantly reduces the relative abundance of the Man<sub>8</sub>GlcNAc<sub>2</sub> peak compared with the Man<sub>5</sub>GlcNAc<sub>2</sub> peak (Figure 4). Quantification of seven independent experiments revealed the Man<sub>8</sub>GlcNAc<sub>2</sub>/Man<sub>5</sub>GlcNAc<sub>2</sub> ratio of  $0.71 \pm 0.15$  in wildtype cells and  $0.24 \pm 0.06$  in *ALG9*-overexpressing cells. Interestingly, we did not observe a concomitant increase in the level of the complete Glc<sub>3</sub>Man<sub>9</sub>GlcNAc<sub>2</sub> product upon *ALG9* overexpression. This phenotype has to be expected in a biosynthetic pathway where the flux through the pathway is controlled by the level of the product. Overexpression of biosynthetic enzymes in such a system results in an alteration of relative intermediate concentrations but not in an increase of the product level. Therefore, we concluded that Alg9p can add the ninth mannose *in vivo* as the bottleneck at the



**Fig. 3.** Exo- $\alpha$ -1,2-mannosidase digest analysis of elongation assay products. HPLC analysis of peaks isolated from a  $\text{Man}_7\text{GlcNAc}_2\text{-PP-Dol}$  elongation assay (as in Figure 2) or of  $\text{Man}_9\text{GlcNAc}_2$  isolated from a  $\Delta\text{alg5}$  strain, either mock incubated (–) or digested with  $\alpha$ -1,2 specific exo-mannosidase from *T. reesei* (+). (A) Oligosaccharide standard mix of [ $^3\text{H}$ ]mannose-labeled lipid-linked oligosaccharide (LLO) from yeast. (B) M7 peak isolated from  $\text{Man}_7\text{GlcNAc}_2\text{-PP-Dol}$  input material after hydrolysis. (C) M9 peak isolated from the elongation assay with wildtype membranes (as in Figure 2C). (D) M8 peak isolated from the elongation assay with wildtype membranes (as in Figure 2C). (E) M8 peak isolated from the elongation assay with  $\Delta\text{alg9}$  membranes (as in Figure 2D). (F)  $\text{Man}_9\text{GlcNAc}_2$  peak isolated from LLO of [ $^3\text{H}$ ]mannose-labeled  $\Delta\text{alg5}$  yeast.

$\text{Man}_8\text{GlcNAc}_2$  to  $\text{Man}_9\text{GlcNAc}_2$  step can be alleviated by *ALG9* overexpression.

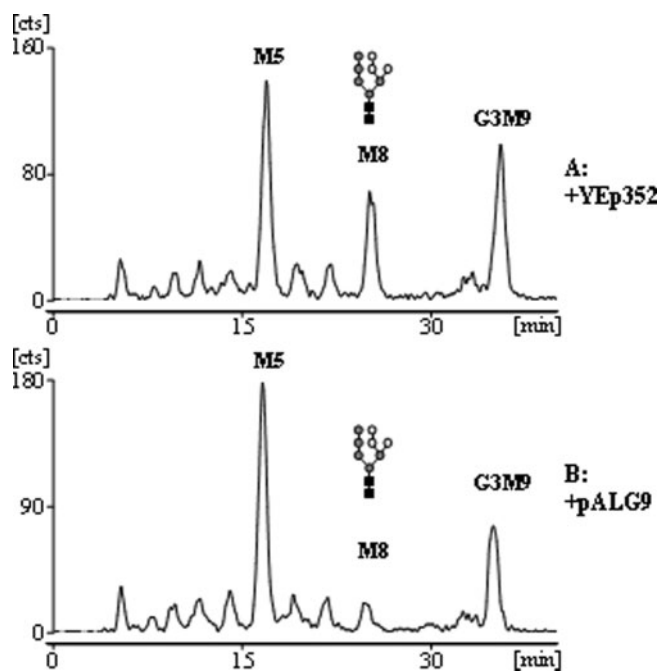
## Discussion

We provided both biochemical and genetic evidence that Alg9p is a Dol-P-Man-dependent  $\alpha$ -1,2 mannosyltransferase with dual function: the enzyme transfers the seventh mannose residue on the B-arm (i) (Burda *et al.*, 1996) as well as the ninth mannose residue on the C-arm (k) to the LLO.

We have recently identified a mutation of the human *ALG9* homologue as the cause of a novel type of CDG (CDG-1I; Frank *et al.*, 2004). Analysis of the LLO from the patient cells revealed that both *ALG9* substrates  $\text{Man}_6\text{GlcNAc}_2$  and  $\text{Man}_8\text{GlcNAc}_2$  accumulated, together with detectable amounts of full-length  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ . In agreement with this observation, expression of human *ALG9* cDNAs in yeast mutant strains indicated that the mutated

*ALG9* retained residual activity. This shows that the dual role of *ALG9* is conserved also in mammals. *ALG9* might also be bifunctional in amoebae, as Freeze and co-workers previously isolated a *Dictyostelium* glycosylation mutant accumulating  $\text{Man}_6\text{GlcNAc}_2$ -,  $\text{Man}_8\text{GlcNAc}_2$ -, and  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2\text{-PP-Dol}$  (HL244, Figure 4 in [Freeze *et al.*, 1989]). This mutant was not studied further, but on the basis of our findings most likely the *ALG9* locus is affected.

Trypanosomatids lack glycosylated LLOs and transfer unglycosylated oligosaccharides to protein (Parodi, 1993). Depending on the species, the mature LLO is  $\text{Man}_6\text{GlcNAc}_2\text{-PP-Dol}$ ,  $\text{Man}_7\text{GlcNAc}_2\text{-PP-Dol}$ , or  $\text{Man}_9\text{GlcNAc}_2\text{-PP-Dol}$ . Interestingly, no Trypanosoma species has been identified so far that transfers  $\text{Man}_8\text{GlcNAc}_2$  to protein (Bosch *et al.*, 1988). Our model predicts that cells of the  $\text{Man}_6\text{GlcNAc}_2\text{-PP-Dol}$  synthesizing species do not have *ALG9* activity, that those producing  $\text{Man}_7\text{GlcNAc}_2\text{-PP-Dol}$  have *ALG9* activity but no *ALG12* activity, and that the  $\text{Man}_9\text{GlcNAc}_2\text{-PP-Dol}$ -producing cells have both



**Fig. 4.** Effect of *ALG9* overexpression on the lipid-linked oligosaccharide (LLO) profile of wildtype cells. HPLC analysis of LLO from [<sup>3</sup>H]mannose-labeled yeast cells transformed with (A) YEp352, empty multicopy plasmid and (B) pALG9, multicopy plasmid carrying *ALG9*. Selected oligosaccharide species are indicated as M5 (Man<sub>5</sub>GlcNAc<sub>2</sub>), M8 (Man<sub>8</sub>GlcNAc<sub>2</sub>), and G3M9 (Glc<sub>3</sub>Man<sub>9</sub>GlcNAc<sub>2</sub>).

activities. As the presence of *ALG9* is required to form Man<sub>8</sub>GlcNAc<sub>2</sub>-PP-Dol (as without *ALG9* biosynthesis stops at Man<sub>6</sub>GlcNAc<sub>2</sub>-PP-Dol), *ALG9* will always elongate it further to Man<sub>9</sub>GlcNAc<sub>2</sub>-PP-Dol. This explains why no species with Man<sub>8</sub>GlcNAc<sub>2</sub>-PP-Dol as the mature LLO has been observed.

Removal of mannose i from protein-linked Man<sub>9</sub>GlcNAc<sub>2</sub> by ER mannosidase I (which yields the Man<sub>8</sub>GlcNAc<sub>2</sub> isomer B, different from the Man<sub>8</sub>GlcNAc<sub>2</sub> isomer C formed during LLO biosynthesis [Camirand *et al.*, 1991; Weng and Spiro, 1996]) has an important function in glycoprotein folding quality control in the ER (Helenius *et al.*, 1997; Helenius and Aebi, 2004). This trimming step is thought to act as a timer for the decision on whether a glycoprotein may be exported to distal compartments or if it has to be degraded via ER-associated degradation (Helenius *et al.*, 1997; Jakob *et al.*, 1998). It is therefore important that this Man<sub>8</sub>GlcNAc<sub>2</sub> isomer B only arises from trimming of protein-bound Man<sub>9</sub>GlcNAc<sub>2</sub> and is not formed during LLO biosynthesis and is transferred to protein. How is this achieved?

The specificities of the responsible glycosyltransferases ensure that the sequence of monosaccharide additions is following a sequential order: after translocation of Man<sub>5</sub>GlcNAc<sub>2</sub>-PP-Dol into the lumen (Helenius *et al.*, 2002), the B-arm (mannose h and i) is synthesized followed by the assembly of the C-arm (mannoses j and k). The oligosaccharide is completed with the triglucosyl cap on the A-arm (glucoses l–n). Interestingly, all the three branches of the mature Glc<sub>3</sub>Man<sub>9</sub>GlcNAc<sub>2</sub> oligosaccharide precursor terminate in

$\alpha$ -1,2-linked sugars. This suggests that capping with an  $\alpha$ -1,2-linked monosaccharide signals completion of a branch.

Formation of the Man<sub>8</sub>GlcNAc<sub>2</sub> isomer B during LLO biosynthesis is prevented by the substrate specificity of the  $\alpha$ -1,6 mannosyltransferase Alg12p that adds mannose j to LLO (Burda *et al.*, 1999; Cipollo and Trimble, 2002). This transferase requires an acceptor structure with the complete B-arm for optimal activity (Burda *et al.*, 1999). Thus, substrates for Alg9p with a Man<sub>7</sub>GlcNAc<sub>2</sub> structure composed of residues a–h and j are not formed.

Another key factor that ensures that only fully assembled Glc<sub>3</sub>Man<sub>9</sub>GlcNAc<sub>2</sub> oligosaccharide is transferred to protein is the selectivity of the OST for glucosylated LLO (Murphy and Spiro, 1981; Burda and Aebi, 1998; Spiro, 2000; Karaoglu *et al.*, 2001) in combination with the specificity of the Alg6p glycosyltransferase. Alg6p is required for addition of the first glucose (l), the rate-limiting step in LLO glucosylation (Reiss *et al.*, 1996). Analysis of LLO glucosylation in various *alg* mutants indicates that Man<sub>9</sub>GlcNAc<sub>2</sub>-PP-Dol is the optimal substrate for Alg6p (Verostek *et al.*, 1993; Burda *et al.*, 1999; Cipollo and Trimble, 2000). Thus, capping with  $\alpha$ -1,2-linked mannoses by Alg9p serves as a signal for Alg6p for completion of the LLO.

The relaxed specificity of Alg9p toward the mannose-acceptor structure rises an interesting question whether Alg9p can add an  $\alpha$ -1,2-linked mannose (at position f) to the Man<sub>3</sub>GlcNAc<sub>2</sub>-PP-Dol observed in  $\Delta$ *alg11* cells (Cipollo *et al.*, 2001). We have not carried out further studies on the substrate specificity so far. However, some conclusions on limitations of the substrate spectrum can be drawn from previously reported analyses of mutants affected in GPI-anchor biosynthesis. Besides Alg9p, two other members of the Man-P-Dol-dependent  $\alpha$ -1,2 mannosyltransferase family, Smp3p (Grimme *et al.*, 2001) and Gpi10p (Canivenc-Gansel *et al.*, 1998; Sutterlin *et al.*, 1998), are known in yeast. Both are involved in GPI-anchor biosynthesis, act on a lumenally oriented substrate, and might have evolved from a common ancestor with Alg9p (Oriol *et al.*, 2002). Overall sequence similarity to Alg9p is low (Gpi10p 20% identical, 36% similar over 616 amino acids; Smp3p 19% identical, 36% similar over 555 amino acids), but they share a similar topology as judged from the hydrophobicity profiles (Kyte-Doolittle hydrophathy algorithm). The *gpi10-1* mutant is affected in addition of the third mannose to GPI-anchor precursors but has a normal LLO profile (Canivenc-Gansel *et al.*, 1998). As demonstrated by the inviability of *SMP3*- or *GPI10*-deletion mutants (Sutterlin *et al.*, 1998), they are not redundant to each other. Assuming *SMP3* and *GPI10* do not have additional unknown functions indicates that the relaxed LLO substrate specificity of Alg9p does not extend to GPI-anchor intermediates, as it could otherwise compensate for loss of *SMP3* or *GPI10*.

## Materials and methods

### Yeast strains and media

Standard yeast media and genetic techniques were used (Guthrie and Fink, 1991). Yeast strains used in this study are listed in Table I.

**Table 1.** Yeast strains used in this study

Strain	Genotype	Source
SS328	<i>MATα ade2-101 his3Δ200 lys2-801 ura3-52</i>	Vijayraghavan <i>et al.</i> (1989)
YG414	<i>MATα ade2-101 ura3-52 his3Δ200 lys2-801 Δalg9::kanMX</i>	Burda <i>et al.</i> (1996)
YG840	<i>MATα ade2-101 ura3-52 his3Δ200 lys2-801 Δalg12::kanMX4</i>	Burda <i>et al.</i> (1999)

### Plasmids

pALG9 (Jakob *et al.*, 1998) is a derivative of YEp352 (Hill *et al.*, 1986) and contains a 2.5 kbp *BglII-KpnI* fragment of yeast genomic DNA encompassing the *ALG9* ORF plus 0.5 kbp upstream sequence in the *BamHI-KpnI* sites.

### HPLC analysis of [<sup>3</sup>H]-labeled oligosaccharides

HPLC analysis was carried out as described (Zufferey *et al.*, 1995).

### Isolation of [<sup>3</sup>H]Man<sub>7</sub>GlcNAc<sub>2</sub>-PP-Dol substrate

*Δalg12* yeast were grown in YPD medium at 30°C to late log phase and 80 OD<sub>546</sub> cells pulse labeled for 12 min with 125 μCi [<sup>3</sup>H]mannose (20 Ci/mmol; ICN Pharmaceuticals, Costa Mesa, CA) as described previously (Zufferey *et al.*, 1995). Workup of LLO was stopped after the CHCl<sub>3</sub> : CH<sub>3</sub>OH : H<sub>2</sub>O (10:10:3) (v/v/v) extraction, and the glycolipids were stored at -20°C until use.

### [<sup>3</sup>H]mannose labeling of ALG9-overexpressing cells

Wildtype yeast (SS328) transformed with either pALG9 or YEp352 were grown in 50 mL YPD at 30°C to mid-log phase (OD<sub>546</sub> 1.0 ± 0.1) and pulse labeled for 12 min with 25 μCi [<sup>3</sup>H]mannose as described previously (Zufferey *et al.*, 1995). Half of the isolated hydrolyzed oligosaccharides were analyzed by HPLC (see Results).

For the assessment of the overexpression effect, individual peak areas were quantified with the FLO-One software (Packard Biosciences, Meridan, CT), and the ratios of the Man<sub>8</sub>GlcNAc<sub>2</sub> and Man<sub>5</sub>GlcNAc<sub>2</sub> peaks (M8/M5) were calculated for each trace.

### Preparation of membranes

Yeast strains were grown to mid-log phase in YPD medium at 30°C.

200 OD<sub>546</sub> cells were harvested and washed once in washing buffer (50 mM HEPES/NaOH pH 7.5, 3 mM MgCl<sub>2</sub>). The cells were resuspended in cold lysis buffer (washing buffer supplemented with 1 mM DTT and protease inhibitors: 1 mM PMSF, 2 μg/mL Leupeptin, and 1 μg/mL E-64) and lysed by vortexing for 10 min with glass beads. The liquid was collected by puncturing the tubes with a hot needle and centrifugation at 1000 × *g* for 1 min. Intact cells, cell debris, and nuclei were removed by centrifugation for 5 min at 1000 × *g*. This supernatant was then centrifuged for 45 min at 50,000 × *g* to obtain the membrane

pellet. The pellet was resuspended and homogenized in storage buffer (lysis buffer supplemented with 35% [v/v] glycerol). Membranes were shockfrozen in liquid nitrogen and stored at -80°C until use. Protein concentration was determined using the bicinchoninic acid method.

### In vitro assay for elongation of [<sup>3</sup>H]Man<sub>7</sub>GlcNAc<sub>2</sub>-PP-Dol

Membranes (340 μg protein) were incubated with 1 mM GDP-Man and 1 mM CTP in 44 mM HEPES/NaOH pH 7.5, 3 mM MgCl<sub>2</sub>, 1 mM DTT, 30% (v/v) glycerol in a volume of 20 μL.

After incubation for 10 min at 25°C, 100 μL of [<sup>3</sup>H]Man<sub>7</sub>GlcNAc<sub>2</sub>-PP-Dol substrate (60,000–80,000 cpm) in 50 mM HEPES/NaOH pH 6.5, 60 mM NaCl, 5.8 mM MgCl<sub>2</sub>, 4.8 mM CaCl<sub>2</sub>, 4.8 mM MnCl<sub>2</sub>, 1 mM DTT, 0.3% (w/v) NP40 was added. The reaction was stopped after incubation at 25°C after 20 min by addition of 800 μL CHCl<sub>3</sub> : MeOH (1:1) (v/v) and thorough vortexing. The supernatant after centrifugation for 5 min at 9,000 × *g*/4°C was saved, and the pellet was re-extracted with 800 μL CHCl<sub>3</sub> : MeOH : H<sub>2</sub>O (10:10:3) (v/v/v). Both supernatants were combined, dried under nitrogen, and subjected to mild acid hydrolysis and analyzed by HPLC as described previously (Zufferey *et al.*, 1995).

### Oligosaccharide digestion with exo-α-1,2 mannosidase

Single peaks from HPLC analysis of assay products or *in vivo* labeling were collected, dried, and resuspended in incubation buffer (100 mM HOAc/NaOAc pH 5.0). The samples were split and incubated with or without 10 μU *Trichoderma reesei* exo-α-1,2 mannosidase (a gift from R. Contreras, Ghent) for 96 h at 37°C in a volume of 30 μL. After heat inactivation of the enzyme for 5 min at 95°C, the samples were spin-filtered and analyzed by HPLC as described above. Initial experiments were performed with an exo-α-1,2 mannosidase from *Aspergillus saitoi* (a gift from Dr. T. Butters, Oxford).

### Protein sequence alignments

The protein sequences used have the accession numbers NP\_014180 (Alg9p), NP\_011373 (Gpi10p), NP\_014792 (Smp3p), and NP\_014350 (Alg11p). Sequences were aligned using the Clustal W algorithm with a BLOSUM scoring matrix.

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### Abbreviations

ER, endoplasmic reticulum; GlcNAc, *N*-acetylglucosamine; LLO, lipid-linked oligosaccharide; OST, oligosaccharyl-transferase; P-Dol, dolichylphosphate; PP-Dol, dolichylpyrophosphate.

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