The *ALG10* locus of *Saccharomyces cerevisiae* encodes the α–1,2 glucosyltransferase of the endoplasmic reticulum: the terminal glucose of the lipid-linked oligosaccharide is required for efficient N-linked glycosylation

Patricie Burda and Markus Aebi[1]

Mikrobiologisches Institut, ETH Zürich, CH-8092 Zürich, Switzerland

[1]To whom correspondence should be addressed at: Mikrobiologisches Institut, ETH Zentrum, LFV E20, 8092 Zürich, Switzerland

The biosynthesis of the lipid-linked oligosaccharide substrate for N-linked protein glycosylation follows a highly conserved pathway at the membrane of the endoplasmic reticulum. Based on the synthetic growth defect in combination with a reduced oligosaccharyltransferase activity (*wbp1*), we have identified *alg10* mutant strains which accumulate lipid-linked $Glc_2Man_9GlcNAc_2$. We cloned the corresponding wild-type gene and show in a novel *in vitro* assay that Alg10p is a dolichyl-phosphoglucose-dependent glucosyltransferase which adds the terminal α-1,2 glucose to the lipid-linked $Glc_2Man_9GlcNAc_2$ oligosaccharide. Hypoglycosylation of secreted proteins in *alg10* deletion strains demonstrates that the terminal α-1,2-linked glucose residue is a key element in substrate recognition by the oligosaccharyltransferase. This ensures that primarily completely assembled oligosaccharide is transferred to protein.

Key words: lipid-linked oligosaccharides/glucosyltransferase/ endoplasmic reticulum

Introduction

Assembly of the lipid-linked core oligosaccharide $Glc_3Man_9GlcNAc_2$ occurs at the membrane of the endoplasmic reticulum and is a highly conserved process in eukaryotic cells (Kornfeld and Kornfeld, 1985; Tanner and Lehle, 1987; Herscovics and Orlean, 1993). In the central reaction of N-linked protein glycosylation the lipid-linked core oligosaccharide is transferred *en bloc* to selected asparagine residues of nascent polypeptide chains, a process catalyzed by the oligosaccharyltransferase (Kaplan *et al.*, 1987; Silberstein and Gilmore, 1996).

The core oligosaccharide is formed by the sequential addition of the sugars N-acetylglucosamine (GlcNAc), mannose (Man) and glucose (Glc) from their activated derivates to the lipid carrier dolichyl-pyrophosphate (Kornfeld and Kornfeld, 1985; Hirschberg and Snider, 1987; Verbert *et al.*, 1987; Abeijon and Hirschberg, 1992; Herscovics and Orlean, 1993). The addition of three glucose units to one antenna of lipid-linked $Man_9GlcNAc_2$ oligosaccharide forming two α-1,3 and one α-1,2 linkage are the final reactions in the oligosaccharide assembly. The glucose donor for these reactions is dolichyl-phosphoglucose (Dol-P-Glc). Genetic studies in yeast showed that mutations in the *ALG5* locus encoding the Dol-P-Glc synthase (Runge *et al.*, 1984; te Heesen *et al.*, 1994), the

ALG6 (Runge *et al.*, 1984; Reiss *et al.*, 1996) and *ALG8* locus (Runge and Robbins, 1986; Stagljar *et al.*, 1994), each encoding a putative α-1,3 glucosyltransferase, affect the glucosylation steps of the lipid-linked oligosaccharide. The phenotype of mutations in these *ALG* loci, namely, hypoglycosylation of secreted proteins *in vivo*, as well as additional biochemical data show that glucosylation of the oligosaccharide is important for the affinity of the oligosaccharyltransferase towards the lipid-linked oligosaccharide (Kornfeld and Kornfeld, 1985; Trimble and Verostek, 1995). Non- or partially glucosylated oligosaccharides can be transferred to protein, albeit with a reduced efficiency. Removal of the three terminal glucose residues by glucosidase I (Ray *et al.*, 1991; Shailubhai *et al.*, 1991; Kalz-Füller *et al.*, 1995) and glucosidase II (Burns and Touster, 1982; Trombetta *et al.*, 1996) are the first modifications that occur in the processing of protein bound oligosaccharide. The trimming of these glucose residues is involved in the quality control mechanism of protein folding in higher eukaryotic cells (Helenius *et al.*, 1997).

In this study we present the cloning and characterization of the *ALG10* locus, which encodes the α-1,2 glucosyltransferase involved in the terminal glucosylation step of the lipid-linked oligosaccharide. We show that *alg10* mutant strains accumulate lipid-linked $Glc_2Man_9GlcNAc_2$ and that the complete core oligosaccharide structure is necessary for efficient oligosaccharyltransferase activity *in vivo*. We show that the glucosyltransferase activity of Alg10p is dependent on the glucose donor dolichyl-phosphoglucose and that the acceptor oligosaccharide recognized by Alg10p is dolichol-linked $Glc_2Man_9GlcNAc_2$.

We postulate that the terminal α-1,2-linked glucose residue is important for substrate recognition by the oligosaccharyltransferase and in combination with the ordered stepwise assembly of lipid-linked $Glc_3Man_9GlcNAC_2$ it is ensured that only completely assembled oligosaccharide is transferred to protein.

Results

Reduced glycosylation efficiency in alg10 mutant cells

Based on the synthetic lethal phenotype of mutations that affect lipid-linked oligosaccharide biosynthesis in combination with a reduced oligosaccharyltransferase activity (*wbp1* mutant strains; (Stagljar *et al.*, 1994), we have identified novel mutations with altered oligosaccharide biosynthesis (Zufferey *et al.*, 1995). Among others, one novel complementation group defined the *ALG10* locus (Zufferey *et al.*, 1995). To investigate whether *alg10* mutations affect the N-glycosylation pathway, we examined the processing of carboxypeptidase Y (CPY), a vacuolar protease that contains four N-linked oligosaccharides (Burda *et al.*, 1996) (Figure 1a). As reported previously, mutations in *ALG* genes lead to underglycosylation of N-linked glycoproteins, because the oligosaccharyltransferase is provided with suboptimal substrate in these mutant strains (Huffaker and Robbins, 1983; Runge and Robbins, 1986; Burda *et al.*, 1996). Both,
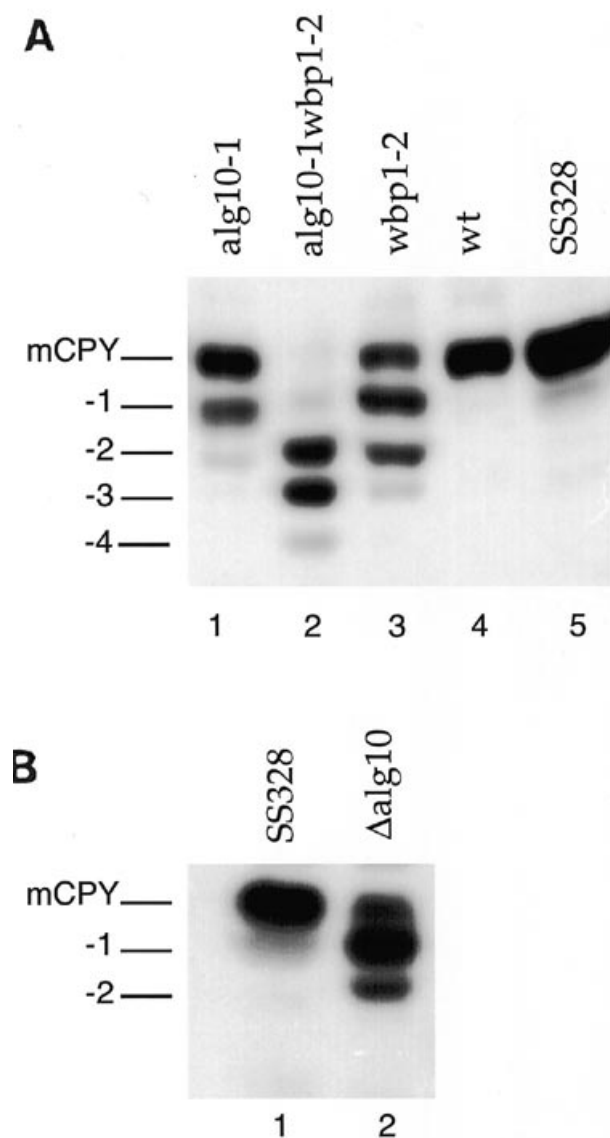
A

B

Fig. 1. *alg10* mutations affect glycosylation *in vivo*. (**a**) Four strains derived from a tetratype tetrad of the cross YG649×SS328 were used for CPY-specific immunoblotting. (**b**) The *ALG10* gene was disrupted using the KanMX-module generating strain YG428 and the processing of CPY was analyzed. 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. Strains: YG651 (*alg10–1*), YG654 (*alg10–1 wbp1–2*), YG652 (*wbp1–2*), YG653 (*wt*), SS328 (wild-type), YG428 (Δ*alg10::kanMX4*).

alg10–1 and *wbp1–2* mutations led to underglycosylation of CPY (Figure 1a, lane 1 and 3). A combination of these two mutations caused a synthetic phenotype and a severe glycosylation defect, resulting in significantly reduced levels of mCPY (Figure 1a, lane 2). This cumulative effect on glycosylation in the double mutant strain as compared to either single mutant strain is also reflected in the growth phenotype: the *alg10–1 wbp1–2* double mutant strain does not grow at 30°C, a temperature permissive for both single mutants (data not shown).

alg10 mutant cells accumulate lipid-linked Glc$_2$Man$_9$GlcNAc$_2$

To investigate whether an *alg10* mutation is affecting the biosynthesis of lipid-linked oligosaccharides, *alg10–2* mutant cells

were labeled *in vivo* with [^{3}H]mannose, lipid-linked oligosaccharides were extracted and the oligosaccharides were analyzed (Figure 2). Wild-type cells accumulated full-length lipid-linked Glc$_3$Man$_9$GlcNAc$_2$ oligosaccharide, minor peaks representing biosynthetic intermediates were also detected (Figure 2A). In *alg10–2* mutant cells, an additional oligosaccharide peak that eluted between Glc$_1$Man$_9$GlcNAc$_2$ and Glc$_3$Man$_9$GlcNAc$_2$ oligosaccharide was observed (Figure 2B–D). The additional peak had the retention time expected for Glc$_2$Man$_9$GlcNAc$_2$, since the biosynthesis of the core oligosaccharide follows a highly ordered assembly (Burda *et al.*, 1996). We therefore postulate that *alg10* mutations affect the glucosyltransferase activity required for the terminal glucosylation step of the lipid-linked core oligosaccharide. This defect in the terminal glucosylation reaction results in the accumulation of lipid-linked Glc$_2$Man$_9$GlcNAc$_2$.

Isolation of the ALG10 locus

The *ALG10* locus was cloned by complementation of the synthetic lethal growth phenotype observed in *alg10 wbp1* double mutant strains, because *alg10* single mutant strains do not show a detectable growth phenotype (data not shown). The *alg10–1 wbp1–2* strain (YG649) was transformed with a plasmid library containing yeast chromosomal DNA integrated into vector YEp352. Among 15,000 transformants, we found 15 strains which grew at 30°C but not at 37°C, indicating complementation of the *alg10* mutation. Plasmids were recovered from 10 of the 15 strains, amplified in *E.coli* and reintroduced into the *alg10–1 wbp1–2* mutant strain (YG649). All plasmids were able to restore growth at 30°C in the double mutant strain. Upon restriction enzyme analysis one plasmid was identified as carrying the *OST2* gene (Silberstein *et al.*, 1995). This gene is known as an allele specific, high copy number suppressor of the *wbp1–2* mutation. Restriction enzyme analysis of the other plasmids revealed a common restriction pattern among six of them, the remaining three plasmids were not further analyzed in this study. The plasmid containing the smallest common insert (1.58 kb) of the six related isolates was termed pALG10–1 and subjected to sequence analysis. Comparison of the insert sequence with available databases revealed one ORF (YGR227w) encoding a protein with a calculated molecular mass of 62 kDa. YGR227w has been previously identified as the *DIE2* locus (Nikawa and Hosaki, 1995). The amino acid sequence predicts three potential N-linked glycosylation sites but no potential N-terminal signal sequence. However, the sequence contains a basic KK motif at the N-terminus, which might serve as an ER-retention signal (Schutze *et al.*, 1994). The putative *ALG10* protein is hydrophobic and has a calculated high isoelectric point of 9.4. These features are also observed in the other two putative ER-glucosyltransferases, Alg6p (Reiss *et al.*, 1996) and Alg8p (Stagljar *et al.*, 1994). Nevertheless, there is no significant sequence similarity between Alg10p and the putative glucosyltransferases Alg6p and Alg8p, but the hydrophobicity pattern of these three proteins appears to be highly similar (data not shown). A search in sequence databases showed that Alg10p has potential homologues of unknown function in *Schizosaccharomyces pombe* (30% amino acids identity, accession no. Z69728) and in *Caenorhabditis elegans* (19% amino acid identity, accession no. Z81131).

To confirm that pALG10–1 contained the gene identified by the *alg10* mutations, we deleted the *ALG10* gene by replacing the *ALG10* ORF with the *KanMX* gene (Wach *et al.*, 1994). The resulting Δ*alg10::kanMX4* strain (YG428) showed no aberrant growth phenotype at all temperatures tested. However, even a more severe hypoglycosylation phenotype of CPY was observed
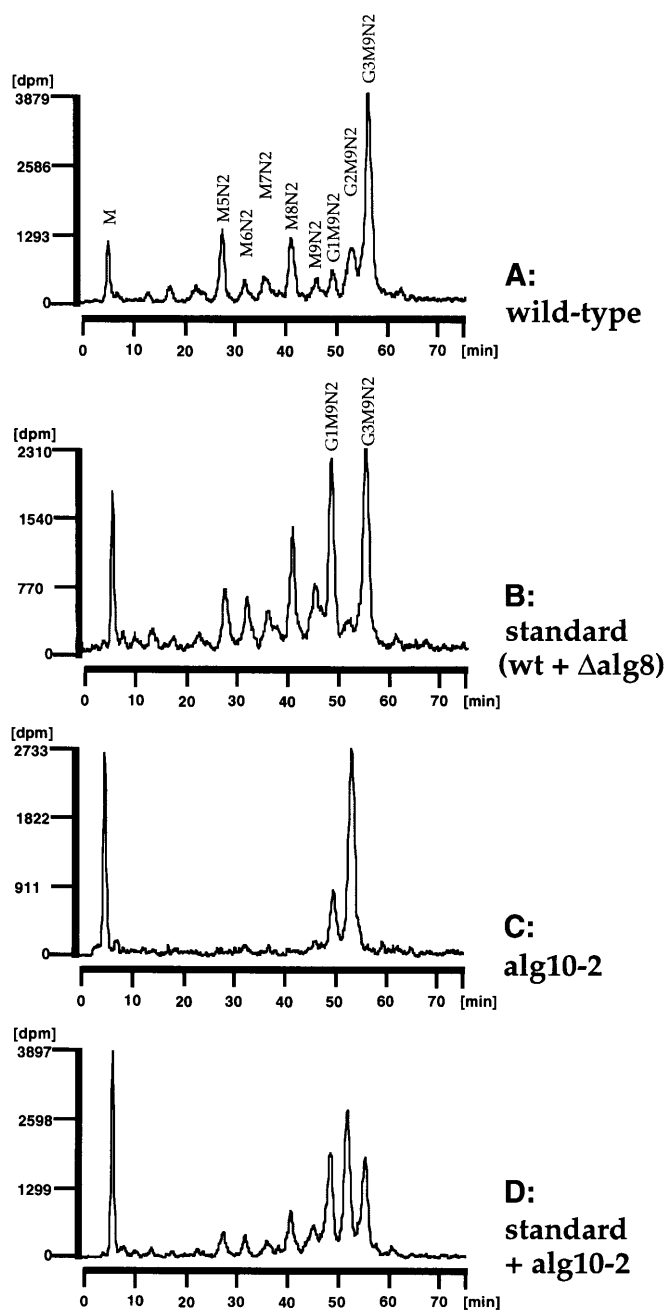
Fig. 2. The *alg10–2* mutant strain (YG650) accumulates lipid-linked Glc$_2$Man$_9$GlcNAc$_2$. Cells were labeled with [^{3}H] mannose. Lipid-linked oligosaccharides were extracted and hydrolyzed, and the liberated oligosaccharides were analyzed by HPLC. Oligosaccharides isolated from strain SS328 (wild-type) and YG125 ($\Delta alg8$) served as standards. The position of mannose (M), Man$_{5–9}$GlcNAc$_2$ (M5–9N2) and Glc$_{1–3}$Man$_9$GlcNAc$_2$ (G1–3M9N2) are indicated. (**A**) Wild-type strain SS328. (**B**) Mixed standard consisting of oligosaccharides derived from wild-type strain SS328 (G3M9N2) and $\Delta alg8$ strain YG125 (G1M9N2). (**C**) *alg10–2* mutant strain YG650. (**D**) Mixture of (**B**) and (**C**).

in the deletion strain (Figure 1b, lane 2) as compared to the *alg10–1* mutant strain (Figure 1a, lane 1). When crossed with the *wbp1–2* mutant strain (MA9-D) the resulting double mutant strain $\Delta alg10::kanMX4$ *wbp1–2* did not grow at 30°C, in agreement with the growth phenotype observed in the original double mutant isolate YG649. To confirm genetically that

alg10–1 and $\Delta alg10::kanMX4$ are alleles of the same gene, we crossed the *alg10–1 wbp1–2* strain with the $\Delta alg10::kanMX4$ strain (Stagljar *et al.*, 1994). Upon analysis of 27 tetrads no *wbp1–2* single mutant was found, indicating that all 27 tetrads were of parental ditype with respect to *alg10–1* and $\Delta alg10::kanMX4$. In addition, both the *alg10–1* and $\Delta alg10::kanMX4$ alleles were found in combination with *wbp1–2*. These results demonstrated genetically the identity of the cloned locus with the *ALG10* gene.

The ALG10 locus encodes a Dol-P-Glc dependent glucosyltransferase

To determine if Alg10p is a glucosyltransferase, we took advantage of the $\Delta alg10$ knockout strain which is unable to synthesize the full length core oligosaccharide and accumulates Glc$_2$Man$_9$GlcNAc$_2$-PP-Dol, the appropriate acceptor oligosaccharide for Alg10p. Microsomal membranes were prepared from wild-type, $\Delta alg10$ and wild-type strain containing pALG10–1 and the membranes were incubated with [^{3}H] mannose labeled Glc$_2$Man$_9$GlcNAc$_2$-PP-Dol. The lipid-linked oligosaccharide product formed was extracted and subjected to acidic hydrolysis, and the liberated oligosaccharides were analyzed by HPLC (Figure 3). When assayed with microsomes deriving from wild-type and wild-type strain containing pALG10–1, we observed a novel dolichol-derived oligosaccharide (Figure 3C,D) which comigrated with full-length Glc$_3$Man$_9$GlcNAc$_2$ oligosaccharide isolated from the wild-type strain SS328 (data not shown). The amount of Glc$_3$Man$_9$GlcNAc$_2$ oligosaccharide formed directly correlated with the expression level of the *ALG10* gene: no formation of Glc$_3$Man$_9$GlcNAc$_2$ was observed in $\Delta alg10$ extracts (Figure 3B), low amount of Glc$_3$Man$_9$GlcNAc$_2$ was present in wild-type microsomes (Figure 3C), whereas a strong production of Glc$_3$Man$_9$GlcNAc$_2$ oligosaccharide was observed in extracts derived from cells overexpressing Alg10p (Figure 3D). We therefore took the formation of the Glc$_3$Man$_9$GlcNAc$_2$ product as a measure for Alg10p activity. To confirm that elevated production of the Glc$_3$Man$_9$GlcNAc$_2$ oligosaccharide indeed correlated to overproduction of Alg10p we constructed a Protein A-tagged version of Alg10p and analyzed the expression levels by Western-blot techniques using α-Protein A antiserum. Expression from the episomal vector YEp352 resulted in an approximately 10- to 20-fold increase of Alg10p as compared to the expression from the genomic locus (data not shown).

The enzymatic activity of Alg10p in the wild-type strain SS328 was found to be linear for up to 10 min and was proportional to the amount of microsomal proteins added, up to 600 µg of protein/assay (data not shown). Microsomes prepared from cells overexpressing the glucosyltransferase activity also exhibited a linearity with time and with the amount of microsomes added (Figure 4A and 4B). The dependence of the Alg10p activity on the concentration of Glc$_2$Man$_9$GlcNAc$_2$-PP-Dol is given in Figure 4C. Under the conditions used, the Alg10p activity followed Michaelis-Menten kinetics (data not shown).

Because we assumed that the Alg10p activity is dependent on endogenous Dol-P-Glc as glucose donor, we wanted to verify the Dol-P-Glc dependence in our *in vitro* reaction. A disruption of the *ALG5* locus causes loss of dolichyl-phosphoglucose synthase activity, cells are therefore devoid of Dol-P-Glc and accumulate lipid-linked Man$_9$GlcNAc$_2$ (Runge *et al.*, 1984; te Heesen *et al.*, 1994). We transformed the $\Delta alg5$ mutant strain with the Alg10p overexpressing plasmid pALG10–1, prepared microsomal membranes and tested in the *in vitro* assay whether we could still measure glucosyltransferase activity. As shown in Table I, almost
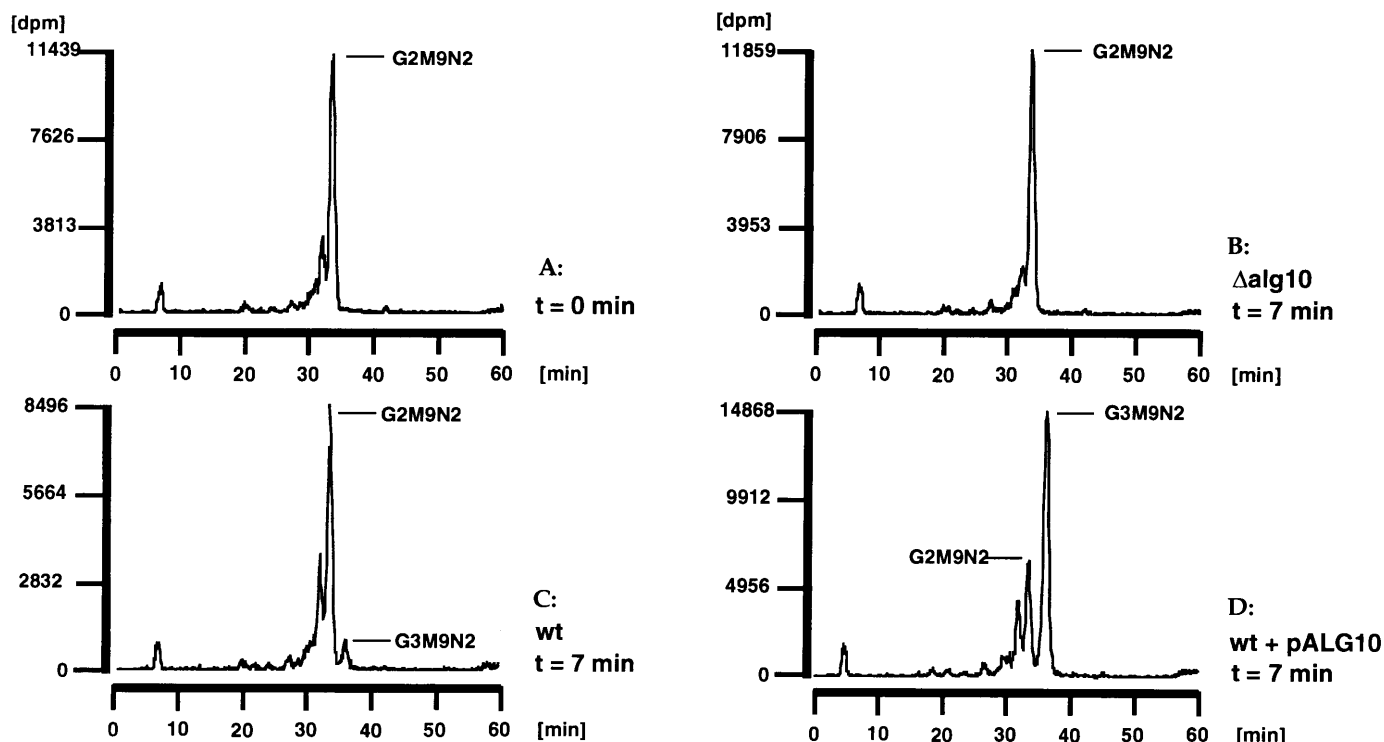
Fig. 3. Glucosyltransferase activity in microsomes derived from Δ*alg10* (YG428), wild-type (SS328) and wild-type strain transformed with pALG10–1 (YG730). Glucosyltransferase activity was determined using [^{3}H] mannose labeled $Glc_2Man_9GlcNAc_2$-PP-Dol as acceptor substrate. The formation of $Glc_3Man_9GlcNAc_2$-PP-Dol was followed by HPLC analysis (G3M9N2, $Glc_3Man_9GlcNAc_2$; G2M9N2, $Glc_2Man_9GlcNAc_2$).

no formation of lipid-linked $Glc_3Man_9GlcNAc_2$ could be observed in a Δ*alg5* background, whereas high activity was seen in an *ALG5* wild-type background. The formation of residual amounts of $Glc_3Man_9GlcNAc_2$ observed with Δ*alg5* microsomes was most probably due to contamination of the acceptor substrate $Glc_2Man_9GlcNAc_2$-PP-Dol with Dol-P-Glc which partially copurifies with the acceptor substrate and serves as glucose donor in the assay. This experiment shows that the glucosyltransferase activity is dependent on an intact dolichyl-phosphoglucose synthase and verifies that *ALG10* encodes a Dol-P-Glc dependent glucosyltransferase. Because Δ*alg10* cells accumulate a lipid-linked oligosaccharide one hexose unit shorter than the fully assembled lipid-linked oligosaccharide (Figure 3B), which can be converted to the complete $Glc_3Man_9GlcNAc_2$-PP-Dol by Alg10p, we conclude that Alg10p is a Dol-P-Glc dependent α-1,2 glucosyltransferase.

Table I. Donor substrate specificity of the ALG10 glucosyltransferase

Time (min)	Strain	% G2M9N2	% G3M9N2
0	Δalg5+ pALG10–1	100	0
10	Δalg5+ pALG10–1	90	10
10	wt + pALG10–1	17	83

The Δ*alg5* strain (YG91) was transformed with pALG10–1, grown to mid-log phase, microsomes were prepared and the glucosyltransferase activity was measured for the time indicated using radiolabeled $Glc_2Man_9GlcNAc_2$-PP-Dol as acceptor substrate. For comparison, the assay was performed with microsomes prepared from wild-type strain SS328 transformed with pALG10–1 (YG730). For the reactions 300 μg microsomes was added. The relative radioactivity present in the substrate (%G2M9N2) and the product (%G3M9N2) is given. (G2M9N2, $Glc_2Man_9GlcNAc_2$; G3M9N2, $Glc_3Man_9GlcNAc_2$).

Table II. Acceptor substrate specificity of the ALG10 glucosyltransferase

Substrate	% G1M9N2	% G2M9N2	% G3M9N2
$Glc_1Man_9GlcNAc_2$-PP-Dol	93	0	7
$Glc_2Man_9GlcNAc_2$-PP-Dol	—	25	75

The Δ*alg10* strain (YG428) was transformed with pALG10–1, grown to mid-log phase, microsomes were prepared and the glucosyltransferase activity was measured using either radiolabeled $Glc_1Man_9GlcNAc_2$-PP-Dol or $Glc_2Man_9GlcNAc_2$-PP-Dol as acceptor substrate. The results represent the average of two independent assays with a 10 min incubation time. The relative radioactivity present in the substrate (%G1M9N2 or %G2M9N2) and the product (%G2M9N2 or %G3M9N2) are given. (G1M9N2, $Glc_1Man_9GlcNAc_2$; G2M9N2, $Glc_2Man_9GlcNAc_2$; G3M9N2, $Glc_3Man_9GlcNAc_2$).

We asked whether the glucosyltransferase encoded by the *ALG10* locus shows a stringent specificity for the acceptor substrate $Glc_2Man_9GlcNAc_2$-PP-Dol. Lipid-linked $Glc_1Man_9GlcNAc_2$ isolated from Δ*alg8* cells (YG125) (Runge and Robbins, 1986; Stagljar *et al.*, 1994) and $Glc_2Man_9GlcNAc_2$-PP-Dol isolated from Δ*alg10* cells were used as substrates in a glucosyltransferase assay with microsomes derived from Alg10p overproducing cells. As presented in Table II, only $Glc_2Man_9GlcNAc_2$-PP-Dol served as efficient acceptor, whereas $Glc_1Man_9GlcNAc_2$-PP-Dol was not converted in significant amounts. The small amount of $Glc_3Man_9GlcNAc_2$ oligosaccharide formed from the $Glc_1Man_9GlcNAc_2$-PP-Dol substrate is believed to be due to the combined Alg8p and Alg10p activity in the microsomes: the presence of Alg8p can convert $Glc_1Man_9GlcNAc_2$-PP-Dol to $Glc_2Man_9GlcNAc_2$-PP-Dol which then serves as a substrate for Alg10p. Because of Alg10p overexpression, no $Glc_2Man_9GlcNAc_2$ intermediate is detected.
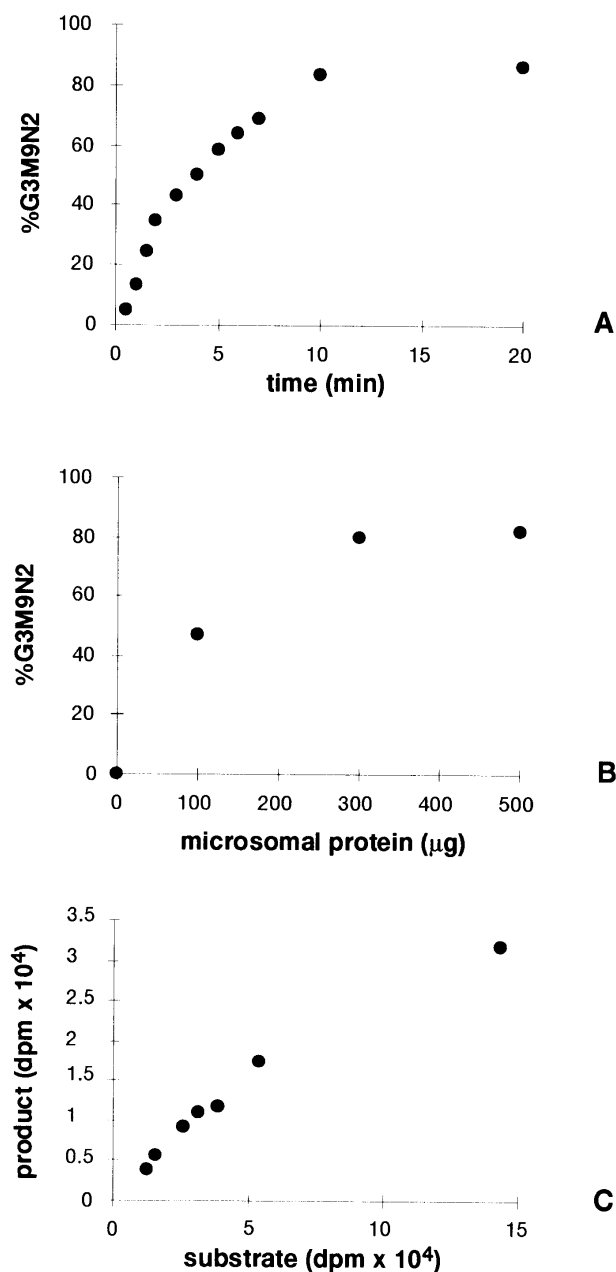
Fig. 4. *In vitro* glucosyltransferase activity in microsomes from wild-type strain overexpressing Alg10p (YG730). The enzymatic activity was measured as described in the method section using [^{3}H] mannose labeled Glc$_2$Man$_9$GlcNAc$_2$-PP-Dol as acceptor substrate. Time and protein concentration dependence of the Alg10p activity is shown in (**A**) and (**B**), respectively. The relative radioactivity present in the product Glc$_3$Man$_9$GlcNAc$_2$-PP-Dol (%G3M9N2) is given. (**C**) shows the effect of concentration of Glc$_2$Man$_9$GlcNAc$_2$-PP-Dol (substrate) on the formation of Glc$_3$Man$_9$GlcNAc$_2$-PP-Dol (product).

When Glc$_1$Man$_9$GlcNAc$_2$-PP-Dol was tested as acceptor oligosaccharide in an assay using $\Delta alg8$ microsomes containing pALG10–1 neither Glc$_3$Man$_9$GlcNAc$_2$ nor Glc$_2$Man$_9$GlcNAc$_2$ oligosaccharide was detected (data not shown). These *in vitro* experiments suggest that lipid-linked Glc$_1$Man$_9$GlcNAc$_2$ oligosaccharide is not a substrate for the *ALG10* transferase. We conclude that Alg10p is a highly specific α-1,2 glucosyltransferase.

Discussion

The addition of three glucose residues to dolichol-linked oligosaccharide is the final modification in the maturation of the carbohydrate substrate prior to transfer to protein. Two α-1,3-linked glucose residues and the terminal α-1,2 glucose are added in a stepwise fashion (Figure 2A) to lipid-linked Man$_9$GlcNAc$_2$ oligosaccharide. Yeast genetic approaches have identified specific mutants deficient in each of these glucosylation reactions: *alg6* mutants (Runge *et al.*, 1984; Reiss *et al.*, 1996) accumulate dolichol-linked Man$_9$GlcNAc$_2$ and are believed to be defective in the transferase that adds the first α-1,3-linked glucose, *alg8* cells (Runge and Robbins, 1986; Stagljar *et al.*, 1994) accumulate dolichol-linked Glc$_1$Man$_9$Glc-NAc$_2$ due to the inability to transfer the second α-1,3 glucose. In this study we report the cloning and characterization of the *ALG10* locus that encodes the α-1,2 glucosyltransferase responsible for the addition of the terminal glucose residue. We established a novel *in vitro* assay to measure the Alg10p activity and our data show that Alg10p transfers a single glucose residue from Dol-P-Glc to Glc$_2$Man$_9$GlcNAc$_2$-PP-Dol. The identification of three individual glucosyltransferases in yeast confirms the observation made in higher eukaryotic cells where three specific glucosyltransferase activities could be separated by hydroxylapatite chromatography (D'Souza-Schorey and Elbein, 1993). The identification of Alg10p homologous proteins in other eukaryotic cells supports the idea that the assembly pathway of lipid-linked core oligosaccharide is highly conserved among eukaryotes (Kornfeld and Kornfeld, 1985; Tanner and Lehle, 1987; Herscovics and Orlean, 1993).

The *ALG10* glucosyltransferase is an enzyme activity of the endoplasmic reticulum and we observe a putative ER retrieval signal at the N-terminus of the protein (Schutze *et al.*, 1994). This ER localization of Alg10p might explain why the same locus was identified as *DIE2* by Nikawa *et al.* (Nikawa and Hosaki, 1995). These authors showed that the *DIE2* product, when expressed in high copy numbers, enhances the expression of *ITR1* (Nikawa *et al.*, 1991) and *INO1* (Hoshizaki *et al.*, 1990). *ITR1* encodes the inositol-transport system I, whereas *INO1* codes for the inositol 1-phosphate synthase. Both gene products are involved in the biosynthesis of phosphatidylinositol, one of the most abundant phospholipids in yeast cells (Carman and Henry, 1989). We explain the regulatory effect of Alg10p/Die2p overproduction on *INO1* and *ITR1* expression as follows: overexpression of ER-resident membrane proteins can induce proliferation of the endoplasmic reticulum (Vergeres *et al.*, 1993; Ohkuma *et al.*, 1995; Parrish *et al.*, 1995) and overexpression of Alg10p/Die2p, of which its hydrophobicity pattern suggests multiple membrane spanning domains, could therefore result in a similar membrane proliferation of the ER. Membrane proliferation would require increased supply of phospholipids thus resulting in an elevated expression of *ITR1* and *INO1*.

All three yeast glucosyltransferases, Alg6p, Alg8p, and Alg10p are highly hydrophobic proteins and seem to contain multiple transmembrane domains. However, we do not find any significant sequence similarity between Alg10p and the two other putative glucosyltransferases, whereas Alg6p and Alg8p share identical sequences (26% identity over 604 amino acids; Reiss *et al.*, 1996). This sequence similarity, located in the more hydrophilic parts of the proteins might reflect the fact that both Alg6p and Alg8p catalyze the addition of an α-1,3 linked glucose residue, whereas Alg10p is an α-1,2 glucosyltransferase. On the other hand Alg6p, Alg8p, and Alg10p all utilize the same glucose

donor, namely dolichyl-phosphoglucose. This substrate is synthesized at the cytoplasmic side of the ER membrane but utilized in the lumenal part, suggesting a flipping of Dol-P-Glc across the membrane. However, our extensive screening for mutants deficient in the biosynthesis of the lipid-linked oligosaccharide has not revealed any mutants with a defect in this flipping reaction, but yielded many mutants with defects in the three glucosyltransferases and the Dol-P-Glc synthase Alg5p (Runge *et al.*, 1984; te Heesen *et al.*, 1994). Putative mutants with a defect in the Dol-P-Glc flippase would be expected to have the same phenotype as *alg5* and *alg6* mutants, namely, the inability to glucosylate the lipid-linked $Man_9GlcNAc_2$ oligosaccharide. The apparent absence of such flippase mutants can be explained in several ways. It is possible that no specific Dol-P-Glc flippase exists and that the transfer of Dol-P-Glc is carried out by each of the Dol-P-Glc-dependent glucosyltransferases: the addition of the glucose residue to the oligosaccharide and the flipping might be a coupled process (Hirschberg and Snider, 1987; Verbert *et al.*, 1987). Alternatively, a putative flippase activity of Alg5p or the presence of multiple Dol-P-Glc flippases would also explain the lack of a complementation group with a defect in the Dol-P-Glc flipping reaction.

An important aspect of our study is the finding that lack of the terminal α-1,2 glucose residue in lipid-linked oligosaccharides results in a reduced transfer of the oligosaccharide to protein *in vivo* (Figure 1b, lane 2). The importance of the terminal glucose for transfer efficiency has already been demonstrated *in vitro* by Spiro and co-workers (Murphy and Spiro, 1981). These results imply that the terminal α-1,2 glucose is a key element in the substrate recognition of the oligosaccharyltransferase (OTase). In combination with the highly ordered, stepwise assembly of the lipid-linked oligosaccharide (Burda *et al.*, 1996) and the high steady state level of complete assembled lipid-linked $Glc_3Man_9GlcNAc_2$ as compared to the biosynthetic intermediates in wild-type cells (see Figure 2A), the specificity of the OTase for lipid-linked $Glc_3Man_9GlcNAc_2$ is sufficient to ensure that only full-length oligosaccharide is transferred to protein. Mannose residues which are added in the lumen of the ER seem to contribute little to the substrate recognition by the OTase because removal of these residues by treatment of the lipid-linked oligosaccharide with α-mannosidase does not alter the affinity of the OTase to the modified substrate (Spiro *et al.*, 1979; Staneloni *et al.*, 1981). However, other determinants than the terminal glucose are recognized by the OTase complex, because incomplete lipid-linked oligosaccharide can be transferred to protein. In addition, glucosylation of the lipid-linked oligosaccharide is not absolutely required in all eukaryotes, because trypanosomatids transfer oligosaccharides onto protein that appear to be devoid of glucose units and contain $Man_{7-9}GlcNAc_2$, depending on the species (Parodi, 1993).

It is interesting to note that the terminal α-1,2 glucose residue is rapidly hydrolyzed from the protein-bound oligosaccharide by glucosidase I immediately after the transfer of the carbohydrate to protein (Hubbard and Robbins, 1979; Chen *et al.*, 1995). Thus, the key element for substrate recognition by the OTase is removed from the protein-bound oligosaccharide preventing the interaction of the OTase complex with its product. If we assume that the hydrolysis of the N-glycosidic linkage (basically the reverse reaction of glycosylation with water instead of dolichyl-pyrophosphate as the acceptor) can also be catalyzed by the OTase complex, the cleavage of the terminal α-1,2 glucose residue by glucosidase I would therefore prevent this reaction. It might ensure that glycosylated N-glycosylation sites of translocating proteins remain glycosylated while the nascent polypeptide might pause in the translocation channel, in the immediate neighborhood of the OTase complex. We suggest that the terminal α-1,2 glucose residue on the lipid-bound oligosaccharide serves as a signal to indicate complete assembly of the core oligosaccharide. Once transferred to protein, the removal of this signal by glucosidase I might ensure that glycosylation sites remain glycosylated and are not deglycosylated by the oligosaccharyltransferase complex. Yeast genetic systems and *in vitro* translocation/glycosylation will make it possible to address this hypothesis directly.

Materials and methods

Yeast strains and media

The following strains of *Saccharomyces cerevisiae* were used in this study: SS328 (*Matα ade2–101 ura3–52 his3Δ200 lys2–801*) (te Heesen *et al.*, 1993), MA9-D (*Mata ade2–101 ura3–52 his3Δ200 lys2–801 wbp1–2*) (te Heesen *et al.*, 1993), YG649 (*Mata ade2–101 ade3 ura3–52 his3Δ200 leu2 wbp1–2 alg10*), YG650 (*Matα ade2–101 ade3 ura3–52 his3Δ200 lys2 wbp1–2 alg10–2 p[CH1122WBP1]*) (Zufferey *et al.*, 1995), YG651 (*Mata ade2–101 ade3 ura3–52 his3Δ200 leu2 alg10–1*), YG652 (*Matα ade2–101 ura3–52 his3Δ200 lys2–801 wbp1–2*), YG653 (*Mata ade2–101 ura3–52 his3Δ200 leu2 lys2–801*), YG654 (*Matα ade2–101 ade3 ura3–52 his3Δ200 alg10–1 wbp1–2*), YG428 (*Matα ade2–101 ura3–52 his3Δ200 lys2–801 Δalg10::kanMX4*), YG125 (*Matα ade2–101 ura3 tyr1 lys2 Δalg8::HIS3*) (Stagljar *et al.*, 1994), YG91 (*Matα ade2–101 his3 ura3 Δalg5::HIS3*) (te Heesen *et al.*, 1994), YG699 (*Matα ade2–101 ura3–52 his3Δ200 lys2–801 pYEp352[ProtA-ALG10]*), YG700 (*Matα ade2–101 ura3–52 his3Δ200 lys2–801 Δalg10::ProtA-ALG10*), YG730 (*Matα ade2–101 ura3–52 his3Δ200 lys2–801 pYEp352[pALG10–1]*). Standard yeast media and genetic techniques were applied (Guthrie and Fink, 1991).

Isolation and disruption of the ALG10 locus

The *ALG10* gene (GenEMBL Accession No. X87941, ORF YGR227w) was isolated by complementation of the temperature-sensitive phenotype of an *alg10 wbp1–2* mutant strain. The principle of the cloning procedure has been described (Stagljar *et al.*, 1994). The disruption of the *ALG10* gene was performed according to the PCR-based gene disruption using the KanMX4-module (Burda *et al.*, 1996) and is described elsewhere (Jakob *et al.*, 1998).

Immunological techniques

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

Preparation of microsomal membranes

Microsomal membranes were prepared according to Reiss *et al.* (Reiss *et al.*, 1997) with the following modification: the pelleted cells were washed and lysed in membrane buffer containing 50 mM HEPES pH 6.5, 1 mM $MgCl_2$, and 1 mM DTT.

Extraction and analysis of lipid-linked oligosaccharides for characterization of the alg10–2 mutant strain

Metabolic labeling with [³H]mannose (20 Ci/mmol; ICN Pharmaceuticals), extraction of lipid-linked oligosaccharides and HPLC analysis of the oligosaccharides were performed as previously described (Zufferey *et al.*, 1995) with the following modifications: the removal of lipids after acidic hydrolysis was done by extraction with $CHCl_3:CH_3OH$ (5:1, v/v). The acidic aqueous phase (containing the oligosaccharides) was neutralized by the addition of saturated sodium carbonate solution prior to drying under nitrogen stream.

Glucosyltransferase assay

The glucosyltransferase activity was measured in microsomal membranes using [³H]mannose labeled dolichyl-linked $Glc_2Man_9GlcNAc_2$ as the acceptor molecule. Dolichyl-phosphoglucose was provided by the membrane fraction. Since the omission of CTP and UDP-Glc (precursors for Dol-P-Glc synthesis) in the transferase assay did not alter the rate of $Glc_3Man_9GlcNAc_2$-PP-Dol production (data not shown) we assumed that the glucose donor Dol-P-Glc is supplied in excess by the microsomes. The conversion of lipid-linked $Glc_2Man_9GlcNAc_2$ to lipid-linked $Glc_3Man_9GlcNAc_2$ was followed by monitoring and analyzing the radiolabeled sugars by HPLC (Zufferey *et al.*, 1995). Radiolabeled dolichyl-linked $Glc_2Man_9GlcNAc_2$ was obtained by scaling up the procedure for isolation of lipid-linked oligosaccharides (see above). The $\Delta alg10$ strain YG428 was used for metabolic labeling. Lipid-linked oligosaccharides were extracted with a mixture of chloroform/water/methanol (10:10:3, v/v/v), dried under a stream of nitrogen, resuspended in 1 ml chloroform/water/methanol (10:10:3, v/v/v) and stored as $Glc_2Man_9GlcNAc_2$-PP-Dol stock solution at -20°C. For a standard glucosyltransferase assay 100 µl of this stock solution (4.5 to 5×10^5 d.p.m.) was dried in a Savant Speed Vac and the lipid-linked oligosaccharides were resuspended for 30 min at 37°C in 75–150 µl buffer containing 50 mM HEPES pH 6.5, 140 mM sucrose, 25 mM KCl, and 0.5% Nikkol. The assay mixture contained together with the resuspended $Glc_2Man_9GlcNAc_2$-PP-Dol 50 mM HEPES pH 6.5, 1 mM $MgCl_2$, 0.25 mM EDTA, 1 mM DTT, 1 mM CTP, 0.5 mM UDP-Glc, 5 mM $CaCl_2$, 105 mM sucrose, 20 mM KCl, and 0.4% Nikkol. The assay was started by the addition of microsomes (100–300 µg) and performed at 25°C for 10 min. The reaction was terminated by the addition of chloroform and methanol to yield a ratio $CHCl_3:CH_3OH:H_2O$ of 10:10:3 (v/v) and lipid-linked oligosaccharides were extracted as described above. The extraction was repeated twice, the supernatants combined and dried under a stream of nitrogen at 37°C. The removal of the lipids and the isolation of the oligosaccharides were performed as described above. The oligosaccharides were dissolved in 70 µl H_2O and resolved by HPLC (Supelco LC-NH2 column) using a gradient of acetonitrile/water (70:30, v/v) to acetonitrile/water (59:41, v/v) over 5 min, followed by acetonitrile/water (48:52, v/v) for 40 min. The column was washed with water for 5 min and reequilibrated with acetonitrile/water (70:30, v/v) for 30 min.

Acknowledgments

Abbreviations

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

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