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

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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 algl0 mutant strains which accumulate lipid-linked Glc2Man9GlcNAc2. 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 Glc2Man9GlcNAc2 oligosaccharide. Hypoglycosylation of secreted proteins in algl0 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 Glc3Man9GlcNAc2 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; Herscovic and Orlean, 1993). In the central reaction of N-linked protein glycosylation, the lipid-linked 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 derivatives to the lipid carrier dolichyl-phosphoglyceraldehyde (Kornfeld and Kornfeld, 1985; Hirschberg and Snider, 1987; Verbert et al., 1987; Abeijon and Hirschberg, 1992; Herscovic and Orlean, 1993). The addition of three glucose units to one antenna of lipid-linked Man9GlcNAc2 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 glycosylation 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 glycosylation 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 glycosylated 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 glycosylation step of the lipid-linked oligosaccharide. We show that algl0 mutant strains accumulate lipid-linked Glc2Man9GlcNAc2 and that the complete core oligosaccharide structure is necessary for efficient oligosaccharyltransferase activity in vivo. We show that the glycosyltransferase activity of Alg10p is dependent on the glucose donor dolichyl-phosphoglucose and that the acceptor oligosaccharide recognized by Alg10p is dolichol-linked Glc2Man9GlcNAc2.

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 Glc3Man9GlcNAc2 it is ensured that only completely assembled oligosaccharide is transferred to protein.

Results

Reduced glycosylation efficiency in algl0 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 algl0 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,
alg10–1 and wbp1–2 mutations led to underglycosylation of CPY (Figure 1a, lane 2). A combination of these two mutations caused a synthetic phenotype and a severe glycosylation defect, resulting in significantly reduced levels of mCPY (Figure 1 a, lane 1 and 3). A combination of these two mutations and wbp1–2 mutations led to underglycosylation of CPY (Figure 1a, lane 2). A combination of these two mutations caused a synthetic phenotype and a severe glycosylation defect, resulting in significantly reduced levels of mCPY (Figure 1 a, lane 1 and 3).

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 [3H]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$_3$Man$_9$GlcNAc$_2$ and Glc$_4$Man$_9$GlcNAc$_2$ oligosaccharide was observed (Figure 2B–D). The additional peak had the retention time expected for Glc$_3$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$_3$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 (Schütze 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 (Staglar 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 Alg10::kanMX4 strain (YG428) showed no aberrant growth phenotype at all temperatures tested. However, even a more severe hypoglycosylation phenotype of CPY was observed.
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 Δalg10::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 Δalg10::kanMX4 are alleles of the same gene, we
crossed the alg10–1 wbp1–2 strain with the Δalg10::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
Δalg10::kanMX4. In addition, both the alg10–1 and
Δalg10::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 Δalg10 knockout strain which is unable to synthesize the full
length core oligosaccharide and accumulates Glc2Man6GlcNAc2-
PP-Dol, the appropriate acceptor oligosaccharide for Alg10p.
Microsomal membranes were prepared from wild-type, Δalg10
and wild-type strain containing pALG10–1 and the membranes
were incubated with [3H]mannose labeled Glc2Man6GlcNAc2-
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-de-
livered oligosaccharide (Figure 3C,D) which comigrated with
full-length Glc2Man6GlcNAc2 oligosaccharide isolated from the
wild-type strain SS328 (data not shown). The amount of
Glc3Man6GlcNAc2 oligosaccharide formed directly correlated
with the expression level of the ALG10 gene: no formation of
Glc3Man6GlcNAc2 was observed in Δalg10 extracts (Figure 3B),
low amount of Glc3Man6GlcNAc2 was present in wild-type
microsomes (Figure 3C), whereas a strong production of
Glc3Man6GlcNAc2 oligosaccharide was observed in extracts
derived from cells overexpressing Alg10p (Figure 3D). We
therefore took the formation of the Glc3Man6GlcNAc2 product
as a measure for Alg10p activity. To confirm that elevated production
of the Glc3Man6GlcNAc2 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 micro-
somes added (Figure 4A and 4B). The dependence of the Alg10p
activity on the concentration of Glc2Man6GlcNAc2-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 Man6GlcNAc2 (Runge et al., 1984; te Heesen et al.,
1994). We transformed the Δalg5 mutant strain with the Alg10p
overexpressing plasmid pALG10–1, prepared microsomal mem-
branes and tested in the in vitro assay whether we could still
measure glucosyltransferase activity. As shown in Table 1, almost
no formation of lipid-linked Glc3Man9GlcNAc2 could be observed in a Δalg5 background, whereas high activity was seen in an ALG5 wild-type background. The formation of residual amounts of Glc3Man9GlcNAc2 observed with Δalg5 microsomes was most probably due to contamination of the acceptor substrate Glc2Man9GlcNAc2-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 Δalg10 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 Glc3Man9GlcNAc2-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

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Strain</th>
<th>% G2M9N2</th>
<th>% G3M9N2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Δalg5+ pALG10–1</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>Δalg5+ pALG10–1</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>wt + pALG10–1</td>
<td>17</td>
<td>83</td>
</tr>
</tbody>
</table>

The Δalg5 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 Glc2Man9GlcNAc2-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, Glc2Man9GlcNAc2; G3M9N2, Glc3Man9GlcNAc2).

We asked whether the glucosyltransferase encoded by the ALG10 locus shows a stringent specificity for the acceptor substrate Glc2Man9GlcNAc2-PP-Dol. Lipid-linked Glc1Man9GlcNAc2 isolated from Δalg8 cells (YG125) (Runge and Robbins, 1986; Stagljar et al., 1994) and Glc2Man9GlcNAc2-PP-Dol isolated from Δalg10 cells were used as substrates in a glucosyltransferase assay with microsomes derived from Alg10p overproducing cells. As presented in Table II, only Glc2Man9GlcNAc2-PP-Dol served as efficient acceptor, whereas Glc1Man9GlcNAc2-PP-Dol was not converted in significant amounts. The small amount of Glc3Man9GlcNAc2 oligosaccharide formed from the Glc1Man9GlcNAc2-PP-Dol substrate is believed to be due to the combined Alg8p and Alg10p activity in the microsomes: the presence of Alg8p can convert Glc1Man9GlcNAc2-PP-Dol to Glc2Man9GlcNAc2-PP-Dol which then serves as a substrate for Alg10p. Because of Alg10p overexpression, no Glc2Man9GlcNAc2 intermediate is detected.

Table II. Acceptor substrate specificity of the ALG10 glucosyltransferase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>% G1M9N2</th>
<th>% G2M9N2</th>
<th>% G3M9N2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glc1Man9GlcNAc2-PP-Dol</td>
<td>93</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Glc2Man9GlcNAc2-PP-Dol</td>
<td>—</td>
<td>25</td>
<td>75</td>
</tr>
</tbody>
</table>

The Δalg10 strain (YG428) was transformed with pALG10–1, grown to mid-log phase, microsomes were prepared and the glucosyltransferase activity was measured using either radiolabeled Glc1Man9GlcNAc2-PP-Dol or Glc2Man9GlcNAc2-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, Glc1Man9GlcNAc2; G2M9N2, Glc2Man9GlcNAc2; G3M9N2, Glc3Man9GlcNAc2).

Fig. 3. Glucosyltransferase activity in microsomes derived from Δalg10 (YG428), wild-type (SS328) and wild-type strain transformed with pALG10–1 (YG730). Glucosyltransferase activity was determined using [3H] mannose labeled Glc2Man9GlcNAc2-PP-Dol as acceptor substrate. The formation of Glc3Man9GlcNAc2-PP-Dol was followed by HPLC analysis (G3M9N2, Glc3Man9GlcNAc2; G2M9N2, Glc2Man9GlcNAc2).
When Glc$_1$Man$_9$GlcNAc$_2$-PP-Dol was tested as acceptor oligosaccharide in an assay using Δalg8 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 transerase. 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$_9$GlcNAc$_2$ 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; Hers-covics 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 (Carnan 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-phosphoglucone. This substrate is synthesized at the cytoplasmic side of the ER membrane but utilized in the luminal 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 flipase would be expected to have the same phenotype as alg5 and alg6 mutants, namely, the inability to glucosylate the lipid-linked ManαGlCNAC2 oligosaccharide. The apparent absence of such flipase mutants can be explained in several ways. It is possible that no specific Dol-P-Glc flipase 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; Verbret al., 1987). Alternatively, a putative flipase activity of Alg5p or the presence of multiple Dol-P-Glc flipases 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 Glc3ManGlCNAC2 as compared to the biosynthetic intermediates in wild-type cells (see Figure 2A), the specificity of the OTase for lipid-linked Glc3ManGlCNAC2 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 Man7–9GlCNAC2, 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-phosphate 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 (Matα ade2–101 ura3–52 his3Δ200 lys2–801 wbp1–2) (te Heesen et al., 1993), YG649 (Matα ade2–101 ade3 ura3–52 his3Δ200 leu2 wbp1–2 alg10), YG650 (Matα ade2–101 ade3 ura3–52 his3Δ200 lys2 wbp1–2 alg10–2 pCH1122WPB1) (Zaffierey et al., 1995), YG651 (Matα ade2–101 ade3 ura3–52 his3Δ200 leu2 alg10–1), YG652 (Matα ade2–101 ura3–52 his3Δ200 lys2–801 wbp1–2), YG653 (Matα ade2–101 ura3–52 his3Δ200 leu2–801), YG654 (Matα ade2–101 ura3–52 his3Δ200 alg10–1–1 wbp1–2), YG428 (Matα ade2–101 ura3–52 his3Δ200 lys2–801 Δalg10::kanMX4), YG125 (Matα ade2–101 ura3–52 his3Δ200 lys2–801 Δalg8::HIS3) (Stagljar et al., 1994), YG91 (Matα ade2–101 his3 ura3–52 his3Δ200 Δalg5::HIS3) (te Heesen et al., 1994), YG699 (Matα ade2–101 ura3–52 his3Δ200 lys2–801 pYEp352[ProtA-AI101]), 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-CYPI 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 MgCl2, 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 CHCl3:CH3OH (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 Glc2Man9GlcNAc2 as the acceptor molecule. Dolichyl-phosphoglucosamine 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 Glc3Man9GlcNAc2 synthesis, it was assumed that the glucose donor Dol-P-Glc is supplied in excess by the microsomes. The conversion of lipid-linked Glc2Man9GlcNAc2 to lipid-linked Glc3Man9GlcNAc2 was followed by monitoring and analyzing the radiolabeled sugars by HPLC (Zufferey et al., 1995). Radiolabeled dolichyl-linked Glc2Man9GlcNAc2 was obtained by scaling up the procedure for isolation of lipid-linked oligosaccharides (see above). The Δalg10 strain YC428 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 at 37°C, and stored as Glc2 Man 9 GlcNAc 2 -PP-Dol stock of nitrogen, resuspended in 1 ml chloroform/water/methanol (10:10:3, v/v/v) for 40 min. The column was washed with water for 5 min and then dried under nitrogen stream. The glucosyltransferase activity was measured in microsomal membranes using [3H]mannose labeled dolichyl-linked Glc2Man9GlcNAc2 as the acceptor molecule. Dolichyl-phosphoglucosamine 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 Glc3Man9GlcNAc2 synthesis, it was assumed that the glucose donor Dol-P-Glc is supplied in excess by the microsomes. The conversion of lipid-linked Glc2Man9GlcNAc2 to lipid-linked Glc3Man9GlcNAc2 was followed by monitoring and analyzing the radiolabeled sugars by HPLC (Zufferey et al., 1995). Radiolabeled dolichyl-linked Glc2Man9GlcNAc2 was obtained by scaling up the procedure for isolation of lipid-linked oligosaccharides (see above). The Δalg10 strain YC428 was used for metabolic labeling. Lipid-linked oligosaccharides were extracted with a mixture of chloroform/water/methanol (10:10:3, v/v/v) and stored as Glc2 Man 9 GlcNAc 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 Glc2Man9GlcNAc2-PP-Dol 50 mM HEPES pH 6.5, 1 mM MgCl2, 0.25 mM EDTA, 1 mM DTT, 1 mM CTP, 0.5 mM UDP-Glc, 5 mM CaCl2, 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 CHCl3:CH3OH:H2O 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 H2O 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.

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Abbreviations

CPY, carboxypeptidase Y; Dol, dolichol; Dol-P-Glc, dolichyl-phosphoglucosamine; ER, endoplasmic reticulum; –PP-Dol, dolichyl-phosphosphate-linked; OTase, oligosaccharidyltransferase.

References


