The biosynthesis of asparagine-linked glycans occurs in an evolutionarily conserved manner with the assembly of the unique lipid-linked oligosaccharide precursor Glc3Man9GlcNAc2-PP-Dol at the ER (endoplasmic reticulum). In the present study we characterize Alg11 from yeast as a mannosyltransferase catalysing the sequential transfer of two α1,2-mannose residues from GDP-mannose to Man5GlcNAc2-PP-Dol and subsequently to Man6GlcNAc2-PP-Dol forming the Man7GlcNAc2-PP-Dol intermediate at the cytosolic side of the ER before flipping to the luminal side. Alg11 is predicted to contain three hydrophobic transmembrane-spanning helices. Using Alg11 topology reporter fusion constructs, we show that only the N-terminal domain fulfils this criterion. Surprisingly, this domain can be deleted without disturbing glycosyltransferase function and membrane association, indicating also that the other two hydrophobic domains contribute to ER localization, but in a non-transmembrane manner. By site-directed mutagenesis we investigated amino acids important for transferase activity. We demonstrate that the first glutamate residue in the EX-E motif, conserved in a variety of glycosyltransferases, is more critical than the second, and loss of Alg11 function occurs only when both glutamate residues are exchanged, or when the mutation of the first glutamate residue is combined with replacement of another amino acid in the motif. This indicates that perturbations in EX-E are not restricted to the second glutamate residue. Moreover, Gly85 and Gly87, within a glycine-rich domain as part of a potential flexible loop, were found to be required for Alg11 function. Similarly, a conserved lysine residue, Lys319, was identified as being important for activity, which could be involved in the binding of the phosphate of the glycosyl donor.

Key words: ALG11, dolichol, lipid-linked oligosaccharide, mannosyltransferase, protein N-glycosylation, Saccharomyces cerevisiae.

INTRODUCTION

The pathway of N-linked protein glycosylation in eukaryotes is highly conserved and starts with the assembly of the common core oligosaccharide donor Glc3Man9GlcNAc2-PP-Dol, the glycan moiety of which is subsequently transferred by the OST (oligosaccharyltransferase) complex on to selected Asn-X-Ser/Thr acceptor sites of the nascent polypeptide chain [1,2]. The initial steps of this multistep pathway up to Man5GlcNAc2-PP-Dol take place at the cytosolic side of the ER (endoplasmic reticulum), using sugar nucleotides as glycosyl donors [3]. The heptasaccharide is then translocated across the bilayer into the lumen of the ER by a specific ATP-independent flipase, the molecular and mechanistic details of which remain yet to be elucidated [4,5]. Subsequently, Man6GlcNAc2-PP-Dol is extended by four mannose and three glucose residues deriving from Man-P-Dol and Glc-P-Dol respectively, in an ordered manner based on differences in the substrate specificity of the various glycosyltransferases [6]. Yeast genetic techniques in the last two decades have opened up new approaches to identify genes of the pathway, among those alg mutants (for asparagine-linked glycosylation), isolated in the pioneering work by Robbins and co-workers, were extremely helpful [7]. Most of these mutants were found to be defective in the assembly of the LLO (lipid-linked oligosaccharide). Likewise various mutant cell lines from mammalian origin have been described that produce truncated LLOs [8–10]. More recent studies of LLO assembly have revealed some quite unexpected findings. The addition of the second GlcNAc residue to form GlcNAc2-PP-Dol is catalysed not by a single subunit enzyme, but by a dimeric complex, in which membrane-bound Alg14 recruits soluble Alg13 to the cytosolic face of the ER to form the active enzyme [11–13]. Biochemical characterization of Alg2 disclosed it as a bifunctional enzyme required for both the transfer of the α1,3- and α1,6-mannose-linked residue to Man6GlcNAc2-PP-Dol to form Man7GlcNAc2-PP-Dol and Man8GlcNAc2-PP-Dol respectively [14,15].

Study of an alg11 mutant from Saccharomyces cerevisiae, which has been isolated as a spontaneous vanadate-resistant mutant, suggested that Alg11 catalyses the fifth, i.e. last, mannosylation step at the cytosolic side of the ER leading to Man8GlcNAc2-PP-Dol [16]. This conclusion arrived from analysis of the LLO and glycoprotein fraction of alg11 cells. However, in vitro biochemical validation to unambiguously define the precise role of Alg11 has been lacking. Because a bioinformatic approach of the yeast database did not reveal an unknown ORF (open reading frame) that might encode an
additional putative mannosyltransferase being involved in LLO synthesis, we considered that ALG11 may have a dual function, i.e. synthesizing both Man₄GlcNAc₂-PP-Dol and Man₅GlcNAc₂-PP-Dol. While the present study was in progress, evidence was presented that a membrane fraction from Escherichia coli, expressing ALG11 from yeast, is able to carry out the transfer of both mannose to the branched pentasaccharide [14]. However, the contribution of native E. coli enzymes could not entirely be ruled out. In the present study, we confirm and extend these findings by further investigating ALG11 in S. cerevisiae. We first established a radioactive in vitro assay and demonstrate that ALG11, immunoprecipitated from detergent extracts of yeast microsomal membranes, catalyses both elongation steps. Furthermore, we investigated the membrane integration of the ALG11 mannosyltransferase. Evidence will be presented that ALG11 is composed only of the N-terminal TMD (transmembrane-spanning domain) out of three predicted ones, whereas the two C-terminal hydrophobic sequences contribute to ER localization merely in a non-transmembrane manner. Surprisingly, the N-terminal TMD is not essential for ALG11 activity and membrane anchoring, as its deletion gives rise to an active transferase localized to the ER.

Glycosyltransferases are classified and grouped into families according to the CAZY and Pfam databases on account of amino acid similarities and structural homologies [17–19]. In many of the retaining transferases, as is also true for ALG11, an EX-E motif can be detected in the C-terminal region. These conserved acidic amino acids have been suggested to be involved in catalysis as a nucleophile stabilizing the donor substrate, but with mixed results. We therefore performed a mutational analysis of this motif, as well as other amino acids, to identify residues required for ALG11 activity.

**EXPERIMENTAL**

**Yeast strains, media and genetic methods**

The following strains were used: SS330 (MATα ade2-101 ura3-52 his3Δ200 tyr1), W303-1A (MATα leu2-3 leu2-112, his3-11 his3-15 ura3-1 ade2-1 trpl-1), BAY2402 (MATα ura3-1 his3-11 leu2-3_12, trplΔ2 ade2-1 can1-100 ALG11::kanΔ MX4) and STY50 (MATα his4-401 leu2-3_112 trpl-1 ura3-52 HOLL1-1 SUC2::LEU2). Strains were grown in YPD medium (1% yeast extract, 2% bacto-peptone and 2% glucose) or in selective YNB (yeast nitrogen base) medium (0.67% YNB, 0.5% casamino acids and 2% glucose) supplemented with amino acids and nucleotide bases, as required. In the case of Δalg11 the medium also contained, if not indicated otherwise, 0.5 M KCl.

To construct the expression plasmids pVT100-Alg11-ZZ and pVT100-Alg11-Bio, genomic ALG11 was amplified from W303-1A by PCR and engineered with HindIII and BamHI restriction sites at the 5’ and 3’ ends respectively, for cloning purposes, and ligated into the HindIII/BamHI cut vectors pVT100-ZZ and pVT100-Bio respectively, placing ALG11 under the control of the constitutive ADH1 (alcohol dehydrogenase 1) promoter. The constructs were sequenced and the functional expression of Alg11 containing two ZZ (Protein A) epitopes or the Bio-epitope in-frame at the C-terminus were verified by immunoblotting and complementation of the growth phenotype of the Δalg11 mutant. For PCR amplification of ALG11-ZZ, the following primers were used: ALG11 fw (5’-CCCAAGCTTAAAAAGGGCGAGTCTTGGACAAA ACTAC-3’) and ALG11 rev (5’-CGGATCCGCCCCCTTCTCTTCT-3’). To generate the truncated Alg11-Bio reporter variants, internal ALG11-specific reverse primers, annealing at the positions as indicated in the corresponding Figures, were used together with the above ALG11 fw primer (primer sequences can be received on request from the corresponding author). For constructing the Alg11–Suc2/His4C topology reporter, plasmid pJK90 was used to engineer the ALG11 variants into the SmaI restriction site and the constructs were transformed into STY50 for analysis. To generate site-specific alg11 mutants, the QuikChange® II XL site-directed mutagenesis kit (Stratagene) was used according to the manufacturer’s instructions. Transformation into yeast and E. coli was carried out using standard techniques.

**In vivo labelling of LLOs with [3H]mannose**

Labelling with [2-3H]mannose (15 Ci/mmol; GE Healthcare) and analysis of LLOs by HPLC were performed as described previously [20]. Cells were grown overnight at 25°C. Subsequently cells were labelled at 25°C (Aalg11) or at 30°C (wild-type) as indicated.

**Isolation of microsomal membranes and preparation of solubilized enzyme extract**

Rough microsomal membranes were isolated as described previously [21]. Membranes were resuspended in 30 mM Tris/HCl buffer (pH 7.5), containing 3 mM MgCl₂, 1 mM DTT (dithiothreitol) and 35% (v/v) glycerol at a concentration of 10 mg/ml protein. For membrane solubilization, Nonidet P40 was added to a final concentration of 1%. After 20 min incubation on ice, the solubilized extract was separated from insoluble material by centrifugation at 150,000 g for 40 min. All steps were carried out at 4°C, unless indicated otherwise.

**Mannosyltransferase assays**

For assay I the activity of solubilized enzyme was determined with Man₄[14C]GlcNAc₂-PP-Dol or [14C]GlcNAc₂-PP-Dol as the acceptor and non-radioactive GDP-Man as the glycosyl donor. The reaction contained the following in a final volume of 0.06 ml: 14 mM Mes (pH 6.0), 0.1% Nonidet P40, 0.1 mM sodium-EDTA, 1 mM DDT, 4 mM potassium citrate, 1 M sucrose, [14C]GlcNAc₂-PP-Dol (3000 c.p.m.) or Man₄[14C]GlcNAc₂-PP-Dol (3000 c.p.m.), 1 mM GDP-Man and solubilized enzyme (equivalent to 70 μg of membrane protein). The reaction was started by the addition of GDP-Man and incubated at 26°C and stopped with chloroform/methanol (3:2) at the times indicated. Labelled glycolipids were then extracted, washed and oligosaccharides were released from the Dol-PP moiety by mild acid and analysed by HPLC as described previously [22]. [14C]GlcNAc₂-PP-Dol was synthesized as described previously [23]; Man₄[14C]GlcNAc₂-PP-Dol was prepared enzymatically by elongating [14C]GlcNAc₂-PP-Dol to Man₄[14C]GlcNAc₂-PP-Dol with a solubilized enzyme extract from Δalg11 membranes. For assay II, determination of enzyme activity in immunoprecipitates of Alg11–ZZ was carried out as follows. Solubilized extract (0.2 ml) was incubated with 0.1 ml of IgG–Sepharose 6 Fast Flow (GE Healthcare) by gently tumbling for 60 min at 4°C. IgG-Sepharose 6 was equilibrated before use with 40 mM Pipes (pH 6.8), 1% Nonidet P40, 1 mM DTT and 15% glycerol. After incubation the affinity matrix was washed five times with equilibration buffer and then three times with 20 mM Mes (pH 6), containing 0.1% Nonidet P40, 1 mM DTT, 10 mM MgCl₂, 4 mM potassium citrate and 1.5 M sucrose. The pellet was resuspended in 50 μl of mannosyltransferase assay I reaction mixture with Man₄[14C]GlcNAc₂-PP-Dol as the acceptor. The reaction was
started by the addition of 1 mM GDP-Man (final concentration), incubated for 45 min and processed as for assay I.

RESULTS
Characterization of the Δalg11-null mutant
Originally, an alg11 mutant was isolated by its resistance to sodium vanadate [16]. This selection procedure has been described previously for the isolation of yeast glycosylation mutants [24], although the biochemical basis for this is not understood. alg11 was shown to be temperature-sensitive and defective in N-glycosylation. That study also implied that Alg11 is involved merely in the addition of the terminal α1,2-linked mannose to Man₅GlcNAc₂-PP-Dol, but enzymatic validation was lacking [16]. To characterize Alg11 in more detail and in particular to define its specific role in LLO synthesis, an Δalg11-null allele has been isolated by sporulation of an ALG11/Δalg11 diploid strain. As we expected that deletion of a gene involved in an early stage of LLO assembly may be lethal or cause poor growth, an osmotic stabilizer such as sorbitol or KCl was added during the sporulation procedure to compensate for a weakened cell wall due to defective glycosylation. In fact, sporulation resulted in a 2:2 segregation with two efficiently growing wild-type cells and two slowly growing Δalg11-null alleles (results not shown). In Figures 1(A) and 1(B) the growth behaviour of Δalg11 is depicted in dependency of temperature, osmotic stabilizer and agents interfering with cell-wall biogenesis, such as CFW (Calcofluor White) or caffeine. Compared with wild-type cells, Δalg11 grew poorly and osmotic stabilization by KCl only slightly improved growth (Figure 1B). Nevertheless, KCl was added throughout the present study. However, deletion of ALG11 caused a temperature-sensitive lethality between 32 °C and 36 °C (Figure 1A). Likewise addition of 5 μg/ml CFW or 5 mM caffeine to the medium abolished growth of cells completely. CFW binds to chitin and interferes with cell-wall assembly [25], whereas the phosphodiesterase inhibitor caffeine is thought to exert its effect via PKC (protein kinase C), a central element in the regulation of cell-wall integrity [26]. Figure 1(C) shows the analysis of LLOs of cells metabolically labelled with [¹⁴C]mannose. Whereas in wild-type yeast the fully assembled core oligosaccharide GlcMan₃GlcNAc₂-PP-Dol and various intermediates occurred, in Δalg11 the largest oligosaccharide had a composition of Man₅GlcNAc₂-PP-Dol and various intermediates occurred, in Δalg11 the largest oligosaccharide had a composition of Man₅GlcNAc₂-PP-Dol. According to the results described below, demonstrating that ALG11 encodes the mannosyltransferase catalysing the formation of both Man₅GlcNAc₂-PP-Dol and Man₅GlcNAc₂-PP-Dol, one would have expected Man₃GlcNAc₂-PP-Dol as the largest oligosaccharide in the deletion mutant. As shown previously [16], Man₅GlcNAc₂-PP-Dol in Δalg11 is an isomeric species structurally different from the normal biosynthetic LLO-Man₂ of the wild-type arising by elongation of the α1,6-arm by luminal mannosyltransferases with Man-P-Dol as the glycosyl donor [16]. However, these shortened and aberrant glycan chains are non-optimal substrates for OST and are less efficiently transferred to the protein, as can be seen from the analysis of the glycosylation status of the vacuolar model glycoprotein carboxypeptidase CPY (Figure 1D) (see also further discussion below). CPY from wild-type cells contains four N-linked glycan chains and migrates as a distinct band, whereas in the Δalg11 strain the glycosylation defect gives rise to CPY forms possessing a higher mobility on SDS gels. Complementation with a plasmid harbouring ALG11 or a variant with a C-terminal ZZ-epitope for detection (see below) restored wild-type glycosylation. This result also indicates that the epitope does not disturb Alg11 activity.

Alg11 is a mannosyltransferase with dual function catalysing both the formation of Man₅GlcNAc₂-PP-Dol and Man₅GlcNAc₂-PP-Dol
To unravel the mannosylation defect of Δalg11 in more detail, we first established an enzymatic test that allowed us to measure early reactions of LLO synthesis from GlcNAc₂-PP-Dol to Man₅GlcNAc₂-PP-Dol involving the participation of Alg1, Alg2 and Alg11 in that order. As can be seen in Figure 2(A), incubation of a detergent-solubilized enzyme extract from microsomal membranes of wild-type yeast with radioactive [¹⁴C]GlcNAc₂-PP-Dol as an acceptor and unlabelled GDP-Man as the glycosyl donor led to elongation of the acceptor oligosaccharide up to Man₅GlcNAc₂-PP-Dol (left-hand and middle panels). In contrast, an extract from Δalg11 elongated only to Man₃GlcNAc₂-PP-Dol, indicating that Alg11 catalyses at least the transfer of the fourth mannose (right-hand panel). Prevention of further elongation beyond Man₅GlcNAc₂-PP-Dol in the detergent extract from wild-type, as compared with in vivo, could be due to the fact that the proper reaction conditions are not given, no Dol-P-Man is generated or the respective transferases are not solubilized or are inactive.

In order to measure exclusively Alg11 activity, we introduced a ZZ-epitope at the C-terminus of plasmid-borne ALG11, allowing us to immunoprecipitate Alg11–ZZ from the solubilized extract with IgG–Sepharose as the affinity matrix. The ALG11–ZZ plasmid and, as a control, a plasmid containing ALG11 without the ZZ-epitope were transformed into Δalg11 without the ZZ-epitope were transformed into Δalg11 and the enzyme activity was determined. In the experiment shown in Figure 2(B), Man₅[¹⁴C]GlcNAc₂-PP-Dol was converted into Man₅[¹⁴C]GlcNAc₂-PP-Dol (middle panel). No transfer on to Man₅[¹⁴C]GlcNAc₂-PP-Dol was measurable in the control experiment in which solubilized extract from membranes of Δalg11 cells expressing the non-tagged Alg11 was incubated with IgG–Sepharose beads (right-hand panel). This means that immunoprecipitation of Alg11 is specific, rather than Alg11 binding non-specifically to the affinity matrix. Assuming that no other interacting protein is pulled down, because of extensive washing of the immunoprecipitate applying detergent, the above results demonstrate that Alg11 is able not only to catalyse the addition of the fourth, but also of the fifth, α1,2-linked mannose residue.

Investigation of the membrane integration of Alg11 reveals only one membrane-spanning domain
Alg11 is a protein with a calculated molecular mass of 63 kDa consisting of 548 amino acids. A hydropathy plot reveals three hydrophobic domains (Figure 3A). Topology predictions suggest either three TMDs (with TMpred and TopPred) or only one helix (with TMHMM and SOSUI). From this, and taking into account that the reactions catalysed by Alg11 must occur at the cytoplasmic face of the ER, because the nucleotide sugar donor is only present in the cytosol, three topology models (models A, B and C) can be proposed, as shown in Figure 3(B). As Alg11 belongs to the GT4 glycosyltransferase family and therefore should present a GT-B fold consisting of two Rossmann domains, models A and B requiring approx. 300 amino acids can most likely be excluded. Nevertheless, to experimentally prove or disprove these predictions, we used two approaches. We designed various fusions between Alg11 and a Bio-tag, or Alg11 and a Suc2/His4C-tag as topology-sensitive reporters (Figure 3C). In the case of the Bio-tag, this domain is a substrate for biotin ligase [27] and will be biotinylated only when orientated to the cytosolic side, as this reaction takes place exclusively in the cytosol. His4C maintains histidinol dehydrogenase activity, the last step in
histidine biosynthesis, and his4 mutants, expressing a Suc2/His4C fusion, are able to grow on minimal histidinol medium only when the His4C domain is at the cytoplasmic side of the ER, but not when targeted to the ER lumen. In addition, in the latter case the chimaeric protein becomes extensively glycosylated due to the presence of N-glycosylation sites [28,29]. As shown in Figure 3(D), lanes 2–5, Alg11 fusions with the Bio-tag engineered C-terminal to the postulated first (Alg111-139), second (Alg111-396) and third (Alg111-548) TMDs were all biotinylated, indicating that these fusions were orientated to the cytosol. These results exclude models A and B with three TMDs, and would favour model C with only one TMD. Such a topology is also in agreement with the HisC4 technique approach. All fusions supported growth on histidinol, implying that the C-terminus of the respective constructs localized to the cytosol (Figure 3E). Moreover, the topology of the constructs has been validated by analysing their glycosylation status. Compared with a control glycoprotein (Figure 3F, lanes 7 and 8), treatment with EndoH (endo-glycosidase H) indicated that all fusions were resistant to enzyme treatment and thus were not glycosylated, and the
Alg11 from yeast, a bifunctional mannosyltransferase

Figure 2 Analysis of Alg11 mannosyltransferase activity

(A) Alg11 activity of solubilized enzyme. Membranes from wild-type and Δalg11 cells were solubilized and tested using assay I with [14C]GlcNAc2-PP-Dol as the glycosyl acceptor as described in the Experimental section. Left-hand panel, incubation without GDP-Man; middle and right-hand panels, incubation with GDP-Man. LLOs were extracted and the oligosaccharide moiety was released by mild acid and separated by HPLC. (B) Alg11 transferase activity in immunoprecipitates (Ipp). Alg11 with a C-terminal ZZ-epitope was immunoprecipitated with IgG–Sepharose beads from solubilized extract and the enzyme activity was determined using assay II (left-hand and middle panels). As a control, an extract from wild-type cells expressing non-tagged Alg11 was immunoprecipitated, where no elongation took place (right-hand panel). The positions of standards are indicated. M1−5, GlcNAc2Man1−5; and GN2, GlcNAc2.

The N-terminal TMD is not essential for Alg11 function

Experiments described above demonstrate that Alg11 consists of only one TMD. Therefore we asked which function this TMD, as well as the two other predicted hydrophobic segments, could serve. Several Alg11 truncations with a C-terminal ZZ-epitope for detection purposes, as illustrated in Figure 4(A), were engineered and their role was investigated with respect to growth, N-glycosylation, membrane anchoring and enzyme activity. The constructs were cloned into a constitutive expression vector and analysed in the Δalg11 strain. Quite unexpectedly, deletion of the N-terminal TMD in the Alg1145−548 variant led to a functionally respective C-terminus must be therefore cytosolically orientated. In order to localize the N-terminus, and also to demonstrate that the first hydrophobic segment indeed acts as a transmembrane-spanning segment, we also made a construct with the Bio-tag at the N-terminus. As can be seen in Figure 3(D), lane 1, this fusion is not biotinylated, indicating ER luminal orientation of the N-terminus. As will be demonstrated below (Figure 4), the middle and the C-terminal hydrophobic regions, although not functioning as TMDs, nevertheless contribute to anchoring Alg11 to the ER in a non-peripheral manner, presumably by integrating into the outer leaflet of the ER membrane. Therefore model D is proposed for the membrane integration of Alg11.
Figure 3  Investigation of the membrane integration of Alg11

(A) Hydrophobicity probability plot of Alg11 generated using the TopPred server. (B) Models for the Alg11 membrane topology. The predicted topology of Alg11 suggests three TMD (models A and B); the experimental data using truncated Alg11–reporter fusions and truncated Alg11 variants (see Figure 4) suggest model D, containing one TMD at the N-terminus with orientation to the ER lumen, and two additional C-terminal hydrophobic regions that integrate, but do not cross the membrane. Model C is excluded on account of the extraction behaviour of Alg11. (C) Schematic representation of Alg11–reporter constructs. Either a Bio-tag or a SUC2::HisC4-tag was fused in-frame at the C-terminal side following amino acids 139, 396 or 548, or in the case of the Bio-epitope, also an N-terminal Bio-Alg111−548 variant; drawings are not to scale. (D) Analysis of Alg11 topology by Alg11–Bio fusions. Membranes from cells expressing plasmid-borne Alg11 constructs, as indicated, were analysed by Western blotting. Note that in lane 1 the Bio-tag is at the N-terminus (Bio–Alg11), whereas all the other constructs are C-terminally tagged (Alg11–Bio). *Biotinylated endogenous yeast proteins. Note that Alg111−396 (lane 3) has the same mobility as one of the endogenous proteins. (E) Analysis of topology by Alg11–Suc2::HisC4 fusions. Strain STY50, containing a his4 mutation, was transformed with truncated Alg11 variants as indicated, and analysed for growth on histidine or histidinol. Only cells with a cytosolic orientation of the epitope are able to grow on histidinol. (F) Analysis of glycosylation of Alg11–Suc2::HisC4 constructs. Membranes from cells expressing the various Alg11–Suc2::HisC4 constructs (lanes 1–6), were isolated and incubated without (−) or with (+) EndoH for 3 h to release the glycan chains, and analysed by Western blotting. In lanes 7 and 8 an Ost21−72 fusion, which is glycosylated, was digested as a control. Constructs were probed with an antibody against the haemagglutinin epitope of the fusion protein. The molecular mass in kDa is indicated on the left-hand side.
active mannosyltransferase, which supported the temperatureresponse-growth defect of Δalg11 (Figure 4D), rescued the underglycosylation of CPY (Figure 4E, compare lanes 1 and 2) and was also active in vitro using an Alg11 immunoprecipitate to elongate Man₆GlcNAc₂-PP-Dol to Man₆GlcNAc₂-PP-Dol (Figure 4F). As shown by Western blot analysis (Figure 4B, lane 4), the TMD-lacking construct still bound to the membrane, indicating that one or both of the two other hydrophobic domains contribute to membrane anchoring as well, even though not in a transmembrane-spanning manner.

To address the membrane association more specifically, we performed an alkaline sodium carbonate extraction of membranes, known to release peripheral attached proteins, and compared it with a 2% Nonidet P40 or 0.2% SDS detergent treatment. As shown in Figure 4(C), there was no significant difference in extraction between the full-length Alg111−548 and the N-terminal truncated Alg1145−548 variant. Even extraction with 2% detergent caused only partial solubilization of the N-terminally truncated version, to a similar extent as in the case of wild-type Alg11. These results clearly do not suggest a peripheral attachment.
of truncated Alg11 lacking the N-terminal TMD. Hence, taken together with the results from the Bio- and His4-localization experiments, a topology is proposed as depicted in model D (Figure 3), positioning the hydrophobic helices into the outer leaflet of the membrane (for further discussion see below).

In contrast with N-terminal truncation, C-terminal deletions were detrimental for Alg11 activity. Removal of the third hydrophobic domain in the Alg11<sup>Δ11–418</sup> construct led to underglycosylation of CPY, similar to the vector control (Figure 4E, lanes 1 and 4), but still allowed some residual cell growth (Figure 4D). However, marginally further truncation removing the conserved EX-E motif, as is the case in the Alg11<sup>Δ11–396</sup> variant, resulted in a complete loss of Alg11 function.

### Analysis of Alg11 activity by site-specific mutagenesis

To identify amino acids important for catalysis and function of Alg11, we performed site-specific mutagenesis in conserved amino acid residues and motifs respectively, discussed in the functional context of glycosyltransferases. As already mentioned, a variety of Pfam GT1F glycosyltransferases contain the C-terminal signature sequence motif EX-E [30]. The two glutamate residues of the motif have been proposed to be involved in catalysis, even though their contributions have been controversially discussed and need to be identified by structural analysis [31–33]. Figure 5(B) shows an alignment of the respective region of Alg11 and Alg2 mannosyltransferases from <i>S. cerevisiae</i> that are closely related and are grouped into family 4 of the CaZY database. In addition, human Alg11 and AceA, a mannosyltransferase from <i>Acetobacter xylinum</i>, are aligned. We replaced both Glu<sup>356</sup> and Glu<sup>313</sup> with alanine and found that mutating the first glutamate residue (Glu<sup>356</sup>) caused an underglycosylation of CPY, whereas altering the second glutamate residue (Glu<sup>313</sup>) had no effect (Figure 5B, lanes 2 and 7). This is in agreement with the hypothesis that the first glutamate residue of the EX-E motif is more critical for activity [31] and is also in agreement with the Alg11 enzyme activity expressed in <i>E. coli</i> [14]. Interestingly, the reduced activity of the E405A mutation was not sufficient to prevent cell growth. This variant was still able to complement the <i>Δalg11</i> growth deficiency at 36°C (Figure 5A).

Also three other selected mutations within the motif, such as H406A, F407A or V412A, were not detrimental for growth, albeit impairment of glycosylation of CPY was observed in the first two mutants. However, an E405A/E413, as well as an E405/H406A double mutant no longer supported growth, indicating that further constraints of Glu<sup>356</sup> are deleterious. The loss of activity is not caused by a failure of Alg11 expression, as proven by Western blot analysis (results not shown).

In addition we mutated several lysine and glutamate residues proposed to be involved in nucleotide sugar donor binding of glycosyltransferases to alanine [31]. From six conserved mutations (see Table 1), Lys<sup>302</sup>, Glu<sup>318</sup>, Lys<sup>319</sup>, Lys<sup>322</sup>, Lys<sup>343</sup> and Glu<sup>356</sup>, only Lys<sup>319</sup> (which is also conserved in AceA) was detrimental and caused loss of Alg11 activity, as revealed from the analysis of cell growth (Figure 5D) and glycosylation of CPY (Figure 5C). The significance of residue Lys<sup>319</sup> is also emphasized by the fact that amino acid exchanges in the immediate adjacent amino acids, Glu<sup>316</sup> or Lys<sup>322</sup>, had no effect. Similarly, mutating various aspartate residues as a potential catalytic base had no effect.

Finally, a conserved glycine-rich motif in the N-terminal region was investigated (Figures 5E and 5F) that may be part of a flexible loop important for catalysis of glycosyltransferases both in the GT-A and GT-B families [17,34–36]. Replacing Gly<sup>41</sup>, Gly<sup>87</sup> or Gly<sup>85</sup> by proline residues that may eventually lead to structural constraints was found to be crucial for activity for Gly<sup>41</sup> and Gly<sup>87</sup>, whereas a conservative substitution by alanine had no effect on CPY glycosylation and growth. Table 1 compiles the various mutations.

### DISCUSSION

In the present study we further characterized Alg11 from <i>S. cerevisiae</i> as a mannosyltransferase with a dual function in the early phase of LLO assembly (Figure 6), investigated its membrane association, as well as amino acid residues important for Alg11 function. The results close a gap in the identification of the last ill-defined glycosyltransferase of the dolichol-mediated reactions of N-linked glycosylation. As this pathway is highly conserved from yeast to humans, the results may be of general relevance and one can expect deficiencies of the Alg11 enzyme as a cause for a new fatal-type of CDG (congenital disorders of glycosylation), as it applies for other defects in LLO biosynthesis [2]. We first established an enzymatic <i>in vitro</i> test for Alg11 transferase and demonstrated that membranes from wild-type cells, or an Alg11 immunoprecipitate from a solubilized membrane extract, catalyse the transfer of two mannose residues from the GDP-Man donor to Man,GlcNAc<sub>2</sub>-PP-Dol, giving rise to Man,GlcNAc<sub>2</sub>-PP-Dol as the final product. These reactions were not taking place when membranes from <i>Δalg11</i> cells were used as the enzyme source. This result clearly demonstrates that Alg11 catalyses both final glycosylation steps on the cytosolic side of the ER. Bifunctionality of glycosyltransferases is rather rare, but not without precedent. It has also been observed for the Alg9 mannosyltransferase adding the terminal seventh and ninth, each α1,2-linked, mannose residues during Man,GlcNAc<sub>2</sub>-PP-Dol synthesis [37]. In the case of the Pgl pathway of protein N-glycosylation in the bacteria <i>Campylobacter jejunii</i>, having similarities to the dolichol pathway, the PglH transferase adds iteratively three terminal 1,4-linked GalNAc residues to an undecaprenyl diphosphate-linked heptasaccharide [38]. Whereas in both examples the same type of linkage is catalysed, in the case of Alg2, acting on the formation of the two mannose additions in LLO synthesis prior to Alg11, bifunctionality comprises even the formation of two different linkages, an α1,3- and α1,6-bond [14,15].

Deletion of <i>ALG11</i> was not lethal, but led to a severe slow growth phenotype. However, additional temperature stress or stress challenged by compounds interfering with cell-wall formation caused lethality in <i>Δalg11</i> (Figure 1). This phenotype is in stark contrast with glycosylation defects affecting luminal steps of LLO synthesis that do not display growth impairments, unless in combination with other defects, e.g. with defects in OST or in the glucosylation of LLO [39]. On the protein level <i>Δalg11</i> displays a severe hypoglycosylation of proteins. In principle this could be attributed, on account of the genetic defect of the mutant, to inefficient flipping of the Man,GlcNAc<sub>2</sub>-PP-Dol and/or reduced transfer to the nascent polypeptide of this or the aberrant Man<sub>α1,7</sub>-GlcNAc<sub>2</sub>-PP-Dol isomers, generated through luminal action or Man-P-Dol-dependent glycosyltransferases. It is known that abnormal and truncated LLOs are poor substrates for OST, leading to reduced glycan transfer to the nascent polypeptide chain. However, it was recently shown that in reconstituted proteoliposomes Man,GlcNAc<sub>2</sub>-PP-Dol is flipped almost as well as Man,GlcNAc<sub>2</sub>-PP-Dol [5]. Whether this also holds true <i>in vivo</i> is not known. It has been found that overexpression in <i>Δalg11</i> of <i>RFT1</i>, an integral essential membrane protein originally proposed to be the flipase, increased the yield of Man,GlcNAc<sub>2</sub>-PP-Dol and diminished the growth defect of the cells; however,
Figure 5  Site-specific mutagenesis of Alg11

(A and B) Exploring the EX7E motif. Mutations were introduced into the conserved EX7E motif of plasmid-borne Alg11–ZZ as indicated and transformed into Δalg11. Their role was investigated with respect to functional complementation of growth of Δalg11 cells (A) and glycosylation of CPY was monitored by Western blot analysis (B). (C and D) Identification of Lys319 as an essential amino acid for Alg11 function. Various conserved lysine, glutamate and aspartate residues, with a potential role in Alg11 function, were exchanged by site-specific mutagenesis as indicated. Plasmid-borne Alg2 was transformed into Δalg11 and tested for complementation of the growth defect of alg2 with regard to underglycosylation of CPY. (E and F) Investigation of a glycine-rich motif. A.x., A. xylinum; H.s., Homo sapiens; S.c., S. cerevisiae; wt, wild-type.

this may no longer tenable on account of recent results. Hence Rft1 may contribute to controlling the access of dolichol-linked oligosaccharides to the flippase. It should be emphasized that our analysis does not allow us to distinguish which of the truncated oligosaccharide chains are transferred. But analysis of glycans of the total mannoprotein fraction revealed that the Man7GlcNAc2 isomer structure is detectable in Δalg11 [16]. Worth mentioning in the context of flipping is also the finding that disruption of ALG2 was shown to be lethal in S. cerevisiae. This gene catalyses the two previous glycosylation steps prior to Alg11 in LLO biosynthesis (Figure 6), and its disruption results in the accumulation of Man,GlCNAC2-PP-Dol. Thus one may speculate that glycan chains smaller than Man,GlCNAC2 may no longer be compatible with viability of S. cerevisiae, because of insufficient flipping or other reasons. On the other hand, in an alg2-null mutant of the zygomycete Rhizomucor pusillus, compared with
Table 1 Site-specific mutagenesis of Alg11

Summary of the results of site-specific mutagenesis. To test the complementation of the Δalg11 growth defect, cells were transformed with mutated ALG11 and incubated at non-permissive temperatures as specified in the various Figures. For the analysis of the glycosylation defect, the glycosylation status of CPY was analysed by Western blotting. In columns five and six, the conservation of the mutated amino acids is summarized comparing Alg11 from yeast and human, and Alg11 and Alg2 from yeast respectively; C, conserved amino acids. The E88A exchange is not shown in Figure 5. One-letter amino acid codes are used.

<table>
<thead>
<tr>
<th>Mutants</th>
<th>Motif</th>
<th>Complementation of glycosylation defect of Δalg11</th>
<th>Complementation of growth defect of Δalg11</th>
<th>Conservation ALG11 yeast/ALG11 human</th>
<th>Conservation ALG11 yeast/ALG2 yeast</th>
</tr>
</thead>
<tbody>
<tr>
<td>G84A</td>
<td>G loop</td>
<td>Yes</td>
<td>Yes</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>G84P</td>
<td>G loop</td>
<td>Yes</td>
<td>Yes</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>G85A</td>
<td>G loop</td>
<td>Yes</td>
<td>Yes</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>G85P</td>
<td>G loop</td>
<td>No</td>
<td>No</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>G87A</td>
<td>G loop</td>
<td>Yes</td>
<td>Yes</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>G87P</td>
<td>G loop</td>
<td>No</td>
<td>No</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>E88A</td>
<td>EX, E</td>
<td>Poor</td>
<td>Yes</td>
<td>C</td>
<td>E/R</td>
</tr>
<tr>
<td>E405A</td>
<td>EX, E</td>
<td>Partial</td>
<td>Yes</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>E407A</td>
<td>EX, E</td>
<td>Poor</td>
<td>Yes</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>V412A</td>
<td>EX, E</td>
<td>Yes</td>
<td>Yes</td>
<td>C</td>
<td>V/L</td>
</tr>
<tr>
<td>E413A</td>
<td>EX, E</td>
<td>Yes</td>
<td>Yes</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>E405A/E413A</td>
<td>EX, E</td>
<td>No</td>
<td>No</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>E405A/H406A</td>
<td>EX, E</td>
<td>No</td>
<td>No</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>K302A</td>
<td>D/E/K</td>
<td>Yes</td>
<td>K/T</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>E318A</td>
<td></td>
<td>Yes</td>
<td>Yes</td>
<td>C</td>
<td>E/F</td>
</tr>
<tr>
<td>K319A</td>
<td></td>
<td>Poor</td>
<td>Poor/No</td>
<td>C</td>
<td>K/E</td>
</tr>
<tr>
<td>K322A</td>
<td></td>
<td>Yes</td>
<td>Yes</td>
<td>K/P</td>
<td>C</td>
</tr>
<tr>
<td>K342A</td>
<td></td>
<td>Yes</td>
<td>Yes</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>E356A</td>
<td></td>
<td>Yes</td>
<td>Yes</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>K381A</td>
<td></td>
<td>Yes</td>
<td>K/I</td>
<td>K/D</td>
<td></td>
</tr>
<tr>
<td>K386A</td>
<td></td>
<td>Yes</td>
<td>Yes</td>
<td>K/E</td>
<td>K/S</td>
</tr>
<tr>
<td>K387A</td>
<td></td>
<td>Yes</td>
<td>Yes</td>
<td>K/E</td>
<td>K/S</td>
</tr>
<tr>
<td>D462A</td>
<td></td>
<td>Yes</td>
<td>Yes</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>D476A</td>
<td></td>
<td>Yes</td>
<td>Yes</td>
<td>D/E</td>
<td>D/E</td>
</tr>
<tr>
<td>D502A</td>
<td></td>
<td>Yes</td>
<td>Yes</td>
<td>D/R</td>
<td>D/N</td>
</tr>
<tr>
<td>D504A</td>
<td></td>
<td>Yes</td>
<td>Yes</td>
<td>D/-</td>
<td>D/I</td>
</tr>
</tbody>
</table>

Figure 6 Pathway of early LLO formation on the cytosolic side of the ER membrane

Biosynthesis starts with the transfer of a GlcNAc-P to Dol-P with formation of the pyrophosphate bond, catalysed by Alg7. The second step is catalysed by the dimeric Alg14–Alg13 complex, whereby membrane-bound Alg14 recruits cytosolic Alg13 to the membrane with formation of the active GlcNAc transferase. Subsequent to the addition of the β1,4-linked mannose catalysed by Alg1, bifunctional Alg2 adds both the α1,3- and α1,6-linked mannose residues. The two final α1,2-mannose residues are transferred by Alg11, before the Man5GlcNAc2-PP heptasaccharide is translocated across the ER membrane to the lumen through the action of an ATP-independent flipase, where further elongation occurs to the full-length core saccharide Glc3Man9GlcNAc2-PP-Dol. All sugar residues at the cytosolic side are donated by sugar nucleotides.
S. cerevisiae a slowly growing fungi, Man,GlcNAc2-PP-Dol was the largest oligosaccharide synthesized and transferred to the protein, and yet the mutant was still viable [40]. Whether this could be due to differences in growth rates and/or the process of flipping is speculative at the moment and remains to be demonstrated.

As mentioned above, Alg11 belongs to glycosyltransferase family 4 (CAZY) and glycosyltransferase 1 family (Pfam) respectively. In many of the retaining transferases, an EX:E motif can be detected in the C-terminal region, as it is also true for Alg11. These conserved acidic amino acids have been suggested to be involved in catalysis as a nucleophile, stabilizing the donor substrate [31–33,38,41,42], but with mixed results. As depicted in Figure 5, exchange of Glu405 in this motif to alanine caused some underglycosylation of CPY but with only a mild effect on cell growth. In contrast, mutation of the second Glu413 did not alter Alg11 function at all. This emphasizes that the first glutamate residue in the motif is more crucial [31]. However, mutating both Glu405 and Glu413 residues to alanine caused lethality. As a Glu405/His406 double mutant was not compatible with survival of cells, whereas the single mutation of His406 to alanine was not detrimental, this may indicate that additional perturbation in the local structure of the motif by a further mutation is not restricted to the second glutamate residue. In this context it is worth mentioning that, also in the case of Alg2, it was shown that single mutations of the glutamic acid residues in the EX:E motif do not disturb enzyme activity or cell viability [15].

In addition we detected Lys319, also conserved in Alg11 from other species as well as in AceA mannosyltransferase, as an important amino acid for Alg11 activity (Figures 5C and 5D). In the case of AceA this lysine residue has been proposed to function in nucleotide sugar binding [31]. Replacement of Lys319 by alanine in Alg11 led to underglycosylation, as well as to slower growth and eventually to loss of viability upon stress provoked by caffeine (Figure 5D). Likewise, conserved aspartate residues were mutated, which have also been discussed as potential nucleophiles by deprotonating the hydroxy group of the acceptor, acting as a catalytic nucleophilic base attacking the C-1 atom of the glycosyl donor. However, none of these residues are required for Alg11 function (Figure 5C and Table 1).

Another severe functional influence was observed exchanging glycine in a glycine-rich domain that could be part of a potential flexible glycine loop. Studies on several glycosyltransferases both from the GT-A and GT-B superfamily have shown that one or two flexible loops at the substrate binding site of the enzyme undergo marked conformational changes from an open to a closed conformation, in which the loop acts as a lid covering the bound glycosyl donor [17,34,35]. We have exchanged glycine residues 84, 85 and 87 of a conserved region both by alanine and by proline. In the latter case, the replacement is expected to have a strong effect on the protein architecture. Whereas substitution by alanine did not affect glycosylation and cell growth, the G84P and G87P variant was critical and caused loss of Alg11 function. The significance of these mutations is underscored by the fact that a G85P mutation was not detrimental. Nevertheless, a final evaluation of the mutational analysis allowing a mechanistic explanation needs support by crystal structure information. We also cannot infer from our analysis whether the different mutants select for transfer of different glycan structures as well as whether Man,GlcNAc2-PP-Dol formation can be uncoupled from Man,GlcNAc2-PP-Dol.

Another important issue of the present study was the examination of the membrane integration of Alg11, which has not been analysed previously. Using two different experimental topology-sensitive reporter fusion approaches, we proved that Alg11 consists of only one TMD with the N-terminus in the lumen of the ER, whereas the two other predicted hydrophobic sequences in the middle and the C-terminus of the protein do not span the membrane, but seem to insert into the outer leaflet of the membrane, as proposed in model D (Figure 3). This conclusion is based on the fact that an N-terminal truncated Alg1145–548 variant, lacking the TMD, was tightly integrated into the membrane rather than being peripherally associated upon treatment with alkaline carbonate or neutral detergent (Figure 4C), and furthermore on the observation that the second and third hydrophobic helix are accessible from the cytosolic face (Figure 3). Although enormous progress has been made to predict structural properties of proteins, there is still not much known about the molecular details of how membrane helices are recognized and integrate into the membrane in the correct way. Membrane-embedded helices can be long or short, cross the membrane at oblique angles, lie flat on the surface, or even span only part of the bilayer and turn back [43]. Our experimental results do not allow definition of the exact mode of integration of the two hydrophobic non-transmembrane domains. A helical wheel projection of membrane segments 2 and 3 (results not shown) reveals a concentration of hydrophobic and of more polar amino acid residues on two different sites of the helix. This may suggest an interaction with the surface and the non-polar interior of the membrane as discussed for Ost2 [44], a subunit of the OST, or the Sec61α and Sec61γ-subunit of the translocon [45,46]. Moreover, insertion of monotopic proteins is often accomplished by hydrophobic helices interacting with one leaflet of the membrane [47–49]. Surprisingly, removal of the N-terminal TMD did not disturb Alg11 function in vivo and in vitro, as well as its localization (Figures 4D–4F). However, C-terminal truncations were detrimental. Whereas deletion of the third hydrophobic domain slightly downstream of the EX:E motif (Alg111–418) caused an underglycosylation and a temperature-growth phenotype (Figures 4D and 4E), further truncation resulting in its removal (Alg111–396) led to an inactive transferase, which was still associated with the ER membrane. The specific role of the N-terminal TMD remains obscure and only speculative for the moment. One possibility is that it contributes, even though not being essential, to interact with other Alg proteins. Thus it has been demonstrated that there exist physical interactions between Alg1, Alg2 and Alg11 [50]. Further work will also be necessary to understand the mechanistic details of membrane integration as well as the mechanism, how Alg11 transferase adds sequentially two manose residues. Potentially it is conceivable that it acts in a processive manner, in which the Man,GlcNAc2-PP-Dol intermediate does not dissociate from the enzyme, or alternatively the intermediate freely dissociates and reassociates as substrate.

**AUTHOR CONTRIBUTION**

Ludwig Lehle and Birgit Absmaner designed the research; Birgit Absmaner, Verena Schmeiser and Michael Kämpf performed the research and analysed data; Ludwig Lehle wrote the paper.

**ACKNOWLEDGEMENTS**

We acknowledge the excellent technical assistance of Angelika Rechenmacher. We are also grateful to Dr. J. Stolt (Technische Universität München, München, Germany) for providing plasmids and helpful discussions.

**FUNDING**

This work was supported by the Deutsche Forschungsgemeinschaft and the Körber-Stiftung.
REFERENCES


