Membrane Topology of the Alg14 Endoplasmic Reticulum UDP-GlcNAc Transferase Subunit

Nicole Averbeck, Sabine Keppler-Ross, and Neta Dean

From the Department of Biochemistry and Cell Biology, Stony Brook University, Stony Brook, New York 11794-5215

N-linked glycosylation begins in the endoplasmic reticulum with the synthesis of a highly conserved dolichol-linked oligosaccharide precursor. The UDP-GlcNAc glycosyltransferase catalyzing the second sugar addition of this precursor consists in most eukaryotes of at least two subunits, Alg14 and Alg13. Alg14 is a membrane protein that recruits the soluble Alg13 catalytic subunit from the cytosol to the face of the endoplasmic reticulum (ER) membrane where this reaction occurs. Here, we investigated the membrane topology of Saccharomyces cerevisiae Alg14 and its requirements for ER membrane association. Alg14 is predicted by most algorithms to contain one or more transmembrane spanning helices (transmembrane domains (TMDs)). We provide evidence that Alg14 contains a C-terminal cytosolic tail and an N terminus that resides within the ER lumen. However, we also demonstrate that Alg14 lacking this TMD is functional and remains peripherally associated with ER membranes, suggesting that additional domains can mediate ER association. These conclusions are based on the functional analysis of Alg13/Alg14 chimeras containing Alg13 fused at either end of Alg14 or truncated Alg14 variants lacking the predicted TMD; protease protection assays of Alg14 in intact ER membranes; and extraction of Alg14-containing ER membranes with high pH. These yeast Alg13-Alg14 chimeras recapitulate the phylogenetic diversity of Alg13-Alg14 domain arrangements that evolved in some protozoa. They encode single polypeptides containing an Alg13 domain fused to Alg14 domain in either orientation, including those lacking the Alg14 TMD. Thus, this Alg13-Alg14 UDP-GlcNAc transferase represents an unprecedented example of a bipartite glycosyltransferase that evolved by both fission and fusion.

Glycosylation is an essential modification that influences protein structure and function. The N-linked glycans are the most common protein modification, and many features of their synthesis have been conserved from bacteria to man (1, 2; for review, see Ref. 3). In eukaryotes, N-linked glycosylation begins in the endoplasmic reticulum (ER) with the assembly of a highly conserved dolichol (dol)-linked oligosaccharide Glc3Man9GlcNAc2 precursor (for review, see Refs. 4 and 5). The synthesis of this lipid-linked oligosaccharide (LLO) proceeds in two stages. In the first stage, seven sugars are added sequentially to dolichol pyrophosphate on the cytosolic face of the ER using cytosolic nucleotide sugar substrates. This dolinked intermediate then flips into the lumen of the ER where the next stage occurs, in which the next seven sugars are added from dolinked sugar substrates (6). Once assembled, the Glc3Man9GlcNAc2 oligosaccharide core is transferred from the lipid to the protein by oligosaccharyltransferase. The protein-linked core oligosaccharide is further modified by ER enzymes and then extended by those that are encountered by nascent glycoproteins as they pass through the Golgi complex. Although mature N-linked glycans vary tremendously among eukaryotes, they all share this common core oligosaccharide whose conserved structure reflects the high degree of conservation of the twelve different Alg (asparagine-linked glycosylation) glycosyltransferases that catalyze LLO assembly.

The second step of LLO synthesis involves the addition of N-acetyl glucosamine (GlcNAc) from UDP-GlcNAc to GlcNAc-PP-dol to produce GlcNAc2-PP-dol on the cytoplasmic surface of the ER membrane (7, 8). The UDP-GlcNAc transferase that catalyzes this reaction is a very unusual glycosyltransferase. It is composed of at least two distinct polypeptides, Alg13 and Alg14, which were discovered by their distant homology to the bacterial MurG UDP-GlcNAc transferase (9–11). MurG is involved in the cytoplasmic phase of bacterial peptidoglycan biosynthesis, catalyzing the transfer of GlcNAc from UDP-GlcNAc to lipid-linked N-acetylmuramoyl pentapeptide prior to its translocation to the periplasmic face of the bacterial membrane (12, 13). The C-terminal catalytic domain of MurG is homologous to Alg13 as well as other UDP-GlcNAc transferases, whereas the N-terminal lipid-acceptor domain is related to Alg14 (9). Although Alg13 contains the highly conserved UDP-GlcNAc transferase catalytic domain, it lacks any hydrophobic membrane-spanning regions. Alg14 lacks any sequences predicted to play a direct role in sugar transfer but unlike MurG, is predicted to be an integral membrane protein containing one or more transmembrane-spanning domains (TMDs). Characterization of the Saccharomyces cerevisiae UDP-GlcNAc transferase has led to the model that Alg14 functions as a membrane anchor that recruits Alg13 to the cytosolic membrane.

Received for publication, May 29, 2007, and in revised form, June 28, 2007. Published, JBC Papers in Press, August 8, 2007, DOI 10.1074/jbc.M704410200

The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1.

© 2007 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
Membrane Topology of Alg14

On the basis of results from membrane extraction experiments, we raised the question of how these proteins can maintain their association with the ER membrane even when they lack the first strongly conserved topological domain. Alg13 and Alg14 physically interact and, under normal conditions, are associated with the ER membrane. This physical interaction appears to be essential for catalysis of sugar transfer, because Alg13 that is not bound to Alg14 is inactive for sugar transfer (10).

Table 1. Yeast strains used in this study

| Strain      | Genotype                       | Source |
|-------------|--------------------------------|--------|
| W303-1A     | MATa his3-11 leu2-3,112 ura3-1 trp1-1 ade2-1 can1-100 sld1-d   | (26)   |
| W303-1B     | MATa his3-11 leu2-3,112 ura3-1 trp1-1 ade2-1 can1-100 sld1-d   | (26)   |
| NAY13       | As in W303-1B but Sphis5<sub>-P<sub>Gal1</sub>-HA<sub>-</sub>Total<sub>GAL13</sub> | This study |
| XGY151      | As in W303-1A but Sphis5<sub>-P<sub>Gal1</sub>-HA<sub>-</sub>Total<sub>GAL14</sub> | (11)   |
| SKY20       | As in W303-1B but Sphis5<sub>-P<sub>Gal1</sub>-HA<sub>-</sub>Total<sub>TEV-VRG4</sub> | This study |
| XGY11       | As in W303-1A but Sphis5<sub>-P<sub>Gal1</sub>-HA<sub>-</sub>Total<sub>TEV-VRG4</sub> | (18)   |

Table 2. Plasmids used in this study

| Name                  | Relevant features                                      | Reference |
|-----------------------|-------------------------------------------------------|-----------|
| YEp352GAP             | 2μ/UR3 plasmid containing TDH3 promoter                | (27)      |
| YEp352GAP1            | 2μ/UR3 plasmid containing TDH3 promoter                | (28)      |
| pRS306                | LEU2 integration plasmid                               | (25)      |
| p2μALG14              | myc-ALG14 expressed from the TDH3 promoter in Yep352GAP | This study |
| p2μALG13              | ALG13-FLAG expressed from the TDH3 promoter in Yep352GAP | This study |
| pOST4-ALG13-FLAG      | OST4-ALG13-FLAG expressed from TDH3 promoter in Yepgapi | This study |
| pALG13-ALG14          | ALG13-ALG14 fusion gene expressed from the TPI1 promoter in prs305 | This study |
| pALG14-ALG13          | ALG14-ALG13 fusion gene expressed from the TPI1 promoter in prs305 | This study |
| pALG13-ALG14ΔTMD      | ALG13-ALG14ΔTMD fusion gene expressed from the TPI1 promoter in prs305 | This study |
| pFLAG-ALG13-ALG14     | FLAG-ALG13-ALG14 fusion expressed from TDH3 promoter in YepGAPI | This study |
| pFLAG-ALG14-ALG13     | FLAG-ALG14-ALG13 fusion expressed from TDH3 promoter in YepGAPI | This study |
| pFLAG-ALG13-ALG14ΔTMD | FLAG-ALG13-ALG14ΔTMD fusion expressed from TDH3 promoter in YepGAPI | This study |

ALG13 and ALG14 genes are highly conserved among eukaryotes. However, some protozoa encode a single polypeptide that contains an Alg13 and Alg14 domain instead of two separate polypeptides. Surprisingly, as described in this study, we found that these Alg13/Alg14 proteins and their unusual domain structure raised the question of how these proteins can maintain their association with the ER membrane and motivated us to examine the membrane topology of Alg14.

Here we describe the analysis of chimeric *S. cerevisiae* Alg13-Alg14 fusion proteins that mimic the proteins that naturally evolved in protozoa. Like their protozoa counterparts, we find that these *S. cerevisiae* chimeras are functional and associate with the ER membrane even when they lack the first strongly predicted Alg14 TMD. Protease protection assays allowed us to construct a tentative topological map of Alg14 that places the C terminus in the cytoplasm and the N terminus in the ER lumen. On the basis of results from membrane extraction experiments, our data also suggest that additional domains are required for the peripheral association of Alg14 with the ER. These findings and how they relate to proposed models of the ER topology of the UDP-GlcNAc transferase in eukaryotes are discussed.

**EXPERIMENTAL PROCEDURES**

Yeast Strains and Media—Standard yeast media and genetic techniques were used for the growth and construction of yeast strains (14). Yeast strains were grown in yeast extract/peptone/adenine sulfate (YPEA) or a minimal medium lacking uracil (-URA) or leucine (-LEU) containing either 2% glucose (D) or galactose (Gal). Yeast strains used in this study are listed in Table 1. W303-1A or W303-1B was used as the parental strain for the construction of all strains. SKY20 contains a replacement of the normal chromosomal ALG14 locus with a GAL1/10 promoter-driven ALG14 gene (p<sub>Gal1</sub>-ALG14) and in addition, contains sequences encoding the tobacco etch virus (TEV) protease recognition sequence (SENLYFQG) followed by a 10-amino acid linker (CQFRSRDLYFI) between the TEV recognition sequence at the junction of an N-terminal triple HA epitope tag and the first methionine of Alg14. Epitope tagging of chromosomal loci used one-step PCR-mediated recombination (15), using a standard set of templates (16) and gene-specific primers.

Plasmid Constructions—Plasmids were constructed using standard molecular biological techniques. Plasmids used in this study and their relevant features are listed in Table 2. The correct DNA sequence of all plasmids generated by PCR was verified by DNA sequence analysis.

Protein Isolation and Immunoblotting—Whole cell protein extracts were prepared from 1 to 5 <i>Δ<sub>alg14</sub></i> units of logarithmic phase cells (harvested at an <i>A<sub>600</sub></i> of 0.2–1) by the NaOH/β-mercaptoethanol method as described (17). For immunoblot analyses, proteins (from equal cell equivalents) were separated on 8% (carboxypeptidase Y (CPY)) or 12% (Alg14 and Vrg4) SDS-PAGE gels, blotted on Immobilon-P polyvinylidene difluoride membrane (Millipore) and processed as described (18). Proteins were detected using the following antibodies: 12CA5.
to detect HA-tagged Alg14 protein; mouse anti-V-ATPase to detect the 60-kDa fragment of ATPase (Molecular Probes, 1:2000); rabbit anti-CY (1:1500) (19); mouse anti-FLAG (Sigma) to detect FLAG-tagged Alg14 or -Alg13-Alg14 fusion proteins. Primary antibodies were visualized with secondary anti-rabbit or anti-mouse antibody conjugated to horseradish peroxidase (Amersham Biosciences) followed by chemiluminescence detection (ECL, GE Healthcare).

Isolation of ER or Golgi-enriched Membrane Fractions—For preparation of whole cell lysates used for membrane isolation, 15–20 A600 units of cells (harvested at an A600 of 1–2) were washed three times in 0.5 ml of cold JR lysis buffer (0.1 M sorbitol, 50 mM potassium acetate, 2 mM EDTA, 20 mM HEPES (pH 7.4), 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) and lysed by vortexing with glass beads in 200 μl of the same buffer by five repeated cycles of vortexing (30 s) and incubating on ice (30 s). Cell debris was removed by centrifugation for 5 min at 3000 × g. ER and vacuolar membranes were pelleted by centrifugation of the resulting lysate at 18,000 × g for 10 min at 4 °C. Golgi membranes were enriched by centrifugation of the resulting supernatant at 100,000 × g for 15 min at 4 °C in a Beckman Optima TL ultracentrifuge as described previously (18). The resulting supernatant contained soluble proteins. For protease protection assays (see below), the membrane pellet was resuspended in 200 μl of JR lysis buffer. For membrane extraction experiments (see below), the pellet was resuspended in 200 μl of TE buffer (10 mM Tris-HCl (pH 7.2), 1 mM EDTA) supplemented with 1 mM phenylmethylsulfonyl fluoride.

TEV Protease Protection Assay—ER- or Golgi-enriched membrane fractions were prepared as described above, and 10–20 μl (representing the yield from ~1.5–2 A600 units of cells) were mixed with 10 units of TEV protease (TEVac, Invitrogen) in a reaction containing 0.1 M sorbitol, 50 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, and 1 mM dithiothreitol, in a final volume of 40 μl. Control reactions contained 0.5% Triton X-100 or 0.1% ZnSO4. Reactions were incubated for 1 h at 25 °C, stopped by the addition of Laemmli SDS loading buffer, and heated for 3 min at 55 °C. Proteins (loading equal cell equivalents) were separated by 12% SDS-PAGE and immunoblotted with anti-HA, anti-FLAG or anti-vATPase60 antibodies as described above.

RESULTS

Diverse Strategies for the Organization of Alg13/Alg14 Proteins—With several notable exceptions, data base searches identified a pair of Alg13 and Alg14 orthologues in almost all eukaryotes. An exception is Entamoeba histolytica, which does not appear to encode any glycosyltransferase involved in N-linked glycosylation (including any of the ALG, oligosaccharyltransferase OST, or calnexin/calreticulin CNE/CRT genes that encode the chaperones that recognize N-glycans in the ER). This result suggested that this single cell fungal-related parasite apparently lacks the N-glycosylation pathway. The other exceptions were some protozoa, including those of the Entamoeba (as noted previously by Bickel et al. (10)), Trypanosoma, and Dicyostelium genera, which encode a single Alg13/Alg14 polypeptide instead of a pair of separate proteins (Fig. 1A). Remarkably, sequence alignments demonstrated that these fusions are found in either orientation (see Fig. 1 and supplemental data for sequence alignments). Trypanosomes encode a single protein containing an Alg14 domain at the N terminus and an Alg13 domain at the C terminus, reminiscent of the organization found in the bacterial MurG protein. In contrast, D. discoideum and E. histolytica encode a protein containing an Alg13 domain at the N terminus and a domain at the...
Membrane Topology of Alg14

A. Alg13-Alg14

Alg14-Alg13

Ost4-Alg13

B. P_GAL1-ALG13

PGAL1-ALG13

PGAL1-ALG14

C. P_GAL1-ALG13

mCPY

CPY1

CPY2

CPY3

CPY4

FIGURE 2. Complementation analysis of alg13 and alg14 by Alg13-Alg14 chimeras. A, a schematic representation of the S. cerevisiae Alg13/Alg14 fusion proteins used to functionally map the localization of Alg14 termini. The black box indicates the relative position of the N-terminal predicted TMD of Alg14. B, plasmid-borne ALG13/ALG14 gene fusions were introduced into strains in which chromosomal ALG13 or ALG14 is controlled by the GAL1 promoter (NAY13 and XGY151, respectively). Strains harboring the empty vector or plasmids encoding Alg13-Alg14, Alg14-Alg13, Ost4-Alg13, Alg13, or Alg14 were grown on galactose or glucose-supplemented media and incubated overnight at 30°C. Each strain was re-streaked three times. Plates representing the third re-streaking are shown. C, glycosylation of CPY in P_GAL1-ALG13 (NAY13) strains. P_GAL1-ALG13 harboring the same plasmids described in B was grown in SD(-Ura) or SD(-Leu) medium to mid logarithmic phase. Equivalent amounts of protein extract (from 0.1 A600 units cells) from these cells were separated by 8% SDS-PAGE and immunoblotted with anti-CPY antisera. The position of fully glycosylated CPY (mCPY) and underglycosylated forms are indicated. Also shown is CPY isolated from the LLO biosynthesis-defective alg11Δ mutant.

C terminus that is homologous to most of Alg14 (Fig. 1A). Notably, these D. discoideum and E. histolytica Alg13/Alg14 proteins lack a region that is homologous to the first N-terminal 33 amino acids of Alg14.

This latter finding was puzzling because this N-terminal domain of Alg14 is strongly predicted by all algorithms to contain a transmembrane spanning helix (TMD), composed of amino acids 5–24 (probability p = 1) (Fig. 1B). Secondary structure algorithms are not in agreement about whether or not there are additional transmembrane helices found in Alg14. Most programs predict only this one TMD (i.e. TMHMM; SOSUI), but there are several additional short hydrophobic domains, centered at amino acids ~130 and ~160 that are considered by some algorithms (i.e. TMPred and HMMTOP) as possible low probability membrane-spanning helices (p ~ 0.4 and p ~ 0.6, respectively) (Fig. 1B).

Because the Alg13 domain catalyzes a reaction on the cytosolic face of the ER, the absence of the first TMD in the Alg13-Alg14 polypeptide of D. discoideum and E. histolytica implies that their Alg14 domain may contain additional TMDs that mediate association with the ER membrane. Alternatively, the Alg14 domain may associate with the ER membrane by additional mechanisms that are independent of this N-terminal TMD, which could explain its absence in the D. discoideum and E. histolytica Alg13-Alg14 proteins.

Alg14 Contains a Luminal N Terminus and a Cytosolic C Terminus—As a first step to determine the membrane topology of Alg14, we analyzed chimeric yeast proteins in which S. cerevisiae Alg13 is fused to the C terminus or N terminus of S. cerevisiae Alg14 (see Fig. 2A, schematic diagram). Because Alg13 catalyzes an essential cytosolic reaction, we reasoned that complementation of a yeast strain lacking ALG13 by either of these fusions would yield information about which end of Alg14 faces the cytosol. To provide support for this experimental rationale, we first tested whether or not simply targeting Alg13 to the ER cytosolic face could complement the loss of ALG13 (Ost4-Alg14, Fig. 2B). The topology of Ost4, which contains a single TMD, a cytosolic C terminus, and a luminal N terminus, was determined previously (20), so we used this protein as a control membrane protein to anchor Alg13 to the cytosolic ER face. A plasmid-borne OST4-ALG13 fusion gene was constructed, which encodes Alg13 fused to the cytosolic C-terminal tail of Ost4. This plasmid (pOST4-ALG13-FLAG) was introduced into a yeast strain (NAY13) containing ALG13 under control of the glucose-repressible GAL1 promoter (P_GAL1). Because ALG13 is an essential gene, the functionality of the OST4-ALG13 chimera was analyzed by its ability to complement the lethality of this P_GAL1-ALG13 strain when grown on glucose-containing medium. The results of this experiment demonstrated that the Ost4-Alg13 chimera complemented the loss of ALG13 and supported growth of the P_GAL1-ALG13 strain on glucose media as a well as the wild-type ALG13 (Fig. 2B). These results suggested a correlation between Alg13 functionality and its cytosolic localization...
and provided support for the feasibility of our experimental rationale. The Ost4-Alg13 chimera was also tested for complementation of the lethality associated with loss of ALG14 in a strain containing a GAL1 promoter-driven ALG14 allele (XGY151). However, OST4-ALG13 failed to complement \( P_{\text{GAL1}}^+\)-ALG14 when this strain was grown on glucose (Fig. 2B), demonstrating that Alg14 has an additional role that is distinct from its Alg13 recruiting activity.

As a test for the topological orientation of the Alg14 termini, we examined the functionality of Alg13-Alg14 chimeras that contained Alg13 fused to the N terminus (Alg13-Alg14) or C terminus (Alg14-Alg13) of Alg14 (Fig. 2A). As described above, each of these plasmid-borne fusion genes were introduced into the \( P_{\text{GAL1}}^+\)-ALG13 (NAY13) strain and tested for complementation of lethality on glucose-containing medium. The results of this experiment demonstrated that the Alg14-Alg13 fusion protein complemented the loss of ALG13 and supported growth of the \( P_{\text{GAL1}}^+\)-ALG13 strain on glucose medium (Fig. 2B). In contrast, the Alg13-Alg14 chimera, containing Alg13 at the N terminus of Alg14, could not. This failure of Alg13-Alg14 to complement the loss of ALG13 was not due to the lack of ALG13-ALG14 expression, because this same plasmid-borne ALG13-ALG14 gene complemented the lethality associated with loss of ALG14 in a \( P_{\text{GAL1}}^+\)-ALG14 strain (XGY151) grown in glucose, although not as well as ALG14-ALG13 (Fig. 2C). Further, Western blot analyses of epitope-tagged versions of these Algl3-Alg14 and Alg14-Alg13 chimeric proteins demonstrated that they accumulated in cells at comparable levels (data not shown).

To confirm that these chimeric proteins were functional during LLO assembly, we examined their glycosylation phenotype by monitoring the gel mobility of CPY, a reporter for glycosylation and LLO assembly in the ER. The four N-linked glycans of CPY are inefficiently transferred from lipid to protein when truncated, resulting in a ladder-like mobility pattern of CPY containing 4, 3, 2, 1, or 0 glycans. We examined CPY mobility by Western blot analyses of extracts from \( P_{\text{GAL1}}^+\)-ALG13 strains that expressed the Ost4-Alg13 or Alg13-Alg14 fusion protein while they were grown in glucose to repress expression of the endogenous ALG13. The result of this experiment demonstrated that CPY, which accumulated in all of the growth-complemented strains, was the mature form (Fig. 2C, lanes 2–4). Taken together, these results demonstrated that the C terminus of Alg14 faces the cytosol and suggested that the N terminus faces the lumen.

To provide further evidence for a luminal orientation of the Alg14 N terminus, a protease protection assay was used to determine whether the N terminus of Alg14 in intact ER vesicles is resistant to added protease. To perform this experiment, a yeast strain was constructed in which the chromosomal allele of ALG14 was replaced by one encoding an N-terminally HA-tagged Alg14 protein that contained the TEV protease recognition site and a 10-amino acid linker at the junction between the TEV site and the first methionine of Alg14. This strain (SKY20), whose sole source of Alg14 is this HA-TEV-10-ALG14 allele, displayed no growth or glycosylation defect (data not shown), suggesting that the tag does not affect normal Alg14 function. If the N terminus of Alg14 faces the ER lumen, then the TEV site should be insensitive to TEV protease added to intact membranes. ER membrane fractions were prepared from this HA-TEV-10-tagged ALG14-expressing yeast strain and subjected to incubation with or without TEV protease, in the presence or absence of 1% Triton X-100 that solubilizes membranes. After digestion, proteins were solubilized, separated by SDS-PAGE, and analyzed by immunoanalysis with anti-HA antibodies (Fig. 3). The result of this experiment demonstrated that the N-terminal HA-TEV-10 tag on Alg14 was resistant to digestion by added TEV protease in the absence of Triton X-100 (Fig. 3, lanes 1 and 2). A control membrane protein with an N-terminal HA-TEV tag (TEV-HA-Vrg4) that faces the cytosol (18), however, was sensitive to TEV digestion (Fig. 3, compare lanes 5 and 6). HA-TEV-10-Alg14 was digested by TEV protease when membranes were incubated in the presence of 1% Triton X-100 (Fig. 3, lane 3) demonstrating that intact membranes are required to protect HA-TEV-10-Alg14 from TEV proteolysis. Neither Alg14 nor Vrg4 were digested in the presence of ZnSO\(_4\), an inhibitor of TEV protease, demonstrating the specificity of TEV cleavage (Fig. 3, lanes 4 and 8). The specificity of TEV for the TEV tag was further demonstrated by the resistance of an anti-HA cross-reacting protein (indicated by the asterisk in Fig. 3) whose constant level also served as a useful loading control that is present in all membranes (Fig. 3). Taken together with the genetic data described above, these results demonstrated that the N terminus of Alg14 faces the lumen of the ER while the C terminus faces the cytosol. These results also demonstrated that Alg14 contains either a single, or an odd number of membrane-spanning domains.

**ALG14 Lacking Its First Transmembrane Domain Remains Only Peripherally Associated with ER Membrane**—The naturally occurring Alg13-Alg14 protozoon fusion proteins of *E. histolytica* and *D. discoideum* are highly homologous along their entire sequence to *S. cerevisiae* Alg13 and Alg14 (\( p = 2\)e~13 for Alg13 and 7e~19 for Alg14) but notably lack the first highly predicted TMD that is found in Alg14 of all other eukaryotes. (An alignment of the *E. histolytica* protein with Alg13 and
Alg14 is shown in the supplemental Fig. S1). Together with our data that demonstrate that Alg14 contains an odd number of membrane-spanning domains, this observation raised the question of whether or not *S. cerevisiae* Alg14 indeed has more than one TMD and/or additional mechanisms to allow tethering to the ER membrane. Thus, we investigated the topology of Alg14 and its association with ER membranes more thoroughly.

We considered three possible models based on our experimentally determined topology (N terminus luminal/C terminus cytosolic) and size of the *S. cerevisiae* Alg14 (25 kDa) (Fig. 4). First, *S. cerevisiae* Alg14 may contain three TMDs. This model predicts that an Alg13-Alg14 fusion protein lacking the first TMD behaves as an integral membrane protein that associates with the ER by virtue of the two remaining TMDs, tethering the N-terminal Alg13 to the cytosolic face of the ER. Second, *S. cerevisiae* Alg14 may contain a single N-terminal TMD (N terminus luminal/C terminus cytosolic). The absence of this TMD predicts that an Alg13-Alg14 fusion protein lacking this TMD is soluble in the cytosol and is recruited to the ER membrane by some other, as yet unidentified, means. Third, *S. cerevisiae* Alg14 contains a single TMD but may contain additional domains that peripherally interact with the membrane. This model predicts that the Alg13-Alg14 fusion protein lacking the first TMD behaves like a peripheral membrane protein, which more loosely associates with the ER.

To distinguish between these models, the functional and physicochemical behavior of an *S. cerevisiae* Alg13-Alg14 fusion protein whose predicted structure mimics the *E. histolytica* and *D. discoideum* UDP-GlcNAc transferase was investigated. This protein (Alg13-Alg14ΔTMD, Fig. 5A) contains Alg13 fused to the N terminus of Alg14 lacking the first 33 amino acids (shown schematically in A) were introduced into strains in which chromosomal *ALG13* (NAY13) or *ALG14* (XY151) is driven by the GAL1 promoter. B, Alg13-Alg14ΔTMD can complement the loss of *ALG13* and *ALG14*. P<sub>GAL1</sub>•Alg13 or P<sub>GAL1</sub>•Alg14 strains harboring the empty vector or plasmids encoding Alg13-Alg14ΔTMD, Alg14-Alg13, Alg13, or Alg14 were grown on galactose or glucose-supplemented medium and incubated overnight at 30 °C. Each strain was re-streaked three sequential times. Plates representing the third re-streaking are shown. C, CPY Western analysis. The same plasmid-bearing P<sub>GAL1</sub>•Alg14 strains described above in B were grown in glucose-containing liquid medium and used for the preparation of whole cell protein extracts. Equal amounts of protein (normalized to cell number) were separated by 8% SDS-PAGE and analyzed by immunoblotting with anti-CPY antibodies. The mobility of CPY from the parental wild-type strain (W303-1A) is also shown.

To determine if this chimeric protein was functional, its ability to rescue the loss of *ALG13* or *ALG14* was assayed. P<sub>GAL1</sub>•Alg13 or P<sub>GAL1</sub>•Alg14 yeast strains harboring a plasmid-borne copy of the *ALG13-alg14ΔTMD* gene were grown on glucose or galactose containing medium. As shown in Fig. 5B,
this ALG13-alg14ΔTMD allele rescued both the loss of ALG13 and ALG14, although, in the latter case, not as well as the wild-type ALG14. This result was also seen when LLO assembly was examined by a CPY mobility shift assay. The majority of CPY in the P_{GAL1}-ALG14 strain expressing ALG13-alg14ΔTMD (grown on glucose) contained four glycans, but under glyco-sylated CPY forms were also detectable (Fig. 5C) at levels comparable to that seen when the Alg13-Alg14 chimera was the sole source of Alg14 in this strain. These results demonstrated that the Alg13-Alg14ΔTMD protein is functional and can sustain almost normal growth in strains lacking ALG13 or ALG14.

The models proposed for Alg14 membrane topology differ with regard to the solubility properties of Alg14. To determine if the Alg13-Alg14ΔTMD protein behaves as a soluble, peripheral, or integral membrane protein, extracts from yeast lacking endogenous ALG14 expressing FLAG-tagged-Alg14 or -Alg13-Alg14ΔTMD were fractionated to separate soluble proteins and membranes, and then the membranes were subjected to treatment with 0.1 M sodium carbonate (pH 11), which strips the membrane of peripheral but not integral membrane proteins. After carbonate treatment, these membrane extracts were further centrifuged into supernatant (S), which contained peripheral membrane proteins, and pellet (P) fractions, containing integral membrane proteins that remain associated with the membranes. These fractions were analyzed by immunoblotting with the anti-FLAG antibody. As controls, we compared the sedimentation pattern of Alg14, as well as the Alg14-Alg13 chimera that contains the TMD, in parallel with Alg13-Alg14ΔTMD. We also analyzed the partitioning of the endogenous vacuolar ATPase 60-kDa subunit whose peripheral association with membranes has been established (21). The presence of V-ATPase_{50} in each extract allowed us to quantitate and normalize the efficacy of carbonate extraction in each fraction. As expected, Alg14 and the Alg14-Alg13 fusion protein sedimented with ER membranes and resisted high pH extraction as seen by their almost quantitative sedimentation with the pellet fraction after carbonate extraction (Fig. 6A). In contrast, like the vacuolar ATPase, a small but significant portion of Alg13-Alg14ΔTMD did not sediment with ER membranes after centrifugation of whole cell lysates, but instead remained in the supernatant (~10–30%) (Fig. 6B). Moreover, of the membrane-associated Alg13-Alg14ΔTMD, a large portion was extracted in the high pH supernatant fraction in a manner similar to that seen for the vacuolar ATPase 60-kDa subunit (~50–80%). These results demonstrate that the Alg13-Alg14ΔTMD behaves like a peripheral membrane protein.

**DISCUSSION**

The Alg13/Alg14 UDP-GlcNAc transferase catalyzes a key step in ER N-linked glycosylation. It is the only glycosyltransferase in this pathway that contains multiple subunits, but the factors that mediate and regulate subunit assembly are not known. A better understanding of this enzyme requires knowledge about the topology of the Alg14 membrane subunit, which we characterized here. Our results from functional in vivo analyses of S. cerevisiae Alg13/Alg14 chimeras and from in vitro protease protection assays demonstrated that Alg14 contains an ER luminal N terminus and a cytosolic C terminus. These results are consistent with the single TMD membrane topology of Alg14 predicted by most algorithms. However, they seemed at odds with the paradoxical observation that some protozoa evolved a single polypeptide containing Alg13 naturally fused to Alg14 that lacks this N-terminal TMD, whereas others contain a single polypeptide with Alg13 fused to the C terminus of Alg14. We propose that the explanation for this paradox is that Alg14 contains a single TMD as well as additional domains that do not cross but peripherally interact with the ER membrane.

Our results support a model in which Alg14 contains a very short (~4 amino acids) ER luminal N terminus, followed by a single TMD (~amino acids 5–24), followed by a cytosolic C-terminal domain that contains additional regions that interact with the ER (Fig. 5, Model 3). Several pieces of evidence support this model. First, we used Alg14/Alg13 chimeras to show that the C terminus of Alg14 faces the cytosol. A fusion protein containing Alg13 at the C terminus of Alg14 was fully functional and rescued the glycosylation defect of both alg13 and alg14 mutants. In contrast, placing Alg13 at the N terminus of Alg14 failed to complement loss of ALG13, suggesting its luminal orientation (Fig. 2). Second, we directly determined the location of the N terminus of Alg14 using TEV-protease protection assays of TEV-tagged Alg14, demonstrating that the N terminus of Alg14 is insensitive to digestion by TEV protease that is added to the outside of intact ER membranes. Third, alkaline extraction of ER membranes demonstrated that, unlike Alg14, which behaves like an integral membrane protein, an Alg14 chimera lacking this TMD is only peripherally associated with ER membranes.

Although we have not directly mapped the membrane topology of the Alg14 C-terminal cytosolic domain, there is evidence that additional signals within this region allow Alg14 to interact with the ER membrane without crossing it. First, the solubility...
Membrane Topology of Alg14

properties of ER-associated Alg13-Alg14ΔTMD yeast fusion protein lacking the N-terminal TMD are not like those of either an integral membrane protein or a soluble protein. Instead, this protein associates with the ER in a manner that parallels that of a peripheral membrane protein (Fig. 6). This yeast Alg13-Alg14ΔTMD protein was designed to closely mimic the E. histolytica and D. discoideum fusion proteins that apparently lack a TMD. Our data suggest that the yeast Alg13-Alg14ΔTMD interacts with the ER as a peripherally associated membrane protein and thus provide an explanation for how these protozoa UDP-GlcNAc transferases are efficiently targeted to the face of the ER.

Our data do not address whether or not the C-terminal domain of Alg14 interacts with another integral membrane protein or partially penetrates a single leaflet of the lipid bilayer directly through additional motifs. Evidence to support the latter possibility comes from the similarity between Alg14 and other bacterial glycosyltransferases of the GT-B family. The best characterized of these is MurG, whose N-terminal domain resembles Alg14. MurG has no TMDs, but associates quite stably with membranes (22). The MurG crystal structure revealed the presence of a hydrophobic patch, surrounded by basic residues, that is formed by the association of residues in the N-domain (23). It has been proposed that this patch mediates membrane association through both hydrophobic and electrostatic interactions (23). In so doing, it pulls to the membrane surface two conserved glycine-rich loops that connect the alternating β strands and α helices of a Rossmann-like fold. This fold is a distinguishing feature of many phosphate-binding domains, and it has been proposed that the juxtaposition of the membrane and these G loops allows them to interact with the diphosphates of the lipid-linked N-acetylmuramoyl pentapeptide acceptor (24).

The overall structures of Alg14 and MurG have been conserved, including the position of the highly conserved glycines (9). It is therefore tempting to speculate that a similar mechanism may augment the membrane association of Alg14. The importance of Alg14 for UDP-GlcNAc transferase activity is underscored by our finding that tethering the Alg13 catalytic subunit to the face of the ER can complement loss of ALG13, but cannot complement the loss of ALG14 (Fig. 2). Thus, Alg14 has a function that is distinct from its Alg13-recruiting activity. An attractive possibility, given the MurG paradigm, is that Alg14 functions in the recognition of its GlcNAc-PP-dol acceptor. If this idea is correct, a more detailed investigation of the interaction between Alg14 and the dolichol anchor of GlcNAc-PP-dol will provide important mechanistic insights about lipid recognition during LLO synthesis, which is understudied and poorly understood.

Acknowledgments—We thank Xiao Dong Gao (Hokkaido University, Sapporo) and Christine Noffz (Stony Brook University) for helpful discussion.

REFERENCES

1. Young, N. M., Brisson, J. R., Kelly, J., Watson, D. C., Tessier, L., Lanthier, P. H., Jarrell, H. C., Cadotte, N., St Michael, F., Aberg, E., and Szymbanski, C. M. (2002) J. Biol. Chem. 277, 42530–42539
2. Wacker, M., Linton, D., Hitchen, P. G., Nita-Lazar, M., Haslam, S. M., North, S. I., Panico, M., Morris, H. R., Dell, A., Wren, B. W., and Aebl, M. (2002) Science 298, 1790–1793
3. Weerapan, E., and Imperiali, B. (2006) Glycobiology 16, 91R–101R
4. Kornfeld, R., and Kornfeld, S. (1985) Annu. Rev. Biochem. 54, 631–664
5. Helenius, A., and Aebl, M. (2004) Annu. Rev. Biochem. 73, 1019–1049
6. Snider, M. D., and Rogers, O. C. (1984) Cell 36, 753–761
7. Sharma, C. B., Lehle, L., and Tanner, W. (1982) Eur. J. Biochem. 126, 319–325
8. Tai, V. W., O’Reilly, M. K., and Imperiali, B. (2001) Bioorg. Med. Chem. 9, 1133–1140
9. Chantret, I., Dancourt, J., Barbat, A., and Moore, S. E. (2005) J. Biol. Chem. 280, 9236–9242
10. Bickel, T., Lehle, L., Schwarz, M., Aebl, M., and Jakob, C. A. (2005) J. Biol. Chem. 280, 34500–34506
11. Gao, X. D., Tachikawa, H., Sato, T., Ijigami, Y., and Dean, N. (2005) J. Biol. Chem. 280, 36254–36262
12. Ikeda, M., Wachi, M., Jung, H. K., Ishino, F., and Matsuhashi, M. (1990) Nucleic Acids Res. 18, 4014
13. Bugg, T. D., and Walsh, C. T. (1992) Nat. Reports 9, 199–215
14. Guthrie, C., and Fink, G. R. (1991) Methods Enzymol. 194, 3–20
15. Baudin, A., Ozier-Kalorogopoulos, O., Denouel, A., Lacroute, F., and Cullin, C. (1993) Nucleic Acids Res. 21, 3329–3330
16. Longtine, M. S., McKenzie, A., 3rd, Demarini, D. J., Shah, N. G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J. R. (1998) Yeast 14, 953–961
17. Chi, J. H., Roos, J., and Dean, N. (1996) J. Biol. Chem. 271, 3132–3140
18. Gao, X. D., and Dean, N. (2000) J. Biol. Chem. 275, 1718–1727
19. Dean, N., and Pelham, H. R. B. (1990) J. Cell Biol. 111, 369–377
20. Kim, H., Yan, Q., Von Heijne, G., Caputo, G. A., and Lenarz, W. J. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 7460–7464
21. Bauerle, C., Ho, M. N., Lindorfer, M. A., and Stevens, T. H. (1993) J. Biol. Chem. 268, 12749–12757
22. Bupp, K., and van Heijenoort, J. (1993) J. Bacterial. 175, 1841–1843
23. Ha, S., Walker, D., Shi, Y., and Walker, S. (2000) Protein Sci. 9, 1045–1052
24. Hu, Y., Chen, L., Ha, S., Gross, B., Falcone, B., Walker, D., Mokhtarzadeh, M., and Walker, S. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 845–849
25. Sikorski, R. S., and Hieter, P. (1989) Genetics 122, 19–27
26. Thomas, B. J., and Rothstein, R. (1989) Cell 56, 619–630
27. Yoko-o, T., Roy, S. K., and Ijigami, Y. (1998) Eur. J. Biochem. 257, 630–637
28. Abe, M., Hashimoto, H., and Yoda, K. (1999) FEBS Lett. 458, 309–312