Alg14 Recruits Alg13 to the Cytoplasmic Face of the Endoplasmic Reticulum to Form a Novel Bipartite UDP-\(\text{N}\)-acetylglucosamine Transferase Required for the Second Step of \(\text{N}\)-Linked Glycosylation*

Received for publication, July 12, 2005, and in revised form, August 12, 2005 Published, JBC Papers in Press, August 12, 2005 DOI 10.1074/jbc.M507569200

Xiao-Dong Gao\(^\dagger\), Hiroyuki Tachikawa\(^\dagger\), Takashi Sato\(^\dagger\), Yoshifumi Jigami\(^\dagger\), and Neta Dean\(^\dagger\)

From the \(^\dagger\)Research Center for Glycoscience, National Institute of Advanced Industrial Science and Technology, Tsukuba 305-8566, Japan, the \(^\dagger\)Laboratory of Biological Chemistry, Department of Applied Biological Chemistry, Faculty of Agricultural and Life Science, Graduate School of Agricultural and Life Science, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan, and the \(^\dagger\)Department of Biochemistry and Cell Biology, State University of New York, Stony Brook, New York 11794-5215

\(\text{N}\)-linked glycosylation requires the synthesis of an evolutionarily conserved lipid-linked oligosaccharide (LLO) precursor that is essential for glycoprotein folding and stability. Despite intense research, several of the enzymes required for LLO synthesis have not yet been identified. Here we show that two poorly characterized yeast proteins known to be required for the synthesis of the LLO precursor, GlcNAc\(_2\)-PP-dolichol, interact to form an unusual hetero-oligomeric UDP-GlcNAc transferase. Alg13 contains a predicted catalytic domain, but lacks any membrane-spanning domains. Alg14 spans the membrane but lacks any sequences predicted to play a direct role in sugar catalysis. We show that Alg14 functions as a membrane anchor that recruits Alg13 to the cytosolic face of the ER, where catalysis of GlcNAc\(_2\)-PP-dol occurs. Alg13 and Alg14 physically interact and under normal conditions, are associated with the ER membrane. Overexpression of Alg13 leads to its cytosolic partitioning, as does reduction of Alg14 levels. Concomitant Alg14 overproduction suppresses this cytosolic partitioning of Alg13, demonstrating that Alg14 is both necessary and sufficient for the ER localization of Alg13. Further evidence for the functional relevance of this interaction comes from our demonstration that the human \(\text{ALG13}\) and \(\text{ALG14}\) orthologues fail to pair with their yeast partners, but when co-expressed in yeast can functionally complement the loss of either \(\text{ALG13}\) or \(\text{ALG14}\). These results demonstrate that this novel UDP-GlcNAc transferase is a unique eukaryotic ER glycosyltransferase that is comprised of at least two functional polypeptides, one that functions in catalysis and the other as a membrane anchor.

Asparagine (\(\text{N}\))-glycosylation is an essential modification that regulates protein folding and stability. Prior to its attachment to protein, the oligosaccharide Glu\(_3\)Man\(_5\)GlcNAc\(_2\) is assembled on the lipid carrier, dolichyl pyrophosphate (dol-PP), in the ER (see Refs. 1–4 for review). The earliest steps of this lipid-linked oligosaccharide (LLO) synthesis begin on the cytoplasmic face of the ER. Seven sugars, (two N-acetylgalcosamines and five mannoses) are sequentially added to dol-PP to form Man\(_{\text{a}}\)GlcNAc\(_2\)-PP-dol by enzymes that have their catalytic domain on the cytosolic side of the ER membrane and use sugar nucleotide substrates (2, 5, 6). The enzymes that catalyze addition of the next seven sugars (four mannoses and three glucose) do so within the lumen of the ER and use dolichol-linked sugar substrates (see Ref. 1 for review). Once assembled, this core oligosaccharide is transferred to protein by oligosaccharyltransferase through an \(\text{N}\)-glycosidic bond to an asparagine that is part of the Asn-X-(Ser/Thr) consensus sequence (7). Protein-linked oligosaccharide is immediately modified by the removal of glucose and mannose by ER glucosidases and mannosidases. Failure to properly synthesize, transfer, or modify the \(\text{N}\)-linked glycan results in glycoproteins that are recognized by the quality control systems that restrict these aberrant proteins from exiting the ER to the Golgi and target their degradation (for review see Refs. 8 and 9).

In the yeast, \(\text{Saccharomyces cerevisiae}\), many of the glycosyltransferases that catalyze synthesis of the core oligosaccharide have been identified. All of the \(\text{ALG}\) genes (asparagine-linked glycosylation) identified thus far (\(\text{ALG1–12}\)) encode membrane-spanning glycosyltransferases that have their catalytic domains on one or the other side of the ER membrane, consistent with the topological constraints of this pathway (10–20). In all organisms studied thus far, \(\text{ALG}\) mutations affecting the cytosolic reactions leading to Man\(_{\text{a}}\)GlcNAc\(_2\)-PP-dol synthesis result in lethality or very severe phenotypes (19, 21, 22). Mutations affecting the later, luminal intermediates of LLO synthesis manifest as a range of phenotypes, from undetectable in \(\text{S. cerevisiae}\) (12, 13, 15, 16, 18, 22, 23) to the heterogenous phenotypes associated with congenital disorders of glycosylation (CDG) in humans (24, 25). All of the \(\text{ALG}\) genes are highly conserved among eukaryotes, although certain unicellular eukaryotes are missing a subset (26). These unicellular organisms consequently produce lipid-linked oligosaccharides that are shorter than Glu\(_3\)Man\(_5\)GlcNAc\(_2\)-PP-dol that is produced in \(\text{S. cerevisiae}\) and in humans. With the exception of \(\text{Giardia lamblia}\) and \(\text{Plasmodium falciparum}\), which produce LLO lacking mannone and glucose, the shortest glycan produced by all eukaryotes that have been examined is Man\(_{\text{a}}\)GlcNAc\(_2\)-PP-dol (26). The evolutionary conservation and essentiality of the proteins synthesizing Man\(_{\text{a}}\)GlcNAc\(_2\)-PP-dol underscore the importance of this glycan and the reactions that occur on the cytosolic face of the ER that lead to its assembly.

Despite its importance, the identity of several of the enzymes that catalyze the reactions on the cytosolic face of the ER remains a mystery. Using a bioinformatics approach, two \(\text{S. cerevisiae}\) ORFs (\(\text{YGL047w}\) and \(\text{YBR070c}\)) were recently identified that are distantly related to the bacterial Mur\(_\text{G}\) UDP-GlcNAc glycosyltransferase involved in peptidogly-
Alg13 and Alg14 Form a Functional ER UDP-GlcNAc Transferase

**TABLE ONE**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>W303a</td>
<td>MATa ade2–1 ura3–1 his3–11 trp1–1 leu2–3,112 can1–100</td>
<td>(37)</td>
</tr>
<tr>
<td>XGY31</td>
<td>As in W303a and Gal1pr-ALG1::kanMX4</td>
<td>(32)</td>
</tr>
<tr>
<td>XGY151</td>
<td>As in W303a and Gal1pr-3HA-ALG14::his5&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>XGY154</td>
<td>As in W303a and Gal1pr-3HA-ALG13::his5&lt;sup&gt;+&lt;/sup&gt; trp1::RPT6-TRP1</td>
<td>This study</td>
</tr>
<tr>
<td>XGY155</td>
<td>As in W303a and ALG13-3FLAG::his5&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>SKY202</td>
<td>As in W303a and ALG13-3HA::his5&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>SKY203</td>
<td>As in W303a and ALG13-FLAG::URA3 Gal1pr-3HA-ALG14::his5&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>XGY156</td>
<td>As in W303a and ALG13-3FLAG::his5&lt;sup&gt;+&lt;/sup&gt; Gal1pr-3HA-ALG14-TRP1</td>
<td>This study</td>
</tr>
<tr>
<td>XGY158</td>
<td>As in W303a and ALG13-3FLAG::his5&lt;sup&gt;+&lt;/sup&gt; GAL1pr-3HA-ALG14-TRP1 trp1::RPT6-TRP1</td>
<td>This study</td>
</tr>
<tr>
<td>XGY166</td>
<td>As in W303a and ADE2 ura3::Kar2-mRFP-HDEL-URA3</td>
<td>This study</td>
</tr>
<tr>
<td>XGY167A</td>
<td>As in W303a and ADE2 ALG13-GFP-his5&lt;sup&gt;+&lt;/sup&gt; ura3::Kar2-mRFP-HDEL-URA3</td>
<td>This study</td>
</tr>
<tr>
<td>XGY168</td>
<td>As in W303a and ADE2 ALG14-GFP-his5&lt;sup&gt;+&lt;/sup&gt; ura3::Kar2-mRFP-HDEL-URA3</td>
<td>This study</td>
</tr>
</tbody>
</table>

Strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>XGY167A</td>
<td>As in W303a and ADE2 ALG13-GFP-his5&lt;sup&gt;+&lt;/sup&gt; ura3::Kar2-mRFP-HDEL-URA3</td>
<td>This study</td>
</tr>
<tr>
<td>XGY168</td>
<td>As in W303a and ADE2 ALG14-GFP-his5&lt;sup&gt;+&lt;/sup&gt; ura3::Kar2-mRFP-HDEL-URA3</td>
<td>This study</td>
</tr>
</tbody>
</table>

can synthesis and that are implicated in playing a role in N-linked glycosylation (27). Attenuated expression of either of these genes affects the second step of lipid-linked oligosaccharide synthesis, the addition of a β1,4-linked GlcNAc to GlcNAc-PP-dol that produces GlcNAc<sub>2</sub>-PP-dol on the cytosolic face of the ER (27). Remarkably, unlike any other eukaryotic ER glycosyltransferases, Ygl047w, which we designate here as Alg13, is predicted to contain a consensus glycosyltransferase catalytic domain but lacks any predicted membrane-spanning domains. The other polypeptide, Ybr070c, which we designate as Alg14, contains several predicted membrane-spanning domains but lacks any sequences that are predicted to participate in sugar transfer. Because Alg13 contains a domain that is strongly related to the catalytic domain found in other UDP-sugar transferases and displays a mutant phenotype that demonstrates its involvement in LLO synthesis, it seems like it is involved in a direct role in UDP-GlcNAc transfer. The role of Alg14 is less clear, particularly because “split” glycosyltransferases are unprecedented among eukaryotes. In this work we have tested the hypothesis that Alg13 and Alg14 interact on the ER face to form a functional hetero-oligomeric UDP-GlcNAc glycosyltransferase whose membrane-spanning domain and catalytic domain are on separate polypeptides. We demonstrate that these enzymes physically interact with one another on the ER membranes, and that Alg14 is required to recruit Alg13 from the cytosol to the ER. Furthermore although neither of the human Alg13 and Alg14 orthologues can interact with their yeast partners, when co-expressed these proteins can functionally complement the loss of ALG13 or ALG14. We speculate that this enzyme has evaded identification because it is an unusual hetero-oligomeric glycosyltransferase, comprised of at least two distinct polypeptides, both of which are required for LLO biosynthesis.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Media**—Yeast strains used in this study are listed in TABLE ONE. W303a (MATa ade2–1 ura3–1 his3–11 trp1–1 leu2–3,112 can1–100) is the parental strain for all the strains used in work. Epitope tagging and replacement of promoters or chromosomal loci employed PCR-mediated recombination (28) using the standard pFA6a-series of plasmids as template (29). The pFA6a-3FLAG-His3MX6, a derivative of pFA6a-3HA-His3MX6 was kindly provided by M. Umemura. Standard yeast media, growth conditions, and genetic techniques were used (30).

In XGY151 and XGY154, the ALG14 and ALG13 chromosomal promoter sequences including the initiating ATG (220 and 202 base pairs upstream the initiating ATG, respectively) were replaced by the GAL1 promoter followed by a start codon and the triple HA epitope marked with the Schizosaccharomyces pombe his5<sup>+</sup> gene. XGY154 also contain an additional copy of the RPT6 gene and its upstream promoter sequence integrated at the trp1 locus. RPT6 is an essential gene that lies adjacent to ALG13 but that is transcribed from the opposite strand of chromosome VII. We found that replacement of the ALG13 promoter with the GAL1 promoter interferes with normal RPT6 expression. Therefore a DNA fragment, containing the RPT6 ORF and 267 bp of 5′-flanking sequence, was cloned into the TRP1 integration vector, pRS304 (31), linearized, and integrated at the trp1 locus in XGY154. SKY202 was created by replacing the ALG13 allele with a C-terminal HA-tagged ALG13 allele, marked with the S. pombe his5<sup>+</sup> gene. The XGY155 strain contains a replacement of the chromosomal ALG13 locus with a C-terminal triple FLAG-tagged ALG13 allele, marked by the S. pombe his5<sup>+</sup> gene. XGY156 was generated from XGY155 by replacing 220 base pairs upstream of the ALG14 chromosomal locus with the GAL1 promoter followed by the triple HA epitope, marked with the TRP1 gene. XGY158 was constructed from XGY156 and contains a 3HA-tagged ALG14 allele under its own promoter integrated at ura3 (by integration of pXG202 (see "Plasmid Construction" below) and in addition contains a 3HA-tagged ALG14 allele driven by the glucose repressible GAL1 promoter. XGY166 contains a normal ADE2 allele integrated at the ade2 locus, and the pTiKmRFP plasmid at ura3 locus. pTiKmRFP expresses Kar2-mRFP-HDEL (see "Plasmid Construction", below). This strain allowed the fluorescent analysis of the Kar2-mRFP-HDEL ER marker using monomeric RFP, without interference of the red pigment that accumulates due to the ade2 mutation. Strains XGY167A and 168 were derived from XGY166 and contain a replacement of the chromosomal ALG13 and ALG14 loci, respectively, with GFP-tagged alleles that are marked with the S. pombe his5<sup>+</sup> gene.

**Plasmid Constructions**—Standard molecular biology techniques were used for all plasmid constructions. The correct sequence of all PCR-amplified products was verified by DNA sequencing. The sequences of primers used in this study are available upon request. To express plasmid borne genes from the ALG13 promoter, the promoter sequence of the ALG13 gene was amplified directly from the yeast genome by PCR, digested with XhoI and EcoRI and cloned into the URA3 integrative plasmid, pRS306 (31), to generate pXG200. The same strategy was used to create pXG201, which contains the ALG14 promoter. This PCR product was cloned into the KpnI/HindIII sites of pRS306. To express the 3HA-ALG14 gene from the ALG14 promoter, the 3HA-ALG14 gene was amplified from strain XGY151 and cloned into the HindIII/Smal sites of pXG201 to create pXG202. Linearization
Alg13 and Alg14 Form a Functional ER UDP-GlcNAc Transferase

of this plasmid with EcoRV within the URA3 gene targets integration at the ura3 locus. pTiKmRFP contains a KAR2-mRFP-HDEL fusion gene (with a ClaI site at the KAR2/mRFP-HDEL junction) whose expression is controlled by the constitutive triose-phosphate isomerase (TPI) promoter. This plasmid contains the 5′-region of the KAR2 ORF, which encodes the first 45 amino acids, in-frame with the mRFP (monomeric red fluorescent protein) gene that lacks its initiating ATG and that contains an HDEL-encoding sequence at the 3′-end, in the SacI/XbaI sites of pTi-Sac.

Human ALG13 (GLT28D1) and ALG14 (MGCI7980) cDNAs were amplified by PCR using Marathon-Ready™ cDNA derived from human whole brain (Clontech, Palo Alto, CA). The amplified fragments were digested with HincIII and XbaI and inserted into expression vector, pFLAG-CMV4 (Sigma) to generate pXG203 (containing FLAG-hALG13) and pXG204 (containing FLAG-hALG14). To express the human ALG13-FLAG-tagged gene from the yeast ALG13 promoter, pXG203 was used as template for PCR to amplify ALG13 with a primer set encoding the FLAG peptide sequence. The product was digested with EcoRI and EcoRV, and cloned into EcoRI/SmaI sites of pXG200. This generated pXG205, a URA3 integration plasmid that expresses FLAG-tagged human ALG13 from the yeast ALG13 promoter. An Xhol/XbaI fragment including the ALG13 promoter and entire hALG13-FLAG fusion gene from pXG205 was cloned into the SalI/XbaI sites of pTi-Sac and marked with the LEU2 allele. Linearization of this plasmid with EcoRV, within the LEU2 gene, targets integration at the leu2 locus. To express the human ALG14 homologue from the GAP1 promoter, pXG204 was used as a template to amplify the ALG14-FLAG ORF, with primers tagged with sequences encoding the FLAG tag. The product was digested with SacI and EcoRV, and cloned into SacI/SmaI sites of pTi-Sac (32) to generate pXG207. To create pRSS04RPT6, the RPT6 gene including its promoter sequence was amplified by PCR, digested with Xhol and EcoRV, and cloned into Xhol/SmaI sites of the TRPI integrative plasmid pRSS04.

Subcellular Fractionation and Western Immunoblotting Analysis—Exponentially growing yeast cells were harvested at an A600 of 1–3 and converted to spheroplasts with lyticase.

To prepare detergent extracts, spheroplasts from 5–6 Absorbance units of cells were resuspended in 500 μl of ice-cold lysis buffer (150 mM NaCl, 10 mM HEPES-KOH, pH 7.5, 5 mM MgCl2) that contained protease inhibitors and 1% digitonin as described (33). Epitope-tagged proteins in digitonin extracts were immunoprecipitated with Anti-HA affinity matrix (Roche Applied Science Co.) or Anti-FLAG M2 affinity gel (Sigma). After fractionation by SDS-PAGE, immunoprecipitates were transferred to Immobilon-PVDF membranes. To detect the FLAG-tagged proteins, the membrane was incubated with a mouse anti-FLAG monoclonal antibody conjugated to alkaline phosphatase (anti-FLAG M2-AP) or anti-FLAG M2 affinity gel (Sigma). After fractionation by SDS-PAGE, immunoprecipitates were transferred to Immobilon-PVDF membranes. To detect the FLAG-tagged proteins, the membrane was incubated with a mouse anti-FLAG monoclonal antibody conjugated to alkaline phosphatase (anti-FLAG M2-AP). To detect the HA-tagged protein, the membrane was incubated with a rabbit anti-HA polyclonal antibody (from Sean Munro). HA-Alg14p, Alg13-HA-FLAG were detected using 12CA5 monoclonal anti-HA antibody. Primary antibody staining was followed by incubation with a secondary anti-rabbit or anti-mouse antibody conjugated to horseradish peroxidase (Amersham Biosciences) followed by chemiluminescence (ECL, Amersham Biosciences).

Co-immunoprecipitation—Exponentially growing yeast cells were harvested at an A600 of 1–3 and converted to spheroplasts with lyticase.

To prepare detergent extracts, spheroplasts from 5–6 Absorbance units of cells were resuspended in 500 μl of ice-cold lysis buffer (150 mM NaCl, 10 mM HEPES-KOH, pH 7.5, 5 mM MgCl2) that contained protease inhibitors and 1% digitonin as described (33). Epitope-tagged proteins in digitonin extracts were immunoprecipitated with Anti-HA affinity matrix (Roche Applied Science Co.) or Anti-FLAG M2 affinity gel (Sigma). After fractionation by SDS-PAGE, immunoprecipitates were transferred to Immobilon-PVDF membranes. To detect the FLAG-tagged proteins, the membrane was incubated with a mouse anti-FLAG monoclonal antibody conjugated to alkaline phosphatase (anti-FLAG M2-AP). To detect the HA-tagged protein, the membrane was incubated with a rabbit anti-HA polyclonal antibody (HA-probe Y-11, Santa Cruz Biotechnology) followed by a secondary anti-rabbit antibody conjugated to horseradish peroxidase (Cell Signaling Technologies).

Analysis of GFP and mRFP Fusion Proteins—To visualize the localization of GFP and mRFP fusion proteins, cells were grown to an A600 of 1–3 in YPAD. After washing the cells with phosphate-buffered saline + 2% glucose, GFP fluorescence was imaged using the NIBA filter (Olympus), and mRFP fluorescence was imaged using the WIG filter (Olympus) on an Olympus BX50 microscope.

RESULTS

Alg13 and Alg14 Physically Interact—Although a role for Alg13 as a glycosyltransferase involved in GlcNAc2-PP dol synthesis requires its association with the ER membrane, it lacks any membrane-spanning domains. Interestingly, ALG14 encodes an uncharacterized membrane protein that when underproduced, leads to phenotypes similar to that of alg13 (27). These data suggest a model in which these proteins pair with one another on the cytosolic face of the ER to form a functional heterooligomeric UDP-GlcNAc transferase that catalyzes the second step of core oligosaccharide synthesis on the ER.

Thus, Alg13 and Alg14 should physically interact. To test this idea, we performed a co-immunoprecipitation assay. Yeast strains were constructed that co-express C-terminally FLAG-tagged Alg13 and N-terminally HA-tagged Alg14 (strain XGY158). This strain also contains an...
ALG14 allele driven by the GAL1-promoter but this allele is repressed when this strain is grown on glucose. Both of these tagged alleles are functional, expressed at physiological levels when grown on glucose, and complement the glycosylation defect and growth phenotype of the alg13 and alg14 mutants, respectively (data not shown). Digitonin-containing protein extracts were prepared from these strains and clarified by centrifugation at 100,000 × g to remove any nonspecific protein aggregation (see “Experimental Procedures”). Alg14 was immunoprecipitated with anti-HA antibodies, the immunoprecipitates were separated by SDS-PAGE, and blotted with rabbit anti-FLAG antibody for the detection of Alg13-HA (lanes 1–6), anti-HA for the detection of Alg14-HA (lane 7), or anti-Wbp1 antibody for the presence of Wbp1 (lane 8), and detected by chemiluminescence.

**Alg13 and Alg14 Are Localized at the ER**—Both Alg13 and Alg14 should be localized on ER membranes if they pair to form the UDP-GlcNAc transferase that catalyzes the formation of GlcNAc_2-PP-dol on the cytosolic face of the ER. To test this idea, we constructed strains that produce GFP-tagged Alg13 and Alg14 at physiological levels to examine their localization in live cells (strains XGY167A and XGY168; see “Experimental Procedures”). As judged by fluorescence microscopy, both of these proteins, at least in part, localized in a perinuclear pattern that is characteristic of the yeast ER (Fig. 2). The identity of this organelle was confirmed by the co-localization with an ER reporter protein, Kar2-mRFP-HDEL (Fig. 2, A and B), which contains the Kar2 signal sequence fused to mRFP bearing a C-terminal HDEL ER retention signal (strain XGY166; see “Experimental Procedures”). While Alg14-GFP was detected primarily in ER membranes by this assay (Fig. 2A), the fluorescence observed in cells expressing ALG13-GFP was more diffuse and more difficult to detect. Nonetheless, a significant proportion of ALG13-GFP co-localized with mRFP-Kar2-HDEL (Fig. 2B), demonstrating that both Alg13 and Alg14 are associated with the ER.

Whereas the analysis of the GFP-tagged Alg13 provided strong evidence for its ER localization, the more diffuse weak GFP-staining pattern suggested that Alg13, which lacks any predicted hydrophobic domains, might also localize in the cytoplasm. To determine if this was the case, the sedimentation properties of Alg13 was analyzed by subcellular fractionation. Yeast strains were constructed that co-express an...
HA-tagged allele of ALG14 and a FLAG-tagged allele of ALG14 (XGY158) that produced proteins at comparable levels (Fig. 3A). Lysates from these cells were subjected to differential centrifugation to separate ER from soluble, cytosolic proteins (see “Experimental Procedures”). Lysates were centrifuged at 13,000 × g to sediment ER membranes. The resulting S13 supernatant was further centrifuged at 100,000 × g to separate lighter membrane components (P100), including the Golgi and small transport vesicles, away from soluble cytosolic proteins that remain in the supernatant (S100). Equivalent amounts of protein from each of these fractions, i.e. the P13, P100, and S100, were separated by SDS-PAGE and immunoblotted with anti-HA and anti-FLAG antibodies to detect Alg14-HA, Alg13-FLAG, Dpm1 (an ER marker), or Anp1 (a Golgi marker).

Alg14 is necessary and sufficient to recruit Alg13 to the ER—Despite the absence of any predicted membrane-spanning regions, our fractionation results show that at physiological levels Alg13 is predominantly associated with ER membranes. However, a different pattern of localization was observed in a strain overexpressing ALG13-FLAG under the TPI1 or GAL1 promoter (Fig. 4 and data not shown). A substantial

![Figure 3. Alg13 and Alg14 associate with ER membranes.](image)

**FIGURE 3.** Alg13 and Alg14 associate with ER membranes. Extracts were prepared from the parental isogenic strain, W303a, as a negative control or a strain co-expressing HA-tagged-ALG14 gene and FLAG-tagged ALG13 under their native promoters (XGY158); by growth in a glucose medium. These extracts were analyzed by immunoblotting with anti-HA or anti-FLAG antibody after separation on 10% SDSPAGE (A) or after subcellular fractionation (B) as described under “Experimental Procedures,” in which 10 μg of proteins from each fraction were separated by SDS-PAGE and analyzed by immunoblotting with antibodies against Alg14-HA, Alg13-FLAG, Dpm1 (an ER marker), or Anp1 (a Golgi marker).

![Figure 4. The membrane association of Alg13 is dependent on Alg14.](image)

**FIGURE 4.** The membrane association of Alg13 is dependent on Alg14. A, overexpression of Alg13 results in its cytosolic and ER localization. Extracts from yeast expressing ALG13-HA (SKY202) or GAL1pr-ALG13-HA (XGY154) grown on medium supplemented with galactose were fractionated to separate membrane and soluble proteins. Equivalent amounts of each fraction were separated by 10% SDS-PAGE (see “Experimental Procedures”), and the amount of Alg13-HA in each fraction was compared after immunoblotting with anti-HA antibody. B, depletion of Alg14 results in the cytosolic partitioning of Alg13. Yeast containing a glucose-repressible/galactose-inducible GAL1pr-HA-ALG14 allele and in addition, a FLAG-tagged ALG13 allele (SKY203) were grown in galactose-containing medium, and at time 0, shifted to glucose-containing medium. At variable times, aliquots of cells were collected, lysed by vortexing with glass beads, and fractionated to separate membrane from soluble proteins, as described under “Experimental Procedures.” C, overexpression of Alg14 suppresses the cytosolic partitioning of Alg13. Yeast cells expressing ALG13-FLAG and both a native and GAL7 promoter-driven ALG14 allele (XGY158) were grown to mid-log phase in medium supplemented with either glucose (left panel) or galactose (right panel). Extracts were fractionated to separate ER (P13) from soluble proteins (S100). 10 μg of protein from each fraction were separated by SDS-PAGE, immunoblotted, and Alg13-FLAG was detected with anti-FLAG antibody.
portion of Alg13-FLAG remained soluble and was found in the S100 fraction whereas similar amounts of Alg13-FLAG co-sedimented in the membrane-containing fraction (Fig. 4A, compare lanes 2 and 4). This result demonstrates that overproduction of Alg13 can shift its localization so that more of it partitions in the cytoplasm. Based on our demonstration of the physical interaction between Alg13 and Alg14 (Fig. 1), one explanation for this result is that the localization of Alg13 to the ER is dependent upon its stoichiometric association with Alg14, that when saturated with excess Alg13 leads to its increased cytosolic localization. If Alg14 is required for the recruitment of Alg13 to the ER, depletion of Alg14 from the ER should result in the re-localization of Alg13 to the cytosol. To test this idea, we analyzed the localization of Alg13 as a function of Alg14 depletion over time. A strain was constructed containing ALG14 under the control of the glucose-repressible GAL1p promoter and in addition, a FLAG-tagged allele of ALG13, driven by its native promoter (SKY203). These cells were grown under non-repressing conditions and at time zero, glucose was added to repress ALG14 expression. Aliquots of cells were removed every 4 h, and lysates prepared from these cells were subjected to differential centrifugation. Membrane and soluble fractions were analyzed by SDS-PAGE and immunoblotted with anti-FLAG antibodies to analyze the sedimentation properties of Alg13 (Fig. 4B). In this strain, which overproduces Alg14, at time zero, Alg13 was found predominantly in the membrane fraction (Fig. 4B, compare lanes 1 and 2). However, concomitant with a loss of ALG14 (not shown), the proportion of Alg13 that fractionated in the cytosolic fraction increased. By 24 h, when cells could no longer divide because of the depletion of Alg14 (data not shown), equivalent amounts of Alg13 were found in both the membrane and cytosolic fractions (Fig. 4B, lanes 7 and 8). Depletion of Alg14 results in the cytosolic partitioning of Alg13, therefore, the ER localization of Alg13 is dependent on Alg14.

We further asked if Alg14 is also sufficient to recruit Alg13 to the ER. To address this question, we examined if a concomitant increase in ALG14 expression can rescue the Alg13 cytosolic localization. We analyzed the localization of Alg13 in a yeast strain containing both its normal ALG14 gene and a GAL1 promoter-driven ALG14 allele that is overexpressed in the presence of galactose. This strain (XGY158) also contained FLAG-tagged ALG13. This strain thus expresses normal levels of ALG14 when grown on glucose, but overexpresses ALG14 when grown in galactose. The affect of ALG14 overexpression on Alg13 localization was assayed by preparing lysates from cells grown in galactose or glucose, separating the membrane and soluble proteins by differential centrifugation, and assaying the amount of Alg13-FLAG in each fraction by immunoblotting (Fig. 4C). When grown in glucose, when ALG14 is not overexpressed, Alg13 was observed in both the P13 ER-enriched fraction and in the S100 soluble protein fraction, at about a 4:1 ratio. In marked contrast, overexpression of ALG14 by growth in galactose resulted in significant enrichment of Alg13-FLAG with the P13 fraction. Alg13 was almost undetectable in the S100 fraction under these conditions. This result is also consistent with our failure to...
Alg13 and Alg14 Form a Functional ER UDP-GlcNAc Transferase

FIGURE 6. Complementation of alg13 or alg14 requires co-expression of the human ALG13 and ALG14 orthologues. A, GAL1p-driven ALG13 and ALG14 strains fail to grow on glucose plate. Isogenic wild-type (W303a) and GAL1p-driven ALG13 (XGY154), ALG14 (XGY151), and ALG1 (XGY31) strains were streaked on YPA plates supplemented with galactose (left panel) and glucose (right panel) and then grown for 2 days at 30 °C. B, co-expression of human ALG13 and ALG14 complement the growth defect of GAL1p-driven ALG13 or ALG14 strains on glucose-containing medium. Isogenic GAL1p-driven ALG13 strains (XGY154) harboring plasmid-borne human ALG13 (pXG205) only or both human ALG13 and ALG14 (pXG205 and pXG207) were streaked on YPA + glucose plates and incubated for 2 days at 30 °C (left panel). Isogenic GAL1p-driven ALG14 strain (XGY151) harboring human ALG14 (pXG207) only or both human ALG13 and ALG14 (pXG207 and pXG206) were also grown on YPA + glucose plates (right panel). The growth properties of all these strains were compared with the parental wild-type strain (W303a).

We also tested whether co-expression of these genes complemented the glycosylation phenotype in yeast. Yeast strains containing GAL1p-driven ALG13 (XGY154) or ALG14 (XGY151) harboring plasmids containing the human ALG13 or ALG14 genes (pXG205 and pXG207, respectively), were grown to mid-log phase in YPA medium supplemented with galactose, shifted into YPA supplemented with galactose, or with glucose to repress expression of the ALG13 and ALG14 genes, and grown for 6–8 h at 30 °C. After three repeated passages of growth on galactose or glucose, cells were harvested and crude protein extracts were prepared as described under "Experimental Procedures." Protein extracts (4 μg) were separated by 10% SDS-PAGE and blotted with an anti-CPY monoclonal antibody. The positions of the mature and hypoglycosylated forms of CPY lacking 1, 2, 3, or 4 N-linked glycans are indicated (37).

FIGURE 7. Co-expression of human ALG13 and ALG14 genes rescues the alg13 or alg14 glycosylation defect. Yeast strains containing GAL1p-driven ALG13 (XGY154) or ALG14 (XGY151) harboring plasmids containing the human ALG13 or ALG14 genes (pXG205 and pXG207, respectively), were grown to mid-log phase in YPA medium supplemented with galactose, shifted into YPA supplemented with galactose, or with glucose to repress expression of the ALG13 and ALG14 genes, and grown for 6–8 h at 30 °C. After three repeated passages of growth on galactose or glucose, cells were harvested and crude protein extracts were prepared as described under "Experimental Procedures." Protein extracts (4 μg) were separated by 10% SDS-PAGE and blotted with an anti-CPY monoclonal antibody. The positions of the mature and hypoglycosylated forms of CPY lacking 1, 2, 3, or 4 N-linked glycans are indicated (37).
Alg13 and Alg14 Form a Functional ER UDP-GlcNAc Transferase

after three passages in glucose, because Alg14 is much more stable than Alg13 (data not shown). Co-expression of the human ALG13 and ALG14 genes in either the GAL-ALG13 or GAL-ALG14 strains resulted in a significant increase in the proportion of fully glycosylated CPY that accumulated in these strains when grown on glucose (Fig. 7, compare lanes 2 and 3, and 6 and 7) although the glycosylation defect was not restored to normal levels. Taken together, these data demonstrate that the human Alg13 and Alg14 proteins cannot productively interact with their yeast partner, but can pair with one another to form a functional UDP-GlcNAc transferase in yeast.

DISCUSSION

Alg13 meets most of the criteria demanded of the key enzyme that catalyzes the second step of LLO oligosaccharide biosynthesis. It contains a consensus UDP-sugar binding site, is required for the synthesis of GlcNAc2-PP-dol (27), displays a severe N-glycosylation defect when underproduced, and is conserved among other eukaryotes. However, its lack of a membrane-spanning domain raised the question of how it can be recruited to the cytosolic face of the ER where this reaction occurs. Our results demonstrate that the ER localization of Alg13 is mediated through its physical interaction with Alg14, an essential ER protein that acts as a membrane anchor. Our data suggest a novel topological arrangement for this glycosyltransferase that contains at least two subunits, including Alg13, which mediates sugar transfer, and Alg14, which acts as a membrane anchor. To our knowledge, this is the first example of such a bipartite eukaryotic glycosyltransferase.

We have shown that Alg13 and Alg14 interact by co-immunoprecipitation assays. Recently, a number of other ER glycosyltransferases, namely Alg1, Alg2 and Alg11, which catalyze several steps of LLO biosynthesis on the cytosolic face of the ER have been shown to interact as hetero-oligomeric complexes that are important for their glycosyltransferase activity (32). We have not determined the stoichiometry of Alg13 and Alg14 within the hetero-oligomer nor have we ruled out the possibility that there may be additional interactions between Alg13 and Alg14 with these or other ER proteins that may modify the activity of the UDP-GlcNAc transferase activity. However, our results demonstrate that Alg14 is both necessary and sufficient for the localization of Alg13 to the ER. The depletion of Alg14 results in loss of Alg13 ER localization, as does Alg13 overexpression. The suppression of this latter phenotype by overexpression of ALG14 provides strong genetic data that are consistent with Alg14 being the sole cohort responsible for Alg13 recruitment.

Further support for the physiological relevance of the Alg13/14 hetero-oligomer comes from our analysis of the ALG13 and ALG14 human orthologues. Surprisingly, we found that complementation of either the alg13 or alg14 mutant phenotype occurs only when both human genes are expressed together. One interpretation of these data is that the ability of these human proteins to properly pair with their yeast counterparts has been lost through evolution but that these human proteins can pair with one another to form a functional hetero-oligomeric UDP-GlcNAc transferase.

The use of secondary structure algorithms and hydropathy analyses predicts that Alg14p contains three transmembrane domains. This suggests that one end of the protein faces the lumen, while the other faces the cytosol. Several observations suggest that the N terminus of Alg14 is within the lumen and the C terminus is in the cytosol. First the first and third hydrophilic domains of Alg14 are predicted to be very short (just four and eleven amino acid residues, respectively). Thus a topological arrangement in which the N terminus is within the lumen and the C terminus is in the cytosol would result in significantly longer Alg14 domains oriented toward the cytosol. This toplogy would favor a more stable interaction with Alg13 on the cytosolic face of the ER, where Alg13 must function to catalyze the formation of GlcNAc2-PP-Dol. Second, Alg13 is homologous to the C terminus of the MurG glycosyltransferase whereas Alg14 shows distant similarity to the N terminus. Third, preliminary data supporting this topology comes from our phenotypic analysis of a functional chimeric protein, consisting of Alg13 fused to the C terminus of Alg14, that can complement loss of either ALG13 or ALG14.4 This result implies that sequences in the C-terminal domains of Alg14 participate in its stable interaction with Alg13. The function of the N-terminal membrane-spanning domain of Alg14 is not known, but one possibility is that this region of the protein not only to tethers Alg13 to the ER membrane but also participates in the requisite recognition of dolichol in the membrane. The inability of the human Alg13 and Alg14 proteins to interact with their yeast partners suggests that important residues in the cytosolic facing domains of Alg14 and/or within the Alg13 protein have diverged sufficiently to destabilize the interaction with their yeast partners. A more careful analysis of the diverged residues within these domains may provide information about amino acids that mediate the interaction between Alg13 and Alg14.

Interestingly, a number of unicellular eukaryotic organisms were recently identified that lack certain ER glycosyltransferases and thus produce truncated N-linked glycans, demonstrating a precise correlation between the existence of defined N-linked glycans and the ALG genes required for their synthesis (26). For instance Giardia lamblia, a protozoan considered to be an “ancient eukaryote,” is missing all previously identified Alg glycosyltransferases except Alg7 (the UDP-GlcNac: dolichol phosphate N-acetylglucosamine-1-phosphate transferase that catalyzes formation of GlcNAc-P-P-dol) and produces N-linked glycans that consist entirely of GlcNAc1-P-P-dol (26). Encephalitozoon cuniculi, a microsporidian eukaryotic parasite that lacks mitochondria, makes no dolichol-PP precursors and does not possess any of the previously identified ALG glycosyltransferase genes. Our model for the postulated dual role of both Alg13 and Alg14 in the second step of the N-linked glycosylation pathway predicts that Giardia should possess both ALG13 and ALG14 genes, and that Encephalitozoon should possess neither. Consistent with this idea, searches of the databases demonstrate the existence of highly related Giardia ALG13 (GenBank™ accession no. EAA42775) and ALG14 (GenBank™ accession no. EAA40038) homologues, but no such related genes in the Encephalitozoon genome.

Whereas there are numerous examples of eukaryotic glycosyltransferases that function as part of larger oligomeric complexes (e.g. see (34)), there are few, if any examples of bipartite eukaryotic glycosyltransferases. Thus Alg13, the soluble, catalytic half of the protein, and Alg14, the membrane anchor, together represent a novel example of a eukaryotic bipartite glycosyltransferase within this large family of enzymes. Given the homology of Alg13 and Alg14 to the bacterial MurG protein, which may represent an ancient fusion of these two proteins, it appears that eukaryotes have evolved two distinct proteins where there was once just one. This raises the question of how such a bipartite arrangement may benefit the eukaryotic cell. One possibility that we are currently investigating is that a cytosolic component of the N-linked glycosylation machinery, particularly one that lies early in the pathway such as Alg13, may serve as a sensor for the metabolic state of the cell. In agreement with this idea, we find that Alg13 is a short-lived protein and overexpressed in yeast.3 The rate of LLO synthesis influences, and is influenced by a variety of parameters affect-

---

3 N. Averbeck and N. Dean, unpublished data.
4 X. Gao, unpublished data.
Alg13 and Alg14 Form a Functional ER UDP-GlcNAc Transferase

The first step of this pathway, the formation of GlcNAc-PP-dol has been described as the first committed step of LLO biosynthesis (35), and there is good evidence that this precursor is strongly inhibited by the formation of GlcNAc₂-PP-dol (36). Thus the enzymes required for synthesis of GlcNAc₂-PP-dol provide attractive targets for the regulation of the N-linked glycosylation pathway. Our characterization of ALG13 and ALG14 provides the framework to examine how the cell regulates the formation of these critical oligosaccharide precursors.

Acknowledgments—We thank Sabine Keppler-Ross for invaluable technical assistance and for performing the experiment shown in Fig. 4B. We would also like to thank Nicole Averbeck for discussions and critical reading of the manuscript. We also thank A. Furusawa (Tokyo University of Agriculture and Technology) for technical assistance. X-D. Gao especially acknowledges his appreciation to Drs. H. Narimatsu (AIST Japan) and N. Takahashi (Tokyo University of Agriculture and Technology). H. Tachikawa thanks Dr. Y. Fukui (The University of Tokyo) for support during this work.

REFERENCES