Interaction between the C Termini of Alg13 and Alg14 Mediates Formation of the Active UDP-N-acetylglucosamine Transferase Complex*

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The second step of eukaryotic N-linked glycosylation in endoplasmic reticulum is catalyzed by an UDP-N-acetylglucosamine transferase that is comprised of two subunits, Alg13 and Alg14. The interaction between Alg13 and Alg14 is crucial for UDP-GlcNAc transferase activity, so formation of the Alg13/14 complex is likely to play a key role in the regulation of N-glycosylation. Using a combination of bioinformatics and molecular biological methods, we have undertaken a functional analysis of yeast Alg13 and Alg14 proteins to elucidate the mechanism of their interaction. Our mutational studies demonstrated that a short C-terminal α-helix of Alg13 is required for interaction with Alg14 and for enzyme activity. Electrostatic surface views of the modeled Alg13/14 complex suggest the presence of a hydrophobic cleft in Alg14 that provides a pocket for the Alg13 C-terminal α-helix. Co-immunoprecipitation assays confirmed the C-terminal three amino acids of Alg14 are required for maintaining the integrity of Alg13/Alg14 complex, and this depends on their hydrophobicity. Modeling studies place these three Alg14 residues at the entrance of the hydrophobic-binding pocket, suggesting their role in the stabilization of the interaction between the C termini of Alg13 and Alg14. Together, these results demonstrate that formation of this hetero-oligomeric complex is mediated by a short C-terminal α-helix of Alg13 in cooperation with the last three amino acids of Alg14. In addition, deletion of the N-terminal β-strand of Alg13 caused the destruction of protein, indicating the structural importance of this region in protein stability.

Protein asparagine N-glycosylation is one of the most frequent and common protein modifications that is important because it required for the structure and function of glycoproteins. The process of the N-glycosylation is initiated with the biosynthesis of a highly conserved dolichol-linked oligosaccharide (LLO) Glc₃Man₇GlcNAc₂ that begins on the cytoplasmic face of the endoplasmic reticulum (ER) and ends within the lumen (1–3). In this process, formation of a 14 sugar oligosaccharide is sequentially catalyzed by conserved ER glycosyltransferases (4). N-Glycosylation is essential for viability, and mutations that affect glycosyltransferases that synthesize early steps of LLO are lethal (5–7).

The second step of LLO synthesis produces GlcNAc₂PP-Dol by transferring an N-acetylglucosamine (GlcNAc) from UDP-GlcNAc to GlcNAc-PP-Dol. This reaction is catalyzed by an unusual eukaryotic glycosyltransferase that is comprised of two separate polypeptides, Alg13 and Alg14 (6, 8, 9). In yeast, both Alg13 and Alg14 subunits are essential for cell viability. Alg14 is a membrane protein that recruits the cytosolic Alg13 protein to the ER to form a hetero-oligomeric complex that catalyzes the biosynthesis of GlcNAc₂-PP-Dol (6). Alg13 contains the catalytic domain of the UDP-GlcNAc transferase, but cytosolic Alg13 is not active unless bound to Alg14 at the ER membrane (9), suggesting the formation of the Alg13/14 complex is crucial for UDP-GlcNAc transferase activity. Formation of Alg13/14 complex has also been suggested to be a target for regulation of N-linked glycosylation. Unassembled excess cytosolic Alg13p inhibits N-linked glycosylation, and is prevented to accumulate intracellularly by proteasomal degradation (10), providing further evidence for the importance of Alg13/14 complex formation.

Alg13 and Alg14 were first identified in silico by their structural homology to the bacterial MurG protein (8). MurG is an UDP-GlcNAc undecaprenyl-PP-MurNAc pentapeptide: N-acetylglucosaminyl transferase that plays an essential role in peptidoglycan biosynthesis of Escherichia coli (11). MurG belongs to the GT-B glycosyltransferases (GTases) superfamily (12) and is one of the few family members whose crystal structure is solved. MurG contain two distinct domains, each of which contains several Rossmann folds that are postulated to be involved nucleotide sugar binding and transfer, as well as lipid acceptor recognition. The C-terminal domain contains the UDP-GlcNAc binding and catalytic and the enzymatic reaction occurs, while the N-terminal domain contains the putative lipid-acceptor recognition domain (13, 14). Alg13 protein is pre-
dicted to contain the conserved catalytic domain, found in the C-terminal domain of MurG, while Alg14 contains the predicted lipid acceptor (and membrane) associating domain, found in the N-terminal domain (8). Recently, a NMR structure of yeast soluble Alg13 (2jzc) has been described. Although the N-terminal half of Alg13 contains an irregular mixed α/β domain rather the predicted motif, this NMR analysis still suggests the presence of a highly conserved Rossmann fold in the C-terminal half of Alg13 (15) consistent with the predicted model (8). These structural similarities between MurG and Alg13/14 complex lead to the idea that eukaryotic LLO synthesis evolved from the bacterial glycosylation process but the questions of why the Alg13/14 UDP-GlcNAc transferase split to two subunits and what regulates their interaction remain. In this study, we report that the interaction between Alg13 and Alg14 rely on remarkably short domains. Complex formation requires only the C-terminal α-helix (comprised of fifteen amino acids) of Alg13 and the last three amino acids of Alg14.

EXPERIMENTAL PROCEDURES

Homology Modeling of the Alg13/Alg14 Complex—A refined three-dimensional homology model of the yeast Alg13/14 complex was first constructed based on the crystal structure of E. coli MurG (PDB code: 1NLM Chain A) using the Modeler 9.1 (16, 17). The sequence/template alignment between the MurG and Alg13/14 subunits was used for the modeling (8). Divisions between the Alg13 and Alg14 sequences were described by using character “/”, and gaps were inserted. The model was constructed in the following three steps: (i) Two hundred models of the first construction were generated using the Automodel class. The model with the best objective score was selected and advanced to next step. (ii) The geometry of its loop regions was roughly optimized using Loopmodel, and the best model in 100 candidates was selected for the next calculation. (iii) To further optimize a large loop in yeast Alg13 subunit (from 53 to 79 residues), the best model selected in the second step was again applied to Loopmodel class. The final three-dimensional model of the Alg13/14 complex was then selected from fifty candidates that had the best objective score. The DOPE scores calculated for the model (yeast Alg13/14 complex) and MurG were −38927.46 and −41430.95, respectively. To reassemble the experimental NMR structure of Alg13 (obtained from the protein data (PDB; 2jzc) the experimentally determined 2jzc structure was used to replace the modeled Alg13 structure using PyMol program code developed by DeLano Scientific LLC. To reconstruct the model of the Alg13/14 complex, orientation of last 12 amino acids (from Ser-191 to Ser-202) including the C-terminal α-helix in 2jzc was re-directed based on the structure information of MurG. The minimization for this re-orientation was conducted by using MOE (Chemical Computing Group Inc.) with Amber94 force fields. The SYBYL 7.1 program package (Tripos, Inc.) was used (18) for representing the molecular surface of Alg14 and the C-terminal region of Alg13 shown in Fig. 4. To present topological diagrams of Alg13 and Alg14 in predicted Alg13/14 complex, the experimentally determined secondary structure of 2jzc (15), and the secondary structure predictions of Alg14 (8) were used.

Plasmids, Yeast Strains, and Media—Plasmids used in this work and their important features are listed in Table 1. Standard molecular biology techniques were used for all plasmid constructions (19). 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. W303a (MATa ade2-1 his3-11 trp1-1 leu2-3, 112 can1-100) is the parental strain for all the strains used in work. XGY151 and XGY154, which contain replacements of the ALG14 and ALG13 promoters with the glucose repressible GALI/10 promoter respectively (6) were used for testing the activity of truncated and mutated Alg13 or Alg14 proteins by monitoring complementation of the lethality associated with the loss of ALG13 or ALG14 function. XGY155 contains a C-terminal triple FLAG-tagged ALG13 allele, marked by the Schizosaccharomyces pombe his5+ gene (6). Standard yeast media, growth conditions, and genetic techniques were used (20).

Preparation of Cell-free Lysates and Western Analysis—Exponentially growing yeast cells were harvested at an A600 of 1–3 and converted to spheroplasts with lyticase. To prepare detergent-solubilized extracts, spheroplasts (6–7 absorbance units) were resuspended in 500 μl of ice-cold lysis buffer (150 mM NaCl, 10 mM HEPES-KOH (pH 7.5), 5 mM MgCl₂) with protease inhibitors and 1% Triton X-100 as described previously (6). To prepare an ER-enriched membrane fraction, 6–7 absorbance units of exponentially growing yeast cells were lysed by glass bead beating in 500 μl of ice-cold lysis buffer. The lysate was centrifuged at 3,000 × g for 5 min to remove the unbroken cells and wall debris. The collected post 3,000 × g supernatant was centrifuged at 20,000 × g for 30 min in a TOMY MX-301 centrifuge. The pellet (P20) was re-suspended in 500 μl of lysis buffer followed by the determination of its protein concentration. This P20 fraction was used as the ER membrane fraction.

Equivalent amounts of protein in each fraction were separated by 12% SDS-PAGE and transferred to Immobilon-PVDF membranes (Millipore). The membrane was blotted with anti-FLAG M2 monoclonal antibody conjugated to alkaline phosphatase (Sigma) or a mouse anti-HA monoclonal antibody conjugated to peroxidase (Anti-[HA]-Peroxidase, Roche Applied Sciences) and detected by chemiluminescence (CDP-Star Detection Reagent, ROCHE or ECL, GE).

Co-immunoprecipitation—Epitope-tagged proteins in 1% Triton X-100 extracts were immunoprecipitated with anti-HA Affinity Matrix (Sigma Aldrich) as described (6). After separating proteins by 12% SDS-PAGE, immunoprecipitates were transferred to immobilon-polyvinylidene difluoride membranes (Millipore). To detect the FLAG-tagged proteins, the membrane was blotted with a rabbit anti-FLAG polyclonal antibody (Rockland, Inc.) followed by a secondary anti-rabbit antibody conjugated to alkaline phosphatase (Chemicon, International).

RESULTS

Stereo Structure Suggests the Importance of Termini for Formation of the Alg13/Alg14 Complex—Primary sequence alignment only shows a low similarity between yeast Alg13, Alg14 proteins, and E. coli MurG. But the experimentally determined Alg13 structure (2jzc) and secondary structure prediction of
Alg14 indicates their structural homology to MurG (Fig. 1, C and D). To gain more information about the Alg13/14 complex, its stereo structure was modeled based on the structure of 2jzc and predicted structure of Alg14 (8) using MurG as a guide (see “Experimental Procedures”). It should be noted that 2jzc structure does not reveal information about the interaction between Alg13 and Alg14, because this NMR structure of Alg13 was determined using soluble monomeric Alg13 (15), which is inactive as a glycosyltransferase (9). To reassemble “2jzc” with Alg14 and model the active complex, the C-terminal α-helix of Alg13 was re-orientated based on the structure of MurG. The ribbon-represented structure and topological diagrams in Fig. 1 show the predicted Alg13/Alg14 complex (Fig. 1A) adopting a GT-B fold with a two-domain architecture that shows marked similarities to MurG (Fig. 1B) except that the N-terminal half of Alg13 fold contains an irregular mixed α/β domain rather the predicted N-terminal Rossmann motif (Fig. 1D) (15). Alg14 contains a classical fold with central seven-stranded parallel β-sheets (Fig. 1, A–C, drawn in yellow) flanked by α-helices (Fig. 1, A–C, drawn in red), suggesting that Alg14 can be well modeled on the N-terminal domain of MurG. Like the C-terminal domain of MurG, Alg13 possesses an extended C-terminal α-helix (Alg13C) that is predicted to closely appose Alg14 (Fig. 1A, drawn in blue). This extended α-helix of MurG is considered to be the major feature of the GT-B fold (Fig. 1B, drawn in blue). Other notable structural differences between the predicted Alg13/Alg14 complex and MurG were also found. Alg14 possesses a transmembrane domain at its N terminus that is absent in MurG (Fig. 1A, indicated by a solid arrow, real structure extends out of the panel; or 1C, shown as a white box). Another major difference is the absence of the linker peptide that connects the two Rossmann fold domains of MurG (Fig. 1B) except that the N-terminal half of Alg13 fold contains an irregular mixed α/β domain rather the predicted N-terminal Rossmann motif (Fig. 1D) (15). Alg14 contains a classical fold with central seven-stranded parallel β-sheets (Fig. 1, A–C, drawn in yellow) flanked by α-helices (Fig. 1, A–C, drawn in red), suggesting that Alg14 can be well modeled on the N-terminal domain of MurG. Like the C-terminal domain of MurG, Alg13 possesses an extended C-terminal α-helix (Alg13C) that is predicted to closely appose Alg14 (Fig. 1A, drawn in blue). This extended α-helix of MurG is considered to be the major feature of the GT-B fold (Fig. 1B, drawn in blue). Other notable structural differences between the predicted Alg13/Alg14 complex and MurG were also found. Alg14 possesses a transmembrane domain at its N terminus that is absent in MurG (Fig. 1A, indicated by a solid arrow, real structure extends out of the panel; or 1C, shown as a white box). Another major difference is the absence of the linker peptide that connects the two Rossmann fold domains of MurG (Fig. 1B, drawn in blue). Other notable structural differences between the predicted Alg13/Alg14 complex and MurG were also found. Alg14 possesses a transmembrane domain at its N terminus that is absent in MurG (Fig. 1A, indicated by a solid arrow, real structure extends out of the panel; or 1C, shown as a white box). Another major difference is the absence of the linker peptide that connects the two Rossmann fold domains of MurG (Fig. 1B, drawn in blue).
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green). According to our predicted model, a consequence of the absence of this linker is that the N terminus of Alg13 and the C terminus of Alg14 are exposed at the surface of the complex (Fig. 1A). Especially, the exposed C terminus of Alg14 positions it near the end of the extended C-terminal α-helix of Alg13, suggesting the possibility that these terminal regions of Alg13 and Alg14 mediate their interaction. To test this model, we performed a series of molecular biological experiments using a yeast cell system to examine the structural and functional importance of these terminal regions.

The Predicted C-terminal α-Helix of Alg13 Is Important for Its Interaction with Alg14—To test if the C terminus of Alg13 is required for enzyme activity and/or complex formation, mutant alleles encoding Alg13 proteins deleted for three (alg13-CΔ3) or fifteen (alg13-CΔ15) C-terminal amino acids were constructed. Truncated proteins were tagged with an N-terminal triple FLAG tag. The C terminus of yeast Alg13 contains hydrophobic amino acids (-FXXLLVXXL-) and is predicted to adopt a α-helical structure (Fig. 2A). Deletion of fifteen amino acids completely disrupts this α-helix. To test their functionality, mutant alg13 alleles were introduced into a P<sub>GAL1</sub>-ALG13 strain (XGY154) containing ALG13 under the control of the glucose-repressible GAL1 promoter. This strain grows normally in galactose, but fails to grow when ALG13 gene expression is repressed by glucose. These complementation experiments demonstrated that while deletion of three amino acids had no effect on Alg13, deletion of fifteen C-terminal amino acids completely impaired Alg13 function since FLAG-alg13-CΔ15 failed to complement growth of P<sub>GAL1</sub>-ALG13 strain XGY154 cells in glucose (Fig. 2B). These results suggested that the C-terminal α-helix domain of Alg13 is essential for its function.

Our structural analysis suggested that the C terminus of Alg13 is involved in its interaction with Alg14. To test if this idea is correct, a co-immunoprecipitation assay was performed. Yeast strains were constructed that co-express N-terminally HA-tagged Alg14 with N-terminally FLAG-tagged Alg13-CΔ15p or wild type Alg13p as a positive control (Fig. 2C). Detergent extracts were prepared from these strains and clarified by centrifugation at 100,000 x g to remove any nonspecific protein aggregates (see “Experimental Procedures”). Alg14 proteins were immunoprecipitated with an anti-HA affinity matrix. The immunoprecipitates were separated by SDS-PAGE, and blotted with anti-FLAG rabbit antibodies to determine if the truncated alg13-CΔ15p bound to Alg14. Unlike wild-type Alg13p, we found that HA-tagged Alg14 failed to bind FLAG-tagged Alg13-CΔ15p (Fig. 2C, lanes 1 and 2). Our failure to detect any bound Alg13-CΔ15p was not caused by lowered intracellular levels of this protein because Alg13 lacking its C-terminal 15 amino acids is as stable and as abundant as wild-type Alg13 protein in detergent extracts (Fig. 2C, lane 5 or 6, lanes 4–6). These results demonstrated that the C-terminal fifteen amino acids of Alg13 is required for the interaction between Alg14 and Alg13 and suggest that the predicted α-helix mediates this interaction.

The C Terminus of Alg14 Is Required for Its Interaction with Alg13—Our computational analysis on stereo structure of yeast Alg14 implied that the C-terminal region is involved in complex formation (Fig. 1). Fig. 3A shows the C-terminal amino acid sequence of yeast Alg14 protein that include the predicted β-strand. To test whether or not this conserved region of Alg14 protein is important for its interaction with Alg13, mutant alleles were constructed that encoded truncated Alg14, lacking three (HA-alg14-CΔ3) or twelve (HA-alg14-CΔ12) C-terminal amino acids. These truncated Alg14 proteins were tagged with an N-terminal triple HA. These truncated Alg14 proteins were expressed in a yeast strain containing ALG14 under the control of the glucose-repressible GAL1 promoter (XGY151) and tested by complementation of loss of Alg14 function in the presence of glucose. Surprisingly, neither HA-alg14-CΔ3 nor HA-alg14-CΔ12 complemented the growth of XGY151 strain in the presence of glucose (Fig. 3B), suggesting that deletion of just three amino acids from the C terminus of Alg14 protein completely impaired Alg14 function.

To study how these C-terminal residues affect Alg14 function, the stability of these truncated Alg14 proteins and their interaction with Alg13 were tested. Strains were constructed that co-express these HA-tagged truncated Alg14 proteins and FLAG-tagged Alg13 proteins. The steady state levels of these mutant proteins (HA-Alg14-CΔ3p or HA-Alg14-CΔ12p) in ER membranes, prepared by differential centrifugation, were compared quantitatively to wild-type HA-Alg14 protein by Western blotting with anti-HA antibody. This experiment demon-
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A) The predicted β-strand region is boxed. B) An Alg14 strain (XGY151) with Alg14 under control of the glucose-repressible GAL1 promoter (pXG202) was transformed with plasmids encoding HA-ALG14 (pSA7), HA-ALG14-CΔ12 (pSA7), or HA-ALG14-CΔ12 (pSA8), and streaked onto YPA plates supplemented with galactose (right panel) or glucose (left panel). Cells were incubated for 2 days at 30 °C, A strain that contains a C-terminal triple FLAG-tagged HA-Alg14-C (pXG202), whole cell detergent extracts (lanes 1–4) or directly analyzed for the presence of triple FLAG-tagged HA-Alg14-C (pXG202). ER-enriched membrane fractions (P20) from the transformed cells were prepared as described under “Experimental Procedures.” Protein (80 μg) from each sample was separated by 12% SDS-PAGE and quantitatively analyzed for the presence of triple FLAG-tagged HA-Alg14-C. Immunoprecipitated proteins or extracts were separated by 12% SDS-PAGE, immunoblotted with rabbit anti-FLAG antibodies, and detected by chemiluminescence as described under “Experimental Procedures.”

B) Immunoprecipitation lanes 1–4. C) Western blotting assay described above. Samples were immunoprecipitated with anti-HA affinity matrix (lanes 1–4) or directly analyzed for the presence of triple FLAG-tagged Alg13p by Western blot (lanes 5–8). Immunoprecipitated proteins or extracts were separated by 12% SDS-PAGE, immunoblotted with mouse anti-FLAG and rabbit anti-HA antibodies, and detected by chemiluminescence as described under “Experimental Procedures.”

C) FIGURE 3. The C-terminal amino acids of Alg14 are required for viability and interaction with Alg13. A, C-terminal sequence of yeast Alg14 protein. The predicted β-strand region is boxed. B, Alg14 strain (XGY151) with Alg14 under control of the glucose-repressible GAL1 promoter (pXG202) was transformed with plasmids containing HA-ALG14 (pSA7), HA-ALG14-CΔ3 (pSA7), or HA-ALG14-CΔ12 (pSA8), and streaked onto YPA plates supplemented with glucose (right panel) or galactose (left panel). Cells were incubated for 2 days at 30 °C, A strain that contains a C-terminal triple FLAG-tagged HA-Alg14-C (pXG202), whole cell detergent extracts (lanes 1–4) or directly analyzed for the presence of triple FLAG-tagged HA-Alg14-C (pXG202). ER-enriched membrane fractions (P20) from the transformed cells were prepared as described under “Experimental Procedures.” Protein (80 μg) from each sample was separated by 12% SDS-PAGE and quantitatively analyzed for the presence of triple FLAG-tagged HA-Alg14-C. Immunoprecipitated proteins or extracts were separated by 12% SDS-PAGE, immunoblotted with mouse anti-FLAG and rabbit anti-HA antibodies, and detected by chemiluminescence as described under “Experimental Procedures.”

D) Unlike wild-type Alg14p, neither HA-Alg14-CΔ12p nor HA-Alg14-CΔ3p precipitated FLAG-tagged Alg13 protein (Fig. 3D, lanes 2–4). These results demonstrated that just the last three amino acids of Alg14 protein are required for its interaction with Alg13. The Hydrophobicity of C-terminal Alg14 Amino Acids Is Implicated in Mediating Its Interaction with Alg13—A close up electrostatic surface view of the predicted yeast Alg13/14 complex from our computational analyses suggested that the C-terminal α-helix of Alg13 (Fig. 4, A and B, shown in scheme) is inserted in a cleft formed in the Alg14 subunit (Fig. 4, A and B, colored with brown inside the white circle). This model suggests that the interaction between Alg13 and Alg14 takes place within a hydrophobic pocket. The back or side view of the C-terminal region of Alg13 show that its C-terminal α-helix (Fig. 4C in window and D) has its hydrophobic face surrounded by Alg14 hydrophobic residues in this binding pocket. These observations raise the possibility that the assembly of Alg13/14 complex occurs through hydrophobic interactions between these two domains. Furthermore, the molecular surface view also supports the idea that last three amino acids of Alg14 are involved in Alg13/14 complex formation. These three hydrophobic amino acids (Fig. 3A) are found at the entrance of the hydrophobic pocket (Fig. 4, A and B, indicated by red arrow), where we hypothesize they could function as a key to lock the C-terminal α-helix of Alg13 into the hydrophobic pocket.

To further understand the involvement of C-terminal residues of Alg14 in complex formation, this domain of Alg14 was...
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The predicted β-strand is for the formation of an activity Alg13/14 complex. Alg14 proteins with the last two or three residues that were replaced by glycine were tested for activity. The P_{GAL1}-ALG14 strain (XGY154) was transformed with plasmids containing ALG13-FLAG (pXG208), alg13-NΔ6-FLAG (pSA1), or alg13-NΔ10-FLAG (pSA2) and streaked onto YPA plates supplemented with galactose (left panel) or glucose (left panel). Cells were grown for 2 days at 30 °C. Whole cell detergent extracts were prepared from wild-type yeast (W303a) containing plasmids that encode triple FLAG-tagged Alg13p (pXG208), alg13-NΔ6p (pSA1), or alg13-NΔ10p (pSA2). Equivalent amounts of protein in each sample were separated by SDS-PAGE, immunoblotted, and detected with anti-FLAG monoclonal antibody conjugated with alkaline phosphatase as described under *Experimental Procedures.*

confirmed by Western blotting using anti-FLAG antibody that these mutations did not affect protein production or stability since each of these mutant proteins was produced at levels comparable to wild-type Alg14p (data not shown). These results demonstrated that the hydrophobicity of the last 3 or 2 C-terminal amino acids of Alg14 protein plays an important role in Alg13/14 function.

The Predicted C-terminal β-strand region of Alg13 is required for viability and protein stability. Alg14 proteins in most eukaryotes contain a similar C terminus that includes an invariant glycine residue (Fig. 5, marked with star). These amino acids are predicted to fold into a β-strand that is followed by a tail consisting of the last 3 amino acids (Fig. 5). The hydrophobicity of this “tail” has been conserved; Fungal Alg14 proteins contain hydrophobic amino acids at their last three positions while plants and animals contain two. This conservation suggested that hydrophobicity of this tail might be important for function.

To test this idea, the last three (ILV) or two (LV) amino acids of yeast Alg14 were altered. Plasmids expressing Alg14p with last two or three residues replaced by G were constructed and introduced into the P_{GAL1}-ALG14 strain (XGY151) to test for complementation of alg14. Replacement of ILV or LV residues to G led to severe Alg14 dysfunction because the P_{GAL1}-ALG14 cells expressing Alg14 ILV or LV to G mutant proteins grew very slowly, even after incubation at 30 °C for 3 days on YPD medium supplemented with glucose (Fig. 6, left panel). Each of these proteins was also FLAG-tagged at the N terminus. We compared in other species (Fig. 5). As shown in Fig. 5, Alg14 proteins in most eukaryotes contain a similar C terminus that includes an invariant glycine residue (Fig. 5, marked with star). These amino acids are predicted to fold into a β-strand that is followed by a tail consisting of the last 3 amino acids (Fig. 5). The hydrophobicity of this “tail” has been conserved; Fungal Alg14 proteins contain hydrophobic amino acids at their last three positions while plants and animals contain two. This conservation suggested that hydrophobicity of this tail might be important for function.

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**DISCUSSION**

The assembly of the Alg13 and Alg14 subunits is critical for the activity of the UDP-α-acetamido-α-glucosamine transferase and contributes to the regulation of N-linked glycosylation in eukaryotic cells. Despite the importance of formation of Alg13/14 complex, little is known about the molecular mechanism that mediates its assembly. Using computational and molecular biological analyses, we report the functional importance of the C termini of Alg13 and Alg14 in mediating complex formation.

*Molecular Mechanism of the Interaction between Alg13 and Alg14*—Despite the low sequence similarity, we found that Alg13 and Alg14 can be modeled on the MurG structure as a threading template. Like the most of GT-B structures, MurG has two distinct domains that are connected directly by a linker peptide (Fig. 1B, drawn in green). Although the NMR structure of Alg13 indicates its unconventional N-terminal Rossmann motif, Alg13 in modeled Alg13/14 complex still possesses structural similarity to the C-terminal domain of MurG (Fig. 1, A, B, and D) (15). Comparing to the Alg13, Alg14 in modeled complex presents a classical Rossmann fold resembling the N-terminal domain of MurG (Fig. 1, A–C). An extended C-terminal α-helix of MurG (Fig. 1B, drawn in blue) has been proposed to function as a connector of the two domains, and in the formation of the two Rossmann folds architecture (21). The Alg13/14 complex shares much of this conserved architecture but lacks the linker peptide to connect the subunits (Fig. 1, A and B). Our computational modeling studies of the Alg13/14 complex suggested a potential conformational role of the terminal regions of both Alg13 and Alg14 in mediating their association. This structural information implicated the C-terminal α-helix of Alg13 on the formation of Alg13/14 complex because it is the only structure capable of tying the Alg13 and Alg14 subunits together (Fig. 1A). Our mutational analyses of Alg13 and Alg14 proteins deleted in these domains demonstrated that these termini are essential for the viability of the cells (Figs. 2, 3, and 7), and for the physical interaction between Alg13 and Alg14 (Figs. 2 and 3). In particular, mutations that affect the C-terminal α-helix of Alg13 completely blocked the interaction between Alg13 and Alg14 subunits (Fig. 2). Furthermore, replacement of just the last three amino acids of Alg14 revealed their involvement in formation of the Alg13/14 complex (Fig. 6). Using the molecular surface view of Alg13/14 complex (Fig. 4) to guide our mutational analyses, our results of the molecular requirements for the interaction between Alg13 and Alg14 can be explained as follow: (i) The C-terminal α-helix of Alg13 interacts with Alg14 at a hydrophobic binding pocket. It is reasonable to think that Alg13 and Alg14 subunits assemble through hydrophobic interaction because the role of hydrophobic residues in protein–protein recognition and the formation of multimeric protein assembly has long been recognized (22, 23). Mutational analyses that exchange the hydrophobic residues inside the hydrophobic pocket of Alg14 or on the surface of the C-terminal α-helix of Alg13 will give clear answer on explanation of this interaction. (ii) The hydrophobic character of the last three residues (ILV) of Alg14 is important for the complex formation. From the position in the predicted model (Fig. 4, A and B, indicated by red arrow) and their mutant phenotypes (Fig. 6), we propose that these three residues of Alg14 work as a key that locks the C-terminal α-helix of Alg13 into the Alg14 hydrophobic pocket to stabilize the connection. These hydrophobic interactions could provide the driving force to lock Alg13 in, while the conserved G residue found at the fourth last position of all Alg14 C-terminal domains (Fig. 5, marked by star) could conceivably provide the flexibility for the movement of the last three residues.

**TABLE 1**

Plasmids used in this study

| Plasmid          | Description                           | Source                |
|------------------|---------------------------------------|-----------------------|
| pRS305           | LEU2/CEN6 yeast integration vector    | (27)                  |
| pRS306           | URA3/CEN6 yeast integration vector    | (27)                  |
| pXG202           | 3HA-ALG14 expressed from the ALG14 promoter in pRS306 | (6)                  |
| pXG208           | ALG13-3FLAG expressed from the ALG13 promoter in pRS306 | This study            |
| pXG211           | 3FLAG-ALG13 expressed from theALG13 promoter in pRS305 | This study            |
| pSA1             | alg13-ΔNΔ10-3FLAG expressed from the ALG13 promoter in pRS306 | This study            |
| pSA2             | 3FLAG-alg13-CΔ3 expressed from the ALG13 promoter in pRS305 | This study            |
| pSA4             | 3FLAG-alg13-CΔ3 expressed from the ALG13 promoter in pRS305 | This study            |
| pSA6             | 3FLAG-alg13-CΔ15 expressed from the ALG13 promoter in pRS305 | This study            |
| pSA7             | 3HA-alg14-CΔ2 expressed from the ALG14 promoter in pRS306 | This study            |
| pSA8             | 3HA-alg14-CΔ12 expressed from the ALG14 promoter in pRS306 | This study            |
| pSA13            | 3HA-alg14-L236G, V237G expressed from the ALG14 promoter in pRS306 | This study            |
| pSA14            | 3HA-alg14-L235G, L236G, V237G expressed from the ALG14 promoter in pRS306 | This study            |

growth of $P_{GAL1}$-ALG13 strain in the presence of glucose (Fig. 7B), suggesting that these ten amino acids are required for Alg13 protein stability or enzyme function, which includes its involvement in complex formation with Alg14.

To distinguish between these two possibilities, we examined the effect of NΔ10 on protein stability. Whole cell detergent extracts were prepared from wild-type yeast (W303a) harboring plasmids that encode triple C-terminally FLAG-tagged Alg13p or mutant Alg13 proteins (Alg13-ΔN6p or Alg13-ΔNΔ10p, see Table 1). The steady state levels of these proteins were compared with the wild type Alg13-FLAG protein by Western blotting with anti-FLAG antibody (Fig. 7C). While deletion of six amino acids from the N terminus of Alg13 had no affect on its stability, deletion of an additional four amino acids (Alg13-ΔNΔ10p) that perturbs the predicted β-strand structure resulted in almost undetectable level of Alg13 (Fig. 7C). These results demonstrate that sequences predicted to comprise a β-strand structure in N terminus of Alg13 are required for its stability and cell viability.
**Function of the Terminal Regions on Stability of Cytosolic, Free Alg13**—Our mutational analysis reveals that the terminal regions of Alg13 protein also play important roles in its free cytosolic form. We found that deletion of the N-terminal β-strand (Alg13-ΔN10) destabilizes the Alg13 protein (Fig. 7C). Because of the undetectably low levels of Alg13-NΔ10p, it is technically not possible to test by co-immunoprecipitation if the N-terminal region of Alg13 is directly involved in interaction with Alg14. But, the NMR structure of Alg13 indicates that this β-strand is intimately involved in the central of β-sheet of Alg13 (15). It can be speculated that the Alg13-NΔ10p is degraded in the cytosol before it be folded correctly for interacting with Alg14. We also found that deletion of the C-terminal α-helix inversely stabilized Alg13 protein (Fig. 2C). It has been clearly demonstrated that the C-terminal domain of Alg13 (including 100 amino acids) can serves as an autonomous degradation signal required for degradation of cytosolic Alg13 (10).

Our current studies are consistent with this previous finding and suggest that this α-helix (11 amino acids) at Alg13 C terminal plays a central role in degradation of cytosolic, free Alg13 protein. While speculative, one idea is that the C-terminal α-helix of Alg13 functions differently depending on the status of Alg13 protein. In Alg13/14 complex, it is embedded in the hydrophobic pocket of Alg14 and functions as connector for the complex formation. In the unassembled, cytosolic Alg13 protein, this α-helix may signal degradation by the proteasome. A more detailed investigation on the role of the C-terminal α-helix during degradation of cytosolic Alg13 will be required to confirm this idea.

Our structural analysis on formation of Alg13/14 complex contributes useful information to the development of antibacterial drugs. The bacterial cell wall is an essential organelle. As a consequence of this essentiality, MurG has emerged as an attractive target for development of new antibiotics that target cell wall biosynthesis (24, 25). It will be extremely important to unravel the mechanism of Alg13/14 complex formation since the similarity between MurG and the Alg13/14 complex, which catalyzes an essential step of N-linked glycosylation, requires the rational design of drugs that specifically inhibit the bacterial enzyme without affecting the human one.

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