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Genetics

Stepwise assembly of the lipid-linked oligosaccharide in the endoplasmic reticulum of *Saccharomyces cerevisiae*: Identification of the *ALG9* gene encoding a putative mannosyl transferase

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ABSTRACT **The core oligosaccharide $Glc_3Man_9GlcNAc_2$ is assembled at the membrane of the endoplasmic reticulum on the lipid carrier dolichyl pyrophosphate and transferred to selected asparagine residues of nascent polypeptide chains. This transfer is catalyzed by the oligosaccharyl transferase complex. Based on the synthetic phenotype of the oligosaccharyl transferase mutation *wbp1* in combination with a deficiency in the assembly pathway of the oligosaccharide in *Saccharomyces cerevisiae*, we have identified the novel *ALG9* gene. We conclude that this locus encodes a putative mannosyl transferase because deletion of the gene led to accumulation of lipid-linked $Man_6GlcNAc_2$ *in vivo* and to hypoglycosylation of secreted proteins. Using an approach combining genetic and biochemical techniques, we show that the assembly of the lipid-linked core oligosaccharide in the lumen of the endoplasmic reticulum occurs in a stepwise fashion.**

N-linked glycosylation is an essential modification of proteins and follows a highly conserved pathway in eukaryotic cells (1–3). The core oligosaccharide $Glc_3Man_9GlcNAc_2$ is assembled on the lipid carrier dolichyl pyrophosphate and transferred to selected asparagine residues of nascent polypeptide chains. This transfer is catalyzed by the enzyme oligosaccharyl transferase (4, 5).

It has been suggested that the assembly of the lipid-linked core oligosaccharide occurs at the membrane of the endoplasmic reticulum (ER) in a stepwise manner (1–3, 5). The first sugars (two GlcNAc and five Man residues) derive from UDP-GlcNAc and GDP-Man, respectively, whereas the next seven sugars (four Man and three Glc residues) are provided by the lipid intermediates dolichol (Dol)-P-Man and Dol-P-Glc, respectively. For each transfer reaction, an individual glycosyltransferase has been postulated (1–3, 5). In the yeast *Saccharomyces cerevisiae alg* (defective in *a*sparagine *l*inked *g*lycosylation), mutations affect different steps in the biosynthesis of the lipid-linked core oligosaccharide (6–8). These mutations lead to accumulation of lipid-linked sugar intermediates and a reduction of N-linked glycosylation of proteins. The reactions that result in the formation of lipid-linked $Man_5GlcNAc_2$ are believed to occur on the cytoplasmic side of the ER membrane with the nucleotide-bound monosaccharides as sugar donors. Translocation of dolichol-bound $Man_5GlcNAc_2$ into the lumen of the ER must take place to allow Dol-P-Man- and Dol-P-Glc-dependent elongation of $Man_5GlcNAc_2$ to the full-length core oligosaccharide $Glc_3Man_9GlcNAc_2$. However, the mechanism of the translocation reaction is not known (2, 3, 5, 9). *alg* mutations affecting the early steps in the assembly pathway (5, 10, 11) or mutations resulting in a deficiency of the biosynthesis of sugar donors (12–14) can be lethal or result in temperature-sensitive lethal phenotypes. In contrast, *alg* mutations affecting late steps in the assembly of the core oligosaccharide do not display any growth defect (6–8, 15). However, the combination of a late *alg* mutation (accumulation of incomplete lipid-linked oligosaccharide, suboptimal substrate of oligosaccharyltransferase) with a mutation affecting the oligosaccharyltransferase activity (*wbp1*) leads to a synthetic lethal phenotype at 30°C (16). Based on this phenotype, we initiated a screen for novel mutations affecting the biosynthesis of the lipid-linked core oligosaccharide (17).

In this report, we present the identification and characterization of the *ALG9* locus, which encodes a novel mannosyl transferase involved in the biosynthesis of lipid-linked oligosaccharides. With the help of *alg9* mutant strains, we were able to define specific steps in the assembly of the lipid-linked oligosaccharide. In particular, we show that the assembly of the core oligosaccharide in the lumen of the ER occurs by an ordered step-by-step addition of monosaccharide residues.

MATERIALS AND METHODS

Yeast Strains and Media. Yeast strains used in this study are listed in Table 1. Standard yeast media and genetic techniques were used (22). G418-resistant strains were grown on yeast extract/peptone/dextrose (YPD) plates containing 200 mg/liter G418 (Geneticin, GIBCO/BRL).

Isolation of the *ALG9* Locus. The *ALG9* locus was isolated as described (16).

Disruption of the *ALG9* Gene. The *ALG9* gene was disrupted according to the PCR-based gene disruption using the KanMX4-module (23). A 1.57-kb-long PCR fragment was amplified by using pFA6a-KanMX4 as template and two primers (primer 1, 5′-GCG CAG CTG TTG TTA TTT TTA ACA AGA GTA TAT ATT CAG CCG **CGT ACG CTG CAG GTC GAC**-3′; primer 2, 5′-GGC ACT AGT AGT CTC AGT TTG TTT TTC AAA CAA ACA GTA ATC **ATC GAT GAA TTC GAG CTC G**-3′) with 18- or 20-nucleotide homology to the pFA6a-KanMX4, respectively (bold sequences). The primers have 36- or 35-nt-long extensions that are homologous either to the region directly downstream of the start codon (primer 1) or to the region directly upstream to the stop codon (primer 2) of the *ALG9* ORF. The resulting linear PCR fragment was used to transform the wild-type strain SS328. To

Abbreviations: CPY, carboxypeptidase Y; Dol, dolichol; Endo H, endo-β-N-acetylglucosaminidase H; ER, endoplasmic reticulum.

Data deposition: The sequence reported in this paper has been deposited in the GeneBank data base (accession no. X96417).

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Table 1. Yeast strains used in this study

Strain	Genotype	Ref.
SS328	*Matα ade2-101 ura3-52 his3Δ200 lys2-801*	18
YG92	*Mata ade2-101 ura3-52 his3Δ200 lys2-801 tyr1 Δalg5::HIS3*	19
YG248	*Mata ade2-101 ura3-52 his3Δ200 lys2-801 Δalg3::HIS3*	20
CEN.PK2	*Matα/a leu2-3,112/leu2-3,112 ura3-52/ura3-52 trp1-289/trp1-289 his3Δ1/his3Δ1*	K. D. Entian (personal communication)
FY 1679	*Matα/a ura3-52/ura3-52 trp1Δ63/+ leu2Δ1/+ his3Δ200/+ GAL2+/GAL2+*	21
YG399	*Mata ade2-101 ade3 ura3-52 his3Δ200 leu2 wbp1-2 alg9-1*	This study
YG401	*Matα ade2-101 ade3 ura3-52 his3Δ200 lys2 wbp1-2 alg9-2 [pCH1122WBP1]*	17
YG402	*Matα ade2-101 ade3 ura3-52 his3Δ200 lys2-801 alg9-1*	This study
YG403	*Matα ade2-101 ade3 ura3-52 his3Δ200 leu2 wbp1-2*	This study
YG404	*Mata ade2-101 ura3-52 his3Δ200*	This study
YG405	*Mata ade2-101 ura3-52 his3Δ200 lys2-801 leu2 wbp1-2 alg9-1*	This study
YG406	*Matα ade2-101 ura3-52 his3Δ200 lys2 tyr1*	This study
YG407	*Mata ade2-101 ura3-52 his3Δ200 lys2 Δalg5::HIS3 Δalg9::kanMX4*	This study
YG408	*Mata ade2-101 ura3-52 his3Δ200 lys2 tyr1 Δalg9::kanMX4*	This study
YG409	*Matα ade2-101 ura3-52 his3Δ200 lys2 Δalg5::HIS3*	This study
YG410	*Mata ade2-101 ura3-52 his3Δ200 lys2-801 Δalg9::kanMX4*	This study
YG411	*Matα ade2-101 ura3-52 his3Δ200 lys2-801*	This study
YG412	*Mata ade2-101 ura3-52 his3Δ200 lys2-801Δalg3::HIS3*	This study
YG413	*Matα ade2-101 ura3-52 his3Δ200 lys2-801 Δalg3::HIS3 Δalg9::kanMX4*	This study
YG414	*Matα ade2-101 ura3-52 his3Δ200 lys2-801 Δalg9::kanMX4*	This study
YG415	*Matα ade2-101 ura3-52 his3Δ200 lys2-801 wbp1-2 Δalg9::kanMX4*	This study

select for Geneticin-resistant transformants, cells were first plated on YPD containing 1 M sorbitol, grown for 24 h at 23°C, and subsequently replicated on YPD plates containing Geneticin. Correct integration of the KanMX4 module into the *ALG9* locus was verified by PCR.

Immunological Methods and Endoglycosidase H Digestions. Yeast cells were grown for 15–20 h at 23°C or 30°C in either minimal medium supplemented with the appropriate amino acids or in YPD medium to an A_{546} between 1.0 and 1.5 cm^{-1}, and 4×10^7 cells were pelleted and resuspended in 100 μl of lysis buffer (50 mM Tris·HCl, pH 7.5/1% SDS/2 mM phenylmethylsulfonyl fluoride). Cells were broken by vigorous vortexing in the presence of acid-washed glass beads, incubated for 5 min at 95°C, and then centrifuged for 10 min. Western blotting techniques have been described (24). Detection of the carboxypeptidase Y (CPY) protein was performed by using CPY-specific antibodies and peroxidase-labeled protein A. Visualization was done with the enhances chemiluminescence system (Amersham). Glycoproteins were digested with endo-β-*N*-acetylglucosaminidase H (Endo H; Boehringer Mannheim) as described (17).

Extraction of Lipid-Linked Oligosaccharides. Yeast cells were grown for 15–20 h at 30°C in either minimal medium supplemented with the appropriate amino acids or in YPD medium to an A_{546} between 1.0 and 1.5 cm^{-1}, and 4×10^7 cells were collected by centrifugation. Extraction and analysis of dolichyl pyrophosphate-linked oligosaccharides have been described (17).

RESULTS

Deficiency in Protein Glycosylation and Assembly of the Lipid-Linked Oligosaccharide in *alg9* Mutant Strains. We developed a screen for the isolation of *alg* mutants that was based on the synthetic lethal phenotype of *alg wbp1* double mutants (17). These mutants were identified using the red/white sectoring assay (25) and classified into different complementation groups. As expected, mutations affecting known *ALG* loci (16) and components required for oligosaccharyl transferase activity were isolated (17). One novel complementation group defined the *ALG9* locus. To study more closely the deficiency caused by the *alg9* mutation, the processing of the vacuolar protease CPY was examined (16, 18). In particular, we analyzed the glycosylation of CPY in *alg9-1*, *wbp1-2*, and *alg9-1 wbp1-2* mutant strains (Fig. 1). For this purpose, the *alg9-1 wbp1-2* mutant strain YG399 was backcrossed to wild-type strain SS328 and sporulated, and a resulting tetratype tetrad was analyzed. The *wbp1-2* single mutant led to underglycosylation of CPY, represented by molecules lacking one or two N-linked oligosaccharides (Fig. 1, lane 1) (18). Yet another hypoglycosylation of CPY was observed in the *alg9-1* strain (Fig. 1, lane 3); none of the CPY molecules comigrated with CPY species isolated from the *wbp1-2* strain. The slowest moving protein band (mCPY*) from *alg9-1* cells migrated between fully glycosylated CPY (mCPY) and CPY from the *wbp1-2* strain that lacks one oligosaccharide. The cumulative effect of the *alg9-1 wbp1-2* double mutation was supported by the strong hypoglycosylation of CPY glycoforms, which comigrated with those observed in the *alg9-1* strain but not in the *wbp1-2* strain. These results indicate that the effect of the *alg9-1* mutation on glycosylation is also detected in the *wbp1-2* background (Fig. 1, lane 4). In addition, the transfer of oligosaccharides is less efficient in the *alg9-1 wbp1-2* double mutant compared with either of the single mutants. These results are compatible with the idea that the *alg9-1* mutation affects the biosynthesis of the lipid-linked oligosaccharides and

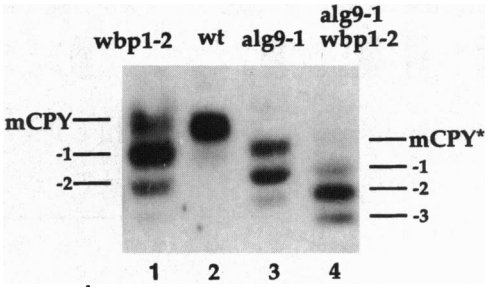

FIG. 1. The *alg9-1* mutation affects glycosylation of CPY. Strain YG399 (*alg9-1 wbp1-2*) was backcrossed to the wild-type strain SS328. The diploid strain was sporulated and tetrads were dissected. One tetrad was used for CPY-specific immunoblotting. The positions of mature CPY (mCPY) and underglycosylated CPY lacking up to two N-linked oligosaccharides (−1 and −2) are indicated. The position of mature CPY derived from strains containing the *alg9-1* mutation (mCPY*) and the different glycoforms are also indicated (−1, −2, and −3). The relevant genotype of the segregants is indicated above the lanes. Lanes: 1, YG403 (*wbp1-2*); 2, YG404 (*wt*); 3, YG402 (*alg9-1*); 4, YG405 (*alg9-1 wbp1-2*).

leads to a severe underglycosylation of CPY in combination with the *wbp1-2* mutation.

Isolation of the *ALG9* Locus. Isolation of the *ALG9* locus was based on the complementation of the synthetic lethal phenotype observed in the double mutants; *alg9 wbp1* double mutant strains do not grow at 30°C, whereas *alg9* and *wbp1* single mutant strains grow at this temperature. The *wbp1* mutation leads to a temperature sensitivity at 37°C (18). The *alg9-1 wbp1-2* strain YG399 (*alg9-1 wbp1-2*) was transformed with a plasmid bank containing yeast chromosomal DNA ligated into vector YEp352. About 15,000 transformants were screened for growth at 30°C. Sixteen transformants were found to grow at this temperature but failed to grow at 37°C. Plasmids of 10 representative strains were recovered in *Escherichia coli*. Upon retransformation, they were able to restore growth of strain YG399 (*alg9-1 wbp1-2*) at 30°C. Due to characteristic restriction sites in the insert, two plasmids could be identified as carrying either the *SWP1* locus (26) or the *OST2* locus (27). It has been shown that these loci are allele-specific suppressors of the temperature-sensitive phenotype caused by the *wbp1-2* mutation. Restriction analysis of the other plasmids revealed an overlapping insert among five of them. The remaining three plasmids were not analyzed further in this study. Of the five plasmids with common insert sequences, the one with the smallest insert (4.2 kb) (p*ALG9-1*) was chosen for sequence analysis. This analysis revealed an ORF (N1295) within the insert encoding a protein of 555 amino acids with a calculated molecular mass of 63.8 kDa (Fig. 2*A*). Subcloning experiments confirmed that this ORF encodes the complementing activity (data not shown). The plasmid p*ALG9-1* not only restored growth of the *alg9-1 wbp1-2* strain YG399 at 30°C but also specifically complemented the underglycosylation of CPY observed in the *alg9-1* strain YG402 (data not shown). Inspection of the amino acid sequence revealed that the putative Alg9 protein contains a predicted cleavable signal sequence at the N terminus (29) (putative cleavage site after position Q-22).

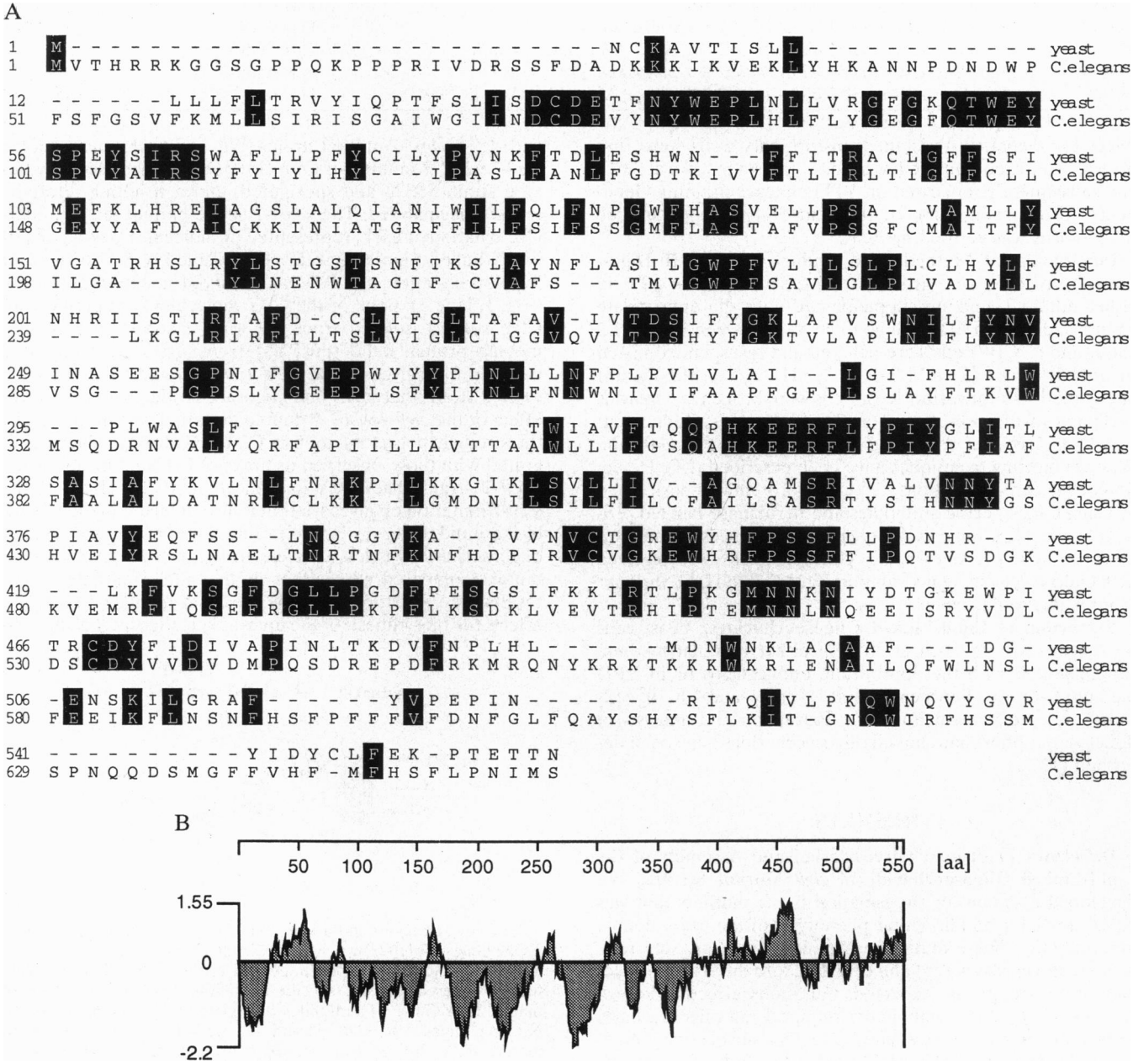

FIG. 2. Analysis of the *ALG9* protein. (*A*) Primary Alg9p sequence (yeast) in comparison with the sequence of a homologous protein from *Caenorhabitis elegans* (accession no. Z49909). The MEGALIGN program (DNASTAR) was used, and identical amino acids are highlighted. (*B*) Hydropathy plot of Alg9p according to Kyte and Doolittle using a window of 19 (28). aa, amino acids.

Alg9p is a very hydrophobic protein with a hydrophilic C-terminal domain (Fig. 2*B*), and its sequence predicts four potential N-linked glycosylation sites. A search for homologous proteins in the data bases revealed a similar protein of unknown function in *C. elegans* (Fig. 2*A*; 29% amino acid identity). Remarkably, the longest stretches of identical amino acids contain a high degree of charged residues (positions 28–63 and positions 312–322) (Fig. 2*A*). We note that in addition to the sequence similarity, both proteins share a similar hydrophobicity pattern (data not shown).

The *ALG9* Locus Is Not Essential for Vegetative Growth. The *ALG9* locus of the diploid strains CEN.PK2 and FY 1679 was replaced by integration of a kanamycin cassette according to the PCR-based gene disruption method (23). Upon sporulation and tetrad analysis, all spores were able to grow, indicating that *ALG9* is not essential for growth, and no growth phenotype was detected at all temperatures tested. To confirm that we have not isolated a high copy number suppressor of the *alg9-1* mutation, the following experiments were performed. First, we demonstrated that the isolated *ALG9* locus encoded the activity affected by the *alg9-1* mutation. To that end, a KanMX4 module (resulting in a Geneticin-resistant phenotype) was used to replace the *ALG9* locus in strain SS328 by homologous recombination. This deletion resulted in the same hypoglycosylation of CPY as did the *alg9-1* mutation (see Fig. 5, lane 3). The Δ*alg9*::*kanMX4 wbp1-2* double mutant strain YG415 had the same growth properties as the *alg9-1 wbp1-2* strain YG399 (data not shown). Second, to confirm genetically that Δ*alg9*::*kanMX4* and *alg9-1* were alleles of the same locus, strain YG415 (Δ*alg9*::*kanMX4 wbp1-2*) was crossed to strain YG399 (*alg9-1 wbp1-2*), and the resulting tetrads were analyzed. Independent segregation of the Δ*alg9*::*kanMX4* and *alg9-1* would be indicated by the appearance of *wbp1-2* single mutants (growth at 30°C). However, all segregants from 19 tetrads analyzed were not able to grow at 30°C, demonstrating that all tetrads were of parental ditype with respect to Δ*alg9*::*kanMX4* and *alg9-1*. Together, these results prove that we have cloned the locus affected by the *alg9-1* mutation.

Lipid-Linked Oligosaccharide Synthesis in *alg9* Mutant Strains. Mutations with a deficiency in the assembly of the lipid-linked oligosaccharide accumulate the substrate of the affected reaction (6). To identify the cause of CPY underglycosylation in the *alg9* mutant strains, cells were labeled with [^{3}H]mannose, and lipid-linked oligosaccharides were analyzed subsequently (Fig. 3). As shown in Fig. 3*C*, the *alg9-2* strain failed to synthesize $Glc_3Man_9GlcNAc_2$ but accumulated a different oligosaccharide. When mixed with a marker containing defined biosynthetic intermediates ($Man_5GlcNAc_2$, $Man_9GlcNAc_2$, and $Glc_3Man_9GlcNAc_2$; Fig. 3*D*), the accumulating oligosaccharide derived from the *alg9-2* strain appeared one sugar moiety larger than $Man_5GlcNAc_2$; these results indicate that *alg9* mutant strains accumulate lipid-linked $Hex_6GlcNAc_2$ (Hex = mannose or glucose). Therefore, we postulate that the *alg9* mutations lead to a deficiency in the assembly of the lipid-linked core oligosaccharide, which results in the accumulation of incomplete lipid-linked oligosaccharide. Possibly, these oligosaccharides are transferred to protein, albeit with reduced efficiency. As a result, different glycoforms of CPY with altered mobility were observed on polyacrylamide gels (Fig. 1, lanes 3 and 4). To define more

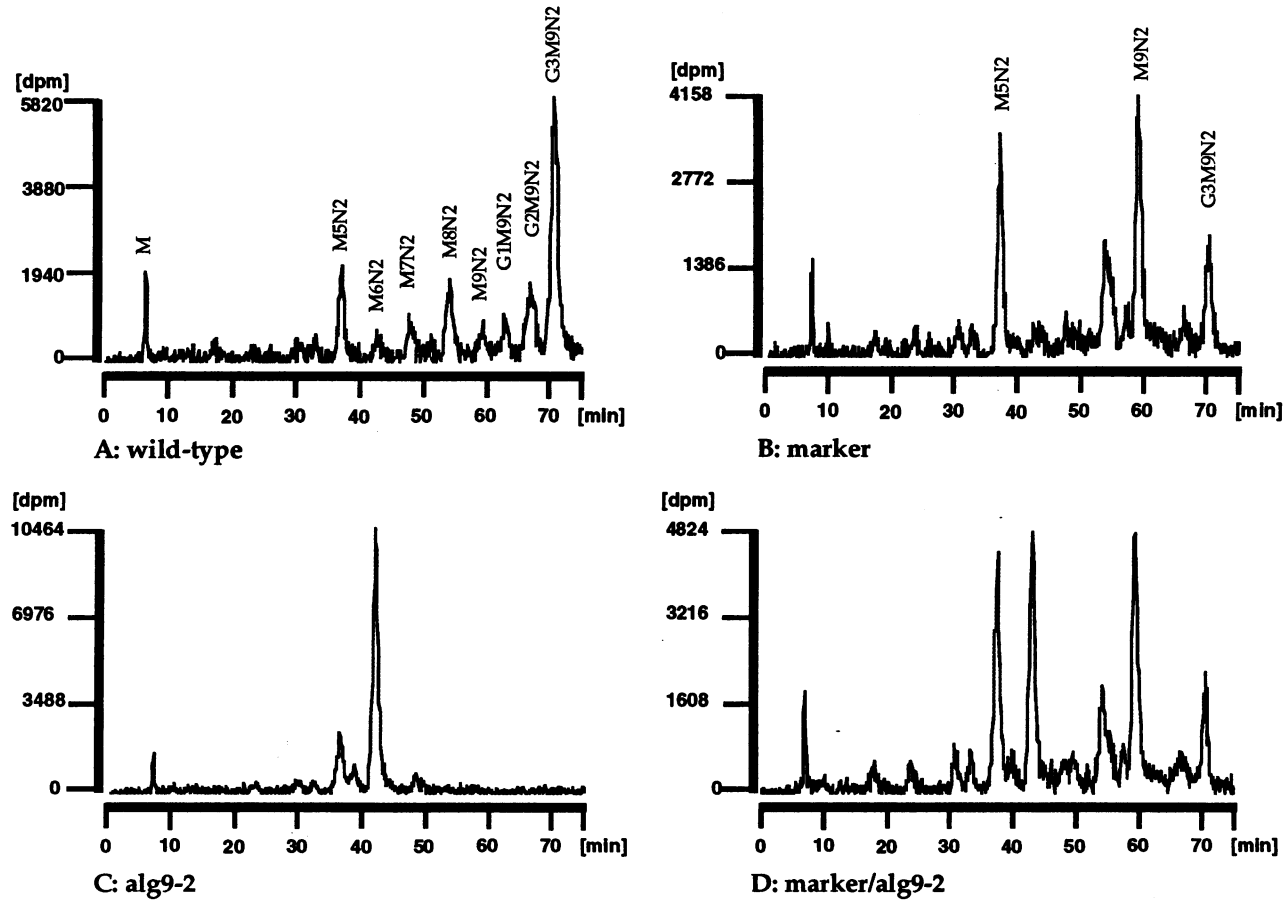

FIG. 3. Analysis of lipid-linked oligosaccharides in wild-type and mutant strains. Cells were labeled with [^{3}H]mannose. Lipid-linked oligosaccharides were extracted, hydrolyzed, and analyzed using HPLC. The position of $Man_{5-9}GlcNAc_2$ (M5-9N2) and $Glc_{1-3}Man_9GlcNAc_2$ (G1-3M9N2) are indicated. (*A*) Wild-type strain YG406. (*B*) Marker, a mixture of extracted oligosaccharides from Δ*alg3* strain YG248 (M5N2), Δ*alg5* strain YG92 (M9N2), and wild-type strain YG406 (G3M9N2). (*C*) *alg9-2* strain YG401. (*D*) Mixture of *A* and *C*.

closely the composition of the lipid-linked oligosaccharide that accumulates in the *alg9* mutant strains, we analyzed lipid-linked oligosaccharides from a Δ*alg9* Δ*alg5* double mutant strain (Fig. 4). The *ALG5* locus encodes Dol-P-Glc synthase (7, 19), and its inactivation results in the inability of cells to synthesize Dol-P-Glc, the donor of glucosyl residues in lipid-linked oligosaccharide biosynthesis (1, 3). In the Δ*alg9* Δ*alg5* double mutant strain, an oligosaccharide accumulated that had the same mobility as that in the Δ*alg9* single mutant strain (Fig. 4 *B* and *C*), whereas lipid-linked $Man_9GlcNAc_2$ was the predominant species accumulating in the Δ*alg5* strain (Fig. 4*D*). A minor oligosaccharide, eluting between $Man_6GlcNAc_2$ and $Man_5GlcNAc_2$, was observed in the Δ*alg9* strain. We have not analyzed this oligosaccharide in detail; however, the presence of a similar peak in the Δ*alg5* strain, migrating between $Man_9GlcNAc_2$ and $Man_8GlcNAc_2$, suggests that this oligosaccharide is derived from the accumulating $Man_6GlcNAc_2$ and might represent an epimerization product created during the isolation procedure (30). Our data show that the accumulating oligosaccharide in the Δ*alg9* strain does not contain glucose residues and that the step affected in the *alg9* mutant precedes the Dol-P-Glc-requiring reactions. Therefore, we conclude that lipid-linked $Man_6GlcNAc_2$ accumulates in the Δ*alg9* mutant strain.

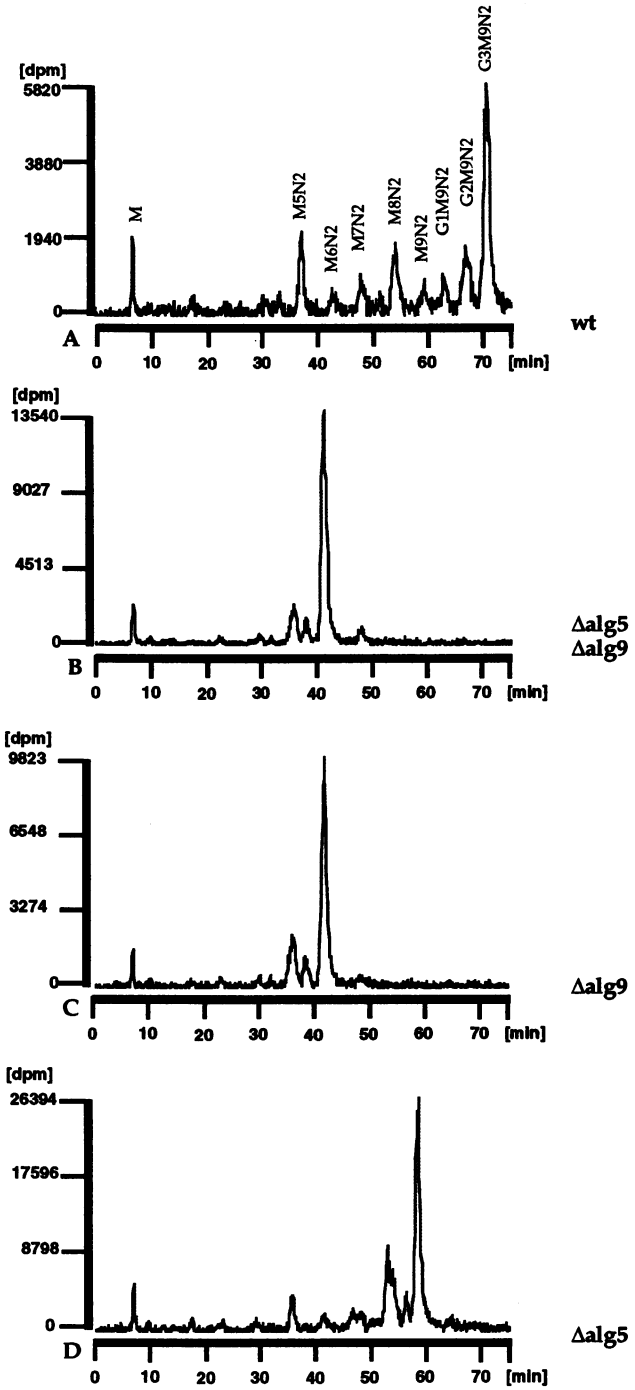

FIG. 4. The Δ*alg9* Δ*alg5* strain accumulates the same lipid-linked oligosaccharide intermediate as the Δ*alg9* strain. The *ALG9* locus was disrupted by insertion of the KanMX4 marker. The resulting strain was crossed with strain YG92 (Δ*alg5*::*HIS3*) and sporulated, and tetrads were dissected. One tetrad, representing a tetratype with respect to Δ*alg5* and Δ*alg9*, was used for analysis of lipid-linked oligosaccharides. After labeling of the cells with [^{3}H]mannose, the lipid-linked oligosaccharides were extracted and analyzed. The position of $Man_{5-9}GlcNAc_2$ (M5-9N2) and $Glc_{1-3}Man_9GlcNAc_2$ (G1-3M9N2) are indicated. (*A*) YG406 (*wt*). (*B*) YG407 (Δ*alg9* Δ*alg5*). (*C*) YG408 (Δ*alg9*). (*D*) YG409 (Δ*alg5*).

Stepwise Assembly of the Lipid-Linked Oligosaccharide. Current models of the biosynthesis of the lipid-linked core oligosaccharide $Glc_3Man_9GlcNAc_2$ suggest an ordered addition of glycosyl residues to the lipid carrier dolichyl pyrophosphate, and it is proposed that individual glycosyl transferases are required for these processes (1). This model of consecutive addition of glycosyl units predicts that mutations affecting the biosynthesis before the *ALG9*-encoded step are epistatic over the Δ*alg9* mutation, whereas Δ*alg9* is epistatic over mutations acting after the addition of the seventh mannose residue. We tested this hypothesis. It has been shown that the *alg3-1* mutation affects the addition of the sixth mannose residue, resulting in the accumulation of lipid-linked $Man_5GlcNAc_2$ and, subsequently, of Endo H-resistant oligosaccharides on secreted proteins (6, 31, 32). In the Δ*alg3* strain, we also observe the Endo H-resistant phenotype of glycoproteins (20) (Fig. 5, lanes 7 and 8). In contrast, Δ*alg9* led to the accumulation of $Man_6GlcNAc_2$ and Endo H-sensitive glycoproteins (Fig. 5, lanes 3 and 4). In the Δ*alg3* Δ*alg9* double mutant, the same hypoglycosylation of CPY was observed as in the Δ*alg3* single mutant (Fig. 5, lanes 7 and 9). In addition, the Endo H-resistant Δ*alg3* phenotype was also observed in the Δ*alg3* Δ*alg9* double mutant (Fig. 5, lanes 8 and 10). These results show that Δ*alg3* is epistatic over Δ*alg9*.

DISCUSSION

We have used a novel approach to identify mutants deficient in the assembly of the lipid-linked core oligosaccharide. Based on the observation that *alg wbp1* double mutants have a

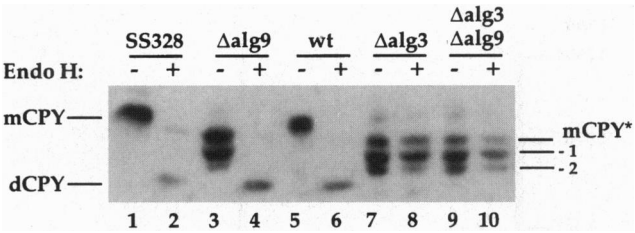

FIG. 5. The Δ*alg9* Δ*alg3* glycosylation is resistant to Endo H digestion. The Δ*alg9* strain YG414 was crossed with YG248 (Δ*alg3*::*HIS3*). The resulting diploid strain was sporulated, and one tetratype tetrad was analyzed using CPY-specific immunoblotting. As indicated, samples were incubated overnight in the presence (+) or absence (−) of Endo H. The positions of the fully glycosylated protein (mCPY) and the completely deglycosylated form (dCPY) are given. The positions of mature CPY derived from strains containing either the Δ*alg9*::*kanMX4* or the Δ*alg3*::*HIS3* knockout (mCPY*) and the different glycoforms are indicated (−1 and −2). Lanes: 1 and 2, SS328 (wild type); 3 and 4, YG410 (Δ*alg9*); 5 and 6, YG411 (*wt*); 7 and 8, YG412 (Δ*alg3*); 9 and 10, YG413 (Δ*alg3* Δ*alg9*).

synthetic lethal phenotype (16), we were able to isolate and characterize the *ALG9* locus. The accumulation of lipid-linked $Man_6GlcNAc_2$ in *Δalg9* strains and the hypoglycosylation of secretory proteins strongly support the idea that the Alg9 protein is required for the elongation of the accumulating lipid-linked oligosaccharide. The incomplete oligosaccharide is transferred to protein in *Δalg9* cells, because CPY glycoforms differing from those observed in wild-type or oligosaccharyltransferase mutant cells are resolved on SDS/PAGE. Interestingly, in the myxomycete *Dictyostelium discoideum*, mutants have been isolated that also accumulate lipid-linked $Man_6GlcNAc_2$ (33). A protein with significant sequence similarity is proposed in *C. elegans*.

Alg9p contains a putative cleavable signal peptide for import into the ER (29) and multiple membrane-spanning domains as predicted by the algorithm of Rost and coworkers (34). It suggests that the Alg9 protein is exposed both to the lumen of the ER as well as to the cytoplasm. Indeed, protease protection assays propose that proteins required for the late stages of lipid-linked oligosaccharide assembly are accessible from the cytoplasmic side even though the catalyzed reactions are suggested to take place in the lumen of the ER (35, 36).

Current models of the lipid-linked oligosaccharide biosynthesis in eukaryotes propose a stepwise assembly pathway on the lipid carrier dolichylpyrophosphate (1). In particular, four dolichylphosphomannose-derived mannose residues are added in the lumen of the ER to the lipid-linked $Man_5GlcNAc_2$ oligosaccharide. The first of these mannose residues is added via an α-1,3 linkage to lipid-linked $Man_5GlcNAc_2$ (37–39). Since $Man_6GlcNAc_2$ accumulates in the *Δalg9* strain and the *Δalg3* mutation is epistatic over the *Δalg9* deletion, our data provide additional evidence that the *alg3* mutation affects the first mannose addition in the lumen of the ER. The addition of the α-1,3 mannose is, therefore, a prerequisite for the *ALG9*-dependent addition of the next mannose residue. The next mannose residue can be added either by an α-1,2 or by an α-1,6 linkage. Whether Alg9p is involved in the addition of the α-1,2 or the α-1,6 mannose remains to be shown. Since Alg3p is a putative mannosyl transferase that catalyzes an α-1,3 linkage, this different catalytic activity might explain the lack of significant sequence similarity with Alg9p (20). It is important to note that the elimination of Dol-P-Glc synthase activity in a *Δalg9* strain (*Δalg9 Δalg5* double mutant) did not alter the accumulation of lipid-linked oligosaccharide or the glycosylation of CPY (data not shown). This result strongly suggests that glucosylation of the $Man_6GlcNAc_2$ oligosaccharide does not occur or only occurs with very low frequency, as it has been demonstrated for the $Man_5GlcNAc_2$ oligosaccharide (31, 32), and that $Man_6GlcNAc_2$ is transferred to protein in *Δalg9* strains. It is likely that the first glucose addition catalyzed by the Alg6 protein (7, 40) requires complete $Man_9GlcNAc_2$ oligosaccharide. Together with the observation that specific intermediates accumulate in different mutant strains affecting the biosynthesis of the branched lipid-linked oligosaccharide, our results provide further evidence that the addition of glycosyl residues in the ER lumen occurs in a defined, stepwise order.

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