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Biochemistry

New phenotype of mutations deficient in glucosylation of the lipid-linked oligosaccharide: Cloning of the *ALG8* locus

(*Saccharomyces cerevisiae*/endoplasmic reticulum/dolichol/glycosylation)

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ABSTRACT **$Glc_3Man_9GlcNAc_2$ is the preferred substrate of the oligosaccharyltransferase of N-linked glycosylation of proteins, but nonglucosylated oligosaccharides can be transferred to proteins in *Saccharomyces cerevisiae*. Mutations affecting the addition of the three terminal glucose residues lead to accumulation of $Man_9GlcNAc_2$ or $Glc_1Man_9GlcNAc_2$ *in vivo* but do not show any detectable growth defect. When these mutations were introduced into a strain with reduced oligosaccharyltransferase activity (due to the *wbp1-1* mutation), a severe growth defect was observed: accumulation of suboptimal lipid-linked oligosaccharide and reduced oligosaccharyltransferase activity resulted in a severe underglycosylation of secreted proteins. This new synthetic phenotype made it possible to isolate the *ALG8* locus, encoding a potential glucosyltransferase of the endoplasmic reticulum. The ALG8 protein is a 63.5-kDa hydrophobic protein that is not essential for the vegetative growth of yeast. However, the lack of this protein resulted in underglycosylation of secreted proteins.**

Asparagine-linked (N-linked) glycosylation of proteins follows a highly conserved pathway in eukaryotic cells. An oligosaccharide precursor is assembled on the lipid carrier dolichol phosphate and transferred to selected asparagine residues of nascent polypeptide chains (1, 2). The stepwise assembly of the lipid-bound oligosaccharide $Glc_3Man_9GlcNAc_2$ occurs at the membrane of the endoplasmic reticulum. The two GlcNAc and five mannose residues (derived from the nucleotide sugars UDP-GlcNAc and GDP-Man) are assembled at the cytoplasmic side, whereas the remaining four mannose residues and the three glucose residues are added in the lumen of the endoplasmic reticulum (3). These four mannose residues and the three glucose residues are transferred from dolichol phosphomannose and dolichol phosphoglucose, respectively (1, 4). The addition of the three glucose residues is the last modification of the lipid-linked oligosaccharide before the transfer to the polypeptide. Three different glucosyltransferases are involved in the glucosylation steps of the lipid-linked oligosaccharide (5). $Glc_3Man_9GlcNAc_2$ is the preferred substrate of the oligosaccharyltransferase. Removal of one, two, or all three glucose residues drastically reduces the transfer rate of the lipid-linked oligosaccharide to protein *in vitro* (4, 6–10).

In the yeast *Saccharomyces cerevisiae*, four different mutations affecting the addition of these glucose residues have been identified: the *alg5* and *dpg1* mutations both affect synthesis of the dolichol phosphoglucose (11, 12), whereas the *alg6* and *alg8* mutant strains accumulate lipid-linked $Man_9GlcNAc_2$ and $Glc_1Man_9GlcNAc_2$, respectively. Based on the accumulation of these biosynthetic intermediates and complementation analysis, it was concluded that the *ALG6* and *ALG8* loci both encode glucosyltransferases (11, 13).

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In contrast to some of the enzymes involved in the biosynthesis of the lipid-linked core oligosaccharide, the oligosaccharyltransferase is an essential enzyme for yeast cell viability. Two components of the yeast oligosaccharyltransferase, Wbp1p and Swp1p, are known (14, 15). Depletion of either protein results in underglycosylation of secreted proteins and growth arrest. Mutations in the *WBP1* locus can lead to reduced oligosaccharyltransferase activity and a temperature-sensitive phenotype (14).

In this study, we report that *alg5*, *alg6*, and *alg8* mutations result in a similar underglycosylation of proteins as a *wbp1* mutation. A combination of these different *alg* mutations with the *wbp1-1* allele caused a severe growth defect, strongly reduced N-linked glycosylation, and a new temperature-sensitive phenotype. This new synthetic phenotype allowed isolation of the *ALG8* locus and cloning of an endoplasmatic glucosyltransferase from eukaryotic cells.‡

MATERIALS AND METHODS

Yeast Strains and Media. The following strains of *S. cerevisiae* were used in this study: SS 328 (*MAT***a** *ade2-101 his3Δ200 ura3-52 lys2-801*) (16), MA7B (*MAT***a** *wbp1-1 ade2-101 his3Δ200 ura3-52 lys2-801*) (14), YG0022 (*MATα ade2-101 his3Δ200 ura3-52 wbp1-1 alg5-1*) (this study), YG0036 (*MATα ade2-101 ura3-52 his3Δ200 wbp1-1 alg6-1*) (this study), YG0100 (*MATα ade2-101 his3Δ200 ura3-52 lys2-801 wbp1-1 alg8-1*) (this study), PRY98 (*MATα ade2-101 ura3-52 alg5-1*) (P. W. Robbins, Massachusetts Institute of Technology), PRY103 (*MAT***a** *ade2-101 ura3-52 alg6-1*) (P. W. Robbins), PRY107 (*MATα ade2-101 ura3-52 alg8-1*) (P. W. Robbins), $M_3$35/2A (*MAT***a** *prp20-11 ade2-101 his3Δ200 tyr1 ura3-52*) (17). Standard yeast media and genetic techniques were used (18).

Isolation of the *ALG8* Locus. Chromosomal DNA was isolated from strain $M_3$35/2A (17) and partially digested with *Sau*3A. DNA fragments between 4 and 8 kb were fractionated by agarose gel electrophoresis and ligated into the vector YEp352 (19). After transformation and amplification in the *Escherichia coli* strain DH5α (20), the plasmid pool was transformed into strain YG0100 and uracil prototrophic transformants were selected at permissive temperature. Temperature-resistant colonies were screened by replica plating on selective minimal medium and incubation of the plates at 30°C and 37°C. Resistant transformants were colony purified, total DNA was extracted, and plasmid DNAs were recovered by transformation into *E. coli* strain DH5α and tested for the ability to transform strain YG0100 to temperature resistance

Abbreviation: CPY, carboxypeptidase Y.

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‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. X75929).

at 30°C or 37°C. Plasmid pALG8-1 was analyzed further and the *alg8-1* complementing fragment was sequenced.

Disruption of the *ALG8* Locus. The small *Eco*RI fragment of the original isolate pALG8-1 was removed, the resulting plasmid pALG8ΔEco was cleaved with *Kpn* I and *Bst*XI and blunted with Klenow polymerase, and the 1.8-kb *Bam*HI fragment of the *HIS3* locus (21) was inserted. Transcription of the *HIS3* locus in the resulting pALG8ΔEcoΔ(KpnBst)HIS3 occurred toward the former *Kpn* I site. This plasmid was cleaved with *Eco*RI and *Xba* I (see Fig. 3) and used to transform the diploid strain SS328 × SS330 to His^+. Transformants were analyzed for proper homologous integration of the Δ*alg8*::*HIS3* fragment by whole-cell PCR (22) using a *HIS3*- and an *ALG8*-specific primer.

Immunological Methods. Labeling of yeast cells and immunoprecipitation of carboxypeptidase Y (CPY) have been described (14, 23).

RESULTS

A New Phenotype of the *alg5-1*, *alg6-1*, and *alg8-1* Mutations. *alg5*, *alg6*, and *alg8* mutants are deficient in different steps of the glucosylation of the lipid-linked core oligosaccharide (11, 13, 24). They accumulate either lipid-linked $Man_9GlcNAc_2$ (*alg5* and *alg6*) or $Glc_1Man_9GlcNAc_2$ (*alg8*), a suboptimal substrate for the oligosaccharyltransferase (7, 9). Underglycosylated invertase is therefore observed in *alg5*, *alg6*, or *alg8* mutant strains (11, 13, 24) but no growth defect is associated with these mutations. To obtain such a selectable phenotype for the *alg5*, *alg6*, and *alg8* mutations, we constructed *alg wbp1-1* double mutants. We reasoned that combining the low activity of the oligosaccharyltransferase in *wbp1* mutants and the suboptimal oligosaccharyltransferase substrates (accumulating in *alg* cells) would result in the synthetic phenotype of severe underglycosylation of proteins. This underglycosylation might then lead to a reduced growth rate at different temperatures. Indeed, in all crosses between *alg5-1*, *alg6-1* or *alg8-1*, and *wbp1-1* strains, we observed segregants displaying a reduced growth rate at permissive temperature (23°C). This reduced growth rate was detected only in segregants carrying the *wbp1-1* allele (identified by the temperature-sensitive phenotype at 37°C). In addition, the slow-growing segregants did not grow on yeast extract/peptone/dextrose (YPD) agar plates at 30°C, a temperature permissive for both parent strains (Fig. 1). Slow-growing segregants were not observed in control crosses of either the *alg* or the *wbp1-1* strains with wild-type strains (data not shown). Genetic analysis was compatible with the conclusion that the slow-growth phenotype and the temperature sensitivity at 30°C were the result of the *alg wbp1-1* combination.

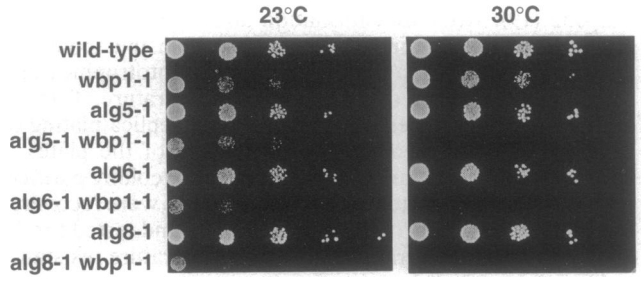

FIG. 1. Synthetic interaction of *alg* and *wbp1-1*. Individual strains (the relevant phenotype is given on the left side of each row) were grown in liquid YPD medium for 16 hr and diluted to the same cell density. Drops of 5 μl of 1:10 dilutions, starting with 2×10^6 cells per ml at the left side of the row, were placed on YPD agar plates and incubated at either 23°C for 4 days or 30°C for 3 days. A reduced growth rate is reflected by a reduced cell density at a given dilution.

To verify that enhanced underglycosylation of secreted proteins in the double mutants occurred, the processing of CPY (25) was analyzed in these strains. Mature CPY contains four N-linked oligosaccharides and has a molecular mass of 61 kDa (26). We have previously shown that a reduced oligosaccharyltransferase activity results in discrete glycoforms of CPY lacking up to all four oligosaccharide moieties (14). As reported previously, underglycosylated CPY was detected in *wbp1-1* cells (Fig. 2, lane 2) (14) and a similar, but less severe, underglycosylation was observed in *alg5-1*, *alg6-1*, or *alg8-1* cells (lanes 3, 5, and 7). CPY molecules lacking one oligosaccharide are the most abundant species found in these strains. In *alg wbp1-1* double mutants, a strong underglycosylation of CPY was observed (lanes 4, 6, and 8). CPY molecules lacking up to all four N-linked oligosaccharides were detected. CPY with only one or two oligosaccharides are formed predominantly in *alg5-1 wbp1-1* and *alg8-1 wbp1-1* cells, whereas CPY lacking three or all four sugars is found most abundantly in the *alg6-1 wbp1-1* strain. We conclude that the growth phenotype of *alg wbp1-1* double mutant strains at 23°C and 30°C was caused by the severe underglycosylation of secreted proteins.

Isolation of the *ALG8* Locus. The synthetic phenotype (temperature sensitivity at 30°C) of *alg wbp1-1* double mutants made it possible to isolate the corresponding wild-type *ALG* loci. As a first goal, we wanted to isolate the *ALG8* locus. For that purpose, an *alg8-1 wbp1-1* double mutant was transformed with a plasmid bank containing partially *Sau*3A cleaved yeast chromosomal DNA inserted into the *Bam*HI site of the vector YEp352 (19). Introduction of the *WBP1* locus into this strain resulted in the *alg8* phenotype (growth at 30°C and 37°C), and addition of the *ALG8* locus was expected to lead to the *wbp1* phenotype (growth at 30°C but not at 37°C), whereas the parent *alg8-1 wbp1-1* strain did not grow at 30°C or 37°C. Therefore 12,000 transformants were tested for growth at both 30°C and 37°C. Five transformants were found to grow at 37°C; they were believed to contain the *WBP1* locus and were not analyzed further. Three transformants were able to grow at 30°C but not at 37°C. The three different plasmids obtained from these transformants were recovered and amplified in *E. coli*. Upon transformation, all of them were able to restore the growth at 30°C of the parental *alg8-1 wbp1-1* strain. Analysis of the yeast DNA inserts in these plasmids revealed the presence of common sequences. The plasmid pALG8-1 carried the smallest (4.2 kb) insert and was analyzed further. Subcloning of different restriction fragments into the vector YEp352 and subsequent testing for the ability to restore growth of the *alg8-1 wbp1-1* strain at 30°C allowed us to locate the complementing activity on a

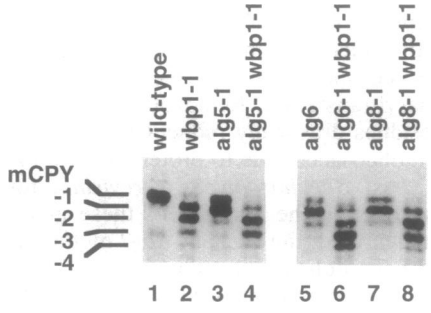

FIG. 2. *alg wbp1* double mutants underglycosylate CPY. The strains SS328 (wild type), MA7B (*wbp1-1*), PRY98 (*alg5-1*), YG0022 (*alg5-1 wbp1-1*), PRY103 (*alg6-1*), YG0036 (*alg6-1 wbp1-1*), PRY107 (*alg8-1*), and YG0100 (*alg8-1 wbp1-1*) were grown in minimal medium at 23°C and incubated for 1 hr in the presence of ^{35}S-labeled amino acids. Cells were lysed and used for CPY-specific immunoprecipitation. Positions of mature CPY (mCPY) and underglycosylated CPY lacking up to four N-linked oligosaccharides are indicated.

3.5-kb DNA fragment (Fig. 3*b*). Sequence analysis of this fragment revealed a single open reading frame that encodes a protein of 577 amino acids with a predicted molecular mass of 63.5 kDa (Fig. 3*a*). The hypothetical protein is very hydrophobic but contains a highly charged region of 50 amino acids at the N terminus (Fig. 3*c*). Two potential N-linked glycosylation sites were present in the primary sequence (Fig. 3*a*), but we have no direct evidence whether or not these sites are glycosylated in the native protein.

pALG8-1 Complements the Underglycosylation Defect of *alg8-1* Mutation. The isolation of pALG8-1 was based on the partial complementation of the growth defect of the *alg8-1 wbp1-1* double mutant. To verify that pALG8-1 complemented more than this synthetic growth defect, we tested whether or not the underglycosylation of proteins observed in *alg8-1* cells was also complemented. Therefore, immunoprecipitation of CPY was performed and analyzed by SDS/PAGE. The wild-type glycosylation of CPY was restored in *alg8-1* cells by introducing the pALG8-1 plasmid (Fig. 4, lane 9), but underglycosylation was not complemented by the overexpression of Wbp1p (lane 8). Vice versa, pALG8-1 did not restore glycosylation in a *wbp1-1* strain (lane 6). As expected, both the pALG8-1 and the *WBP1*-containing plasmids only partially restored the underglycosylation of CPY in an *alg8-1 wbp1-1* strain (lanes 2 and 3). In addition, pALG8-1 was not able to restore underglycosylation of CPY observed in *alg5-1* or *alg6-1* strains (data not shown). We conclude that the pALG8-1 plasmid was able to specifically complement the *alg8-1* mutation.

Deletion of the *ALG8* Locus. The phenotype of the *alg8-1* mutant indicated that this gene might not be essential for the vegetative growth of yeast. To confirm this hypothesis, the *Kpn* I/*Bst*XI fragment of the *ALG8* locus (Fig. 3*b*) was replaced by a *HIS3*-containing DNA fragment and used to exchange one wild-type copy of the putative *ALG8* locus in a diploid strain. Correct integration of the *HIS3*-tagged Δ*ALG8* into the *ALG8* locus was confirmed by PCR analysis (data not shown). Upon sporulation and tetrad dissection, all

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-540  GTTGCGGATTACCAGCCACCGACAAACCAGGGTCTTTCCATTCTGGCGCACTATCCAAGTCTATCTTGTCCGCAAATGAAGCTGTGGTCT
-450  CTAAAAGCTCATTGTCTTTTCTTTCTTCATTTGTAGATATTTGACCAATTGATTCAATTGAATTTGAAACGAGGCTCTGAGTTTTATCTC
-360  CCTCATGATTTAGTTCGTCCCAAACAGGTGCTTCTAGATTATCCTCGTAGTCCATGGCTTGTTATTGCAGGATGTATGAAAGTTTATAAA
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-180  TAAAATTTACTGTTATCACTTAGTTGTTCTTAGCGGACAACTTCCGAAAAGTGAAATTTCATTTTTCAAAACGACAATAGTGGTATAGAA
 -90  TTATAGAATAACATACGTTAAAAATAGCTGAAGTTCATTACAGTTCTGTAGTGGTCAGCAAGGCATTTGACGTCTATTGGATAGCCTACG
  +1  ATGAAAGGTGATCGTTCGAGGCAAAATATGGCTGTGACAAAGAAGGCAAAGTTAAAAAAAAATGACGAGCCAAAAAAGGTTTTGAAGACT
   1  M K G D R S R Q N M A V T K K A K L K K N D E P K K V L K T
 +91  GCCGCGACCGAAAAAGGTGAGGGATCCAAGCGCTACTCCTTATGGAATTTCTGGATAAGCACATTGTTTTTAAAATTACTATTGATTCCT
  31  A A T E K G E G S K R Y S L W N F W I S T L F L K L L L I P
+181  GACTACTTCAGTACAGATTTTGACGTTCATAGAAATTGGCTGGCAATCACCAATAAATTGCCCATCAGCGAATGGTATTATGAGCACACT
  61  D Y F S T D F D V H R N W L A I T N K L P I S E W Y Y E H T
+271  AGTCAATGGACATTGGATTATCCGCCATTTTTTGCTTATTTTGAATGGTTTTTATCGCAATTTGTTCCTAAATCTGTACGTGATGACGGT
  91  S Q W T L D Y P P F F A Y F E W F L S Q F V P K S V R D D G
+361  TGTTTGGATATAGTGGAGATCGGAAAGTTCGGTTTGCCAACAATCGTTTTCCAACGACTCACTGTCATCTTCAGCGAAATTCTTCTATTT
 121  C L D I V E I G K F G L P T I V F Q R L T V I F S E I L L F
+451  GTCATCCTTCAAATATACATTAATACTACAAAGCTAAGTGAAAGATCTCAAAGTTTTGTGGTTGCTTCTAGCATTGTACTATCACCCGGG
 151  V I L Q I Y I N T T K L S E R S Q S F V V A S S I V L S P G
+541  TTTTTAATAATAGATCACATTCACTTCCAATATAATGGTTTTCTTTTTGCGATTCTAATAGGCTCCATTGTCGCCGCCAAGAATAAGAGA
 181  F L I I D H I H F Q Y N G F L F A I L I G S I V A A K N K R
+631  TACATCTTGTGCGCTGTTCTGTATACTACAGCCATATGTTTCAAGCATATATTTTTATATTTGGCGCCATGTTATTTCGTGTTTTTATTG
 211  Y I L C A V L Y T T A I C F K H I F L Y L A P C ▸ Y F V F L L
+721  AGAGCATACGTTCTCAATGTCAACAATTTCAAGTTTAAAAGTTACAAAGATTTTTTGTTCCTGATAAGATGGGCAAATCTTTTGAAGTTG
 241  R A Y V L N V N N F K F K S Y K D F L F L I R W A N L L K L
+811  GCTACCGTTGTTGTTGGAATTTTCACTATTTGTTTCCTTCCATTTGCTCATCAAATGCCGCAAGTGTTGAGCAGGTTATTCCCATTCTCA
 271  A T V V V G I F T I C F L P F A H Q M P Q V L S R L F P F S
+901  AGAGGACTGACGCATGCATATTGGGCACCAAATTTCTGGGCGTTGTATTCTTTTATGGATAAAATACTCACTACGGTAATGTTGAAATTA
 301  R G L T H A Y W A P N F W A L Y S F M D K I L T T V M L K L
+991  CCGTACGTCCACACTTTTGCTACAAAATTTATTAAACCTCCTCTGATCCCTCAGAATATCAAGGAAATTAATGAAAGGCTCGCGGCAAAC
 331  P Y V H T F A T K F I K P P L I P Q N I K E I N E R L A A N
+1081 AACAATGGAAGTAAAGGTCTAGTTCAAGACGTATTTTTTGTTATTCTCCCTCAAATTCCTCCAAAATTAACGTTTATTTTGACGATATTT
 361  N N G S K G L V Q D V F F V I L P Q I P P K L T F I L T I F
+1171 TACCAGGTCTTAGCAGTACTTCCACTTTGTTTGATCCATCGTTCAAAAGATTTGTTGGCTCGTTGACTCTCTGTGGGTTGGCATCGTTT
 391  Y Q V L A V L P L L F D P S F K R F V G S L T L C G L A S F
+1261 TTGTTCGGGTGGCATGTGCATGAGAAAGCTATAATGCTAGTCATCATTCCGTTCACATTTCTTGTTGGATTTGATCGTCGTCTTTTGGTA
 421  L F G W H V H E K A I M L V I I P F T F L V G F D R R L L V
+1351 CCGTTTATGTTAGTTGCCTCAGCTGGTTATGTTTCCTTGTATCCTCTTCTGTACAAAGGCCAAGATTTTTTCATTAAAACATTATACACT
 451  P F M L V A S A G Y V S L Y P L L Y K G Q D F F I K T L Y T
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 481  Y V W C I I Y F A A F R K T T K I S S S V E R R I F F L D R
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 511  L A L T Y I F S L L P I V T V L Q I L D E V K W R Y S F L Q
+1621 AAATTTGAATTCTTAGGATTAATGATATATAGTGTCTATTGTTCATTAGGTATAATCAGCTCGTGGTTTGCCTTATCGTGGTTATATAAT
 541  K F E F L G L M I Y S V Y C S L G I I S S W F A L S W L Y N
+1711 TTTGACGAATTACTATGGCAGTGATACAATATCGCTTCCCTAGGACGTTAAAAAAAAAGTGATATAAAATTATTTATATATGAATCGATT
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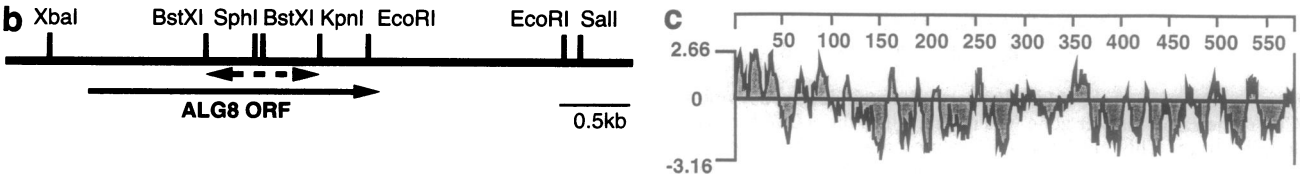

FIG. 3. The *ALG8* locus. (*a*) DNA sequence of the *ALG8* locus. Open reading frame and derived Alg8p amino acid sequence are also shown. Potential N-linked glycosylation sites are underlined. (*b*) Partial restriction map of the *ALG8* locus is shown. Location of the ALG8 open reading frame (solid arrow) and the DNA fragment deleted to produce the Δ*alg8*:*HIS3* strain (dashed arrow) are indicated. (*c*) Hydropathy blot of Alg8p according to Kyte and Doolittle (27).

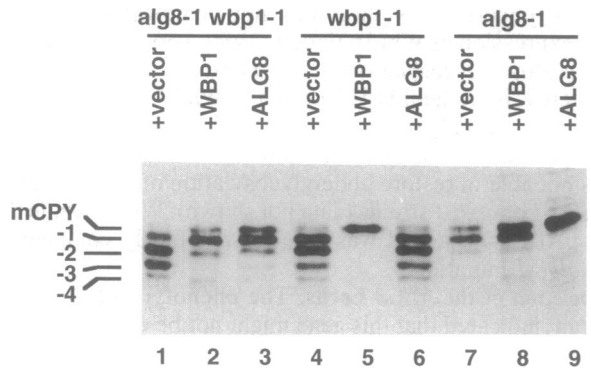

FIG. 4. Complementation of CPY underglycosylation in *alg8-1* strains by pALG8-1. Strains YG0100 (*alg8-1 wbp1-1*), MA7B (*wbp1-1*), and PRY107 (*alg8-1*) were transformed with the plasmids YEp352 (+vector), YEp352/WBP1 (+WBP1), or pALG8-1 (+ALG8). These transformed strains were grown in selective medium at permissive conditions, labeled for 1 hr with ^{35}S-labeled amino acids, and lysed. CPY-specific immunoprecipitation was performed. Positions of mature CPY (mCPY) and underglycosylated CPY lacking up to four N-linked oligosaccharides are indicated.

four products of a given tetrad were able to form colonies. Segregants lacking an intact copy of the *ALG8* locus (marked by the His$^+$ phenotype) did not have any detectable growth defect. To confirm that a deletion in the *ALG8* locus led to the same phenotype as the *alg8-1* mutation, the glycosylation of CPY was analyzed (Fig. 5). Immunoprecipitation of CPY from all segregants of a complete tetrad was performed. Inactivation of the *ALG8* locus (marked by the *HIS3* gene) resulted in the same underglycosylation of CPY as observed in the *alg8-1* strain.

pALG8-1 Encodes the *ALG8* Locus. Although pALG8-1 complements the underglycosylation of CPY in an *alg8-1* strain and disruption of the putative *ALG8* open reading frame results in the same phenotype as the *alg8-1* mutation, we required definitive proof that the *ALG8* locus had been isolated. To that end, we wanted to demonstrate that *alg8-1* and the artificially generated *HIS3*-tagged Δ*ALG8* were alleles of the same gene. The *alg8-1 wbp1-1* strain YG0022 was therefore crossed with the Δ*alg8*::*HIS3* strain YG0098 and sporulated, and resulting tetrads were analyzed. If *alg8-1* and Δ*alg8*::*HIS3* are alleles of the same gene, two segregants of each tetrad were expected to be *alg8 wbp1-1* double mutants. If the *alg8-1* and Δ*algHIS3* mutations affect different loci,

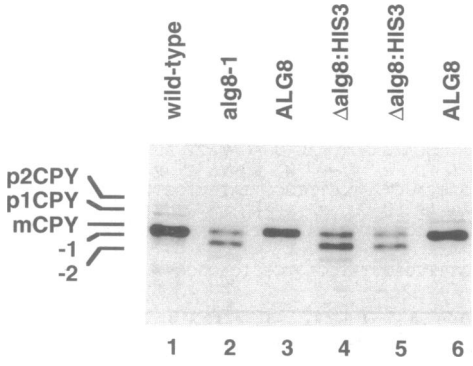

FIG. 5. Deletion of the *ALG8* locus results in underglycosylation of CPY. The *HIS3* marker gene was inserted into the *ALG8* locus in a diploid strain. This strain was sporulated and tetrads were dissected. One tetrad was used for CPY-specific immunoprecipitation. Wild-type (lane 1) and *alg8-1* (lane 2) cells were used as controls. The relevant genotype of the four segregants from one tetrad analyzed in lanes 3–6 is indicated. Positions of mature CPY (mCPY) and underglycosylated CPY lacking one or two N-linked oligosaccharides (−1, −2) and of the p1 and p2 processing forms of CPY are indicated.

wbp1-1 single mutants able to grow at 30°C would be expected as resulting segregants from such a cross. We observed that all of the 27 tetrads tested contained 2 segregants not able to grow at 30°C (*alg8 wbp1-1* double mutants) and no *wbp1* single mutant was obtained. In addition, both the *alg8-1* and the Δ*alg8*::*HIS3* allele were found in combination with *wbp1-1*. This genetic experiment adds the final proof that we have isolated the *ALG8* locus.

DISCUSSION

The three terminal glucose residues of the lipid-linked core oligosaccharide have been shown to be important determinants for substrate affinity to the oligosaccharyltransferase *in vitro* (4, 6–10). *In vivo*, the three yeast mutants accumulating $Man_9GlcNAc_2$ (*alg5*, *alg6*, and *dpg1*) and *alg8* (accumulating $Glc_1Man_9GlcNAc_2$) were found to underglycosylate secreted invertase. Nevertheless, the underglucosylated lipid-linked oligosaccharide was transferred to protein (11, 13). We also detected an underglycosylation of CPY in *alg5*, *alg6*, and *alg8* strains. The general underglycosylation of proteins does not result in a detectable growth defect. On the contrary, mutations in the oligosaccharyltransferase component Wbp1p lead to a more severe underglycosylation of secreted proteins. We have postulated that this underglycosylation results in a temperature-sensitive phenotype (14). In support of this hypothesis, the combination of *alg* and the *wbp1* mutations leads to an even more reduced glycosylation of secreted proteins (visualized by the underglycosylation of CPY) and a more pronounced temperature sensitivity (Fig. 2). This synthetic phenotype of *alg wbp1-1* double mutants adds further support to the notion that the three glucose residues might be important structures for substrate recognition by the oligosaccharyltransferase. However, in *alg3* strains, nonglucosylated $Man_5GlcNAc_2$ seems to be transferred efficiently to protein, indicating a relaxed specificity of the oligosaccharyltransferase (28, 29).

As well as demonstrating the important function of the three glucose residues in the transfer of the lipid-linked core oligosaccharide to polypeptides, the synthetic phenotype (temperature sensitive at 30°C) of *alg wbp1-1* double mutants opened the possibility to isolate the corresponding *ALG* genes. Because of the accumulation of the lipid-linked $Glc_1Man_9GlcNAc_2$, it was postulated that the *ALG8* locus encodes a glucosyltransferase (13). Current models of the biosynthesis of the lipid-linked core oligosaccharide propose that this enzyme is localized at the membrane of the endoplasmic reticulum (2, 3). The hydrophobic nature of Alg8p makes it likely that this protein is localized in the membrane. Besides a hydrophilic N-terminal domain, two long hydrophobic domains separated by a more hydrophilic region are predicted from the primary amino acid sequence of Alg8p (Fig. 3*c*). A potential dolichol recognition sequence located in transmembrane domains of several dolichol-utilizing enzymes has been proposed (30). However, in the *ALG8* glucosyltransferase sequence no region with a high homology to the potential dolichol recognition sequence F(I/V)X(F/Y)XXIPFX(F/Y) (30, 31) was detected. Direct evidence for the involvement of this sequence in the recognition or binding of dolichol is not available, but mutational analysis of this potential dolichol recognition sequence in hamster GlcNAc-1-phosphate transferase revealed that this sequence is essential for enzymatic activity (32). In contrast, deletions of the potential dolichol binding sequence in the yeast enzyme dolichyl-phosphate-mannose synthase (Dpm1p) lead to reduced enzymatic activity. However, this activity was sufficient to support growth of yeast (33).

The observation that mutations affecting the biosynthetic pathway of the lipid-linked core oligosaccharide in combination with a *wbp1* mutation result in a synthetic phenotype will make it possible to isolate other *ALG* loci defined by existing

mutations and lead the way to the isolation of mutants defective in the biosynthesis of the lipid-linked core oligosaccharide. The cloning of the genes encoding glucosyltransferases (*ALG6* or *ALG8*) or the dolichyl-phosphate-glucose synthase (*ALG5*) will also allow us to address the function of the three terminal glucose residues in the pathway of N-linked glycosylation of proteins.

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