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Cloning and characterization of the *ALG3* gene of *Saccharomyces cerevisiae*

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The *Saccharomyces cerevisiae alg3–1* mutant is described as defective in the biosynthesis of dolichol-linked oligosaccharides (Huffaker and Robbins, *Proc. Natl. Acad. Sci. USA*, 80, 7466–7470, 1983). $Man_5GlcNAc_2$-PP-Dol accumulates in *alg3* cells and Endo H resistant carbohydrates are transferred to protein by the oligosaccharyltransferase complex. In this study, we describe the cloning of the *ALG3* locus by complementation of the temperature sensitive growth defect of the *alg3 stt3* double mutant. The isolated *ALG3* gene complements both the defect in the biosynthesis of lipid-linked oligosaccharides of the *alg3*-mutant and the underglycosylation of secretory proteins. The inactivation of the nonessential *ALG3* gene results in the accumulation of lipid-linked $Man_5GlcNAc_2$ and protein-bound carbohydrates which are completely Endo H resistant. The *ALG3* locus encodes a potential ER-transmembrane protein of 458 amino acids (53 kDa) with a C-terminal KKXX-retrieval sequence.

Key words: lipid-linked oligosaccharide/N-glycosylation/synthetic lethality

Introduction

N-Linked glycosylation is an essential process of protein modification in all eukaryotic cells (Kornfeld and Kornfeld, 1985; Tanner and Lehle, 1987; Herscovics and Orlean, 1993). The oligosaccharide $Glc_3Man_9GlcNAc_2$ is assembled on the lipid carrier dolicholpyrophosphate and transferred *en bloc* by the oligosaccharyltransferase complex onto selected asparagine residues of secretory proteins (Hubbard and Ivatt, 1981). Work with CHO cells established that two N-acetylglucosamine and five mannose residues are assembled in a step-wise fashion (Chapman *et al.*, 1979) on the cytoplasmic side of the membrane of the endoplasmic reticulum (Snider and Rogers, 1984). The substrates for these reactions are cytoplasmic UDP-GlcNAc and GDP-Man, respectively. The resulting $Man_5GlcNAc_2$-PP-Dol is then translocated to the lumenal side of the ER membrane, where four mannose residues and three glucose residues are added from Dol-P-Man and Dol-P-Glc, respectively (Chapman *et al.*, 1980; Staneloni *et al.*, 1980; Snider and Rogers, 1984). In the yeast *Saccharomyces cerevisiae*, *alg*-mutants (*alg*, asparagine-linked glycosylation) defective in the biosynthesis of the dolichol-linked $Glc_3Man_9GlcNAc_2$ have been isolated (Huffaker and Robbins, 1981, 1983; Runge *et al.*, 1984; Runge and Robbins, 1986). In the *alg3–1* mutant $Man_5GlcNAc_2$-PP-Dol accumulates (Huffaker and Robbins, 1983) and Endo H resistant saccharides are transferred to protein. The *alg3–1* mutation does not result in a complete block of the biosynthesis of lipid-linked oligosaccharides, because partial synthesis of $Glc_3Man_9GlcNAc_2$ -PP-Dol occurs (Verostek *et al.*, 1993a,b). This 'leaky'-phenotype has hindered the study of the *ALG3*-function, and *alg3–1* mutant cells do not have a known selectable phenotype (Huffaker and Robbins, 1983), which prevented the identification of the *ALG3* locus. The *stt3–3* mutation (Zufferey *et al.*, 1995) affects the activity of the oligosaccharyltransferase and produces a synthetic lethal phenotype in combination with mutations that alter lipid-linked oligosaccharide biosynthesis, such as *alg5*, *alg6* (Runge *et al.*, 1984), and *alg8* (Runge and Robbins, 1986). We concluded that the *stt3–3* mutation alters the affinity of the oligosaccharyltransferase towards incomplete lipid-linked oligosaccharide substrates (Zufferey *et al.*, 1995). *stt3–3* in combination with *alg3–1* results in a temperature-sensitive phenotype (Zufferey *et al.*, 1995). In this work, we used this double mutant to clone the *ALG3* gene by complementation of this temperature-sensitive phenotype. We describe the *ALG3* gene, encoding a putative mannosyltransferase adding the first Dol-P-Man derived mannose in an α1,3 linkage to $Man_5GlcNAc_2$-PP-Dol (Verostek *et al.*, 1991).

Results

Cloning of the ALG3 gene

To clone the *ALG3* gene, an *alg3–1 stt3–3* strain was generated by standard genetic techniques. In contrast to the two single mutants, the double mutant *alg3–1 stt3–3* shows a pronounced underglycosylation of secretory proteins and is temperature sensitive at 37°C (Zufferey *et al.*, 1995). We reasoned that this glycosylation defect produced the temperature sensitive phenotype which would allow the isolation of the *ALG3* locus. Both the wild type *ALG3* and *STT3* genes should complement the temperature sensitive phenotype of the *alg3–1 stt3–3* double mutant. A genomic library of yeast DNA was electroporated into *alg3–1 stt3–3* cells and plasmids were isolated as described previously for the *ALG8* gene (Stagljar *et al.*, 1994). As expected, the *STT3* gene was reisolated (data not shown). Two plasmids, pALG3–1, pALG3–2 contained overlapping DNA-inserts as shown by restriction mapping and sequence analysis. A search of the EMBL sequence database with sequences obtained from the end of each plasmid insert revealed that pALG3–1 and pALG3–2 covered a region of chromosome II (Cusick, 1994; Obermaier *et al.*, 1995) (Figure 1A). One open reading frame was a candidate for the *ALG3* locus because it was positioned in the region of overlap of pALG3–1 and pALG3–2 (Figure 1A).

Properties of the ALG3 locus

ALG3 encodes a hydrophobic protein of 458 amino acids (53.4 kDa) (Figure 1B,C). There are only few hydrophilic parts in the Alg3p sequence. No obvious cleavable leader sequence was

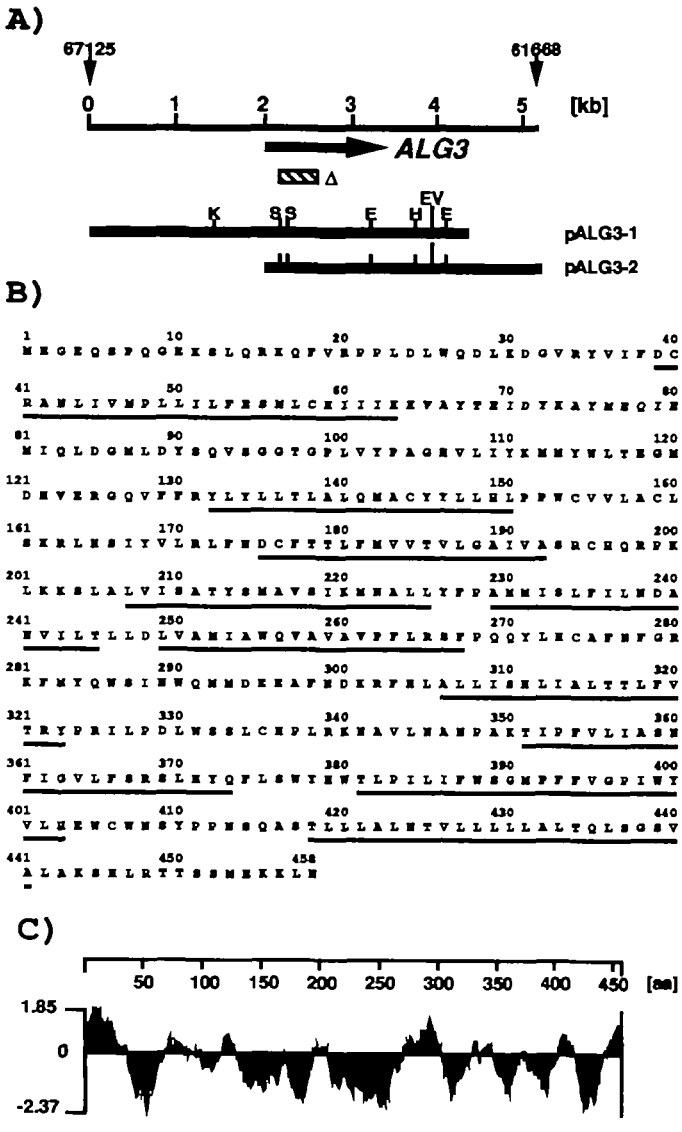

Fig. 1. (**A**) Localization of the genomic clones pALG3–1 and pALG3–2 to the sequence of chromosome II. Shown are the experimentally determined restriction sites (K, *Kpn*I; S, *Sph*I, E, *Eco*RI; EV, *Eco*RV; H, *Hind*III). Both plasmid inserts cover the region from 61,668 to 67,125 (numbering according to Obermaier *et al.*, 1995). The *ALG3* ORF is identical to the ORF YBL0720. The hatched bar indicates the region of *ALG3* replaced by the *HIS3* gene in the Δ*alg3* strain YG247. (**B**) Deduced amino acid sequence of the Alg3p. Underlined are the putative transmembrane helices determined by the method of Rost *et al.* (1995). (**C**) Hydropathy profile according to Kyte and Doolittle (1982), using a window of 19 amino acids.

present in the N-terminus (von Heijne, 1986) and the analysis of putative transmembrane domains revealed 10 potential transmembrane helices (Kyte and Doolittle, 1982; Rost *et al.*, 1995). This suggests that Alg3p is a transmembrane protein with both N- and C-termini located in the cytoplasm. In agreement with the predicted location of Alg3p to the endoplasmic reticulum, the C-terminus contains the sequence KKLN. The sequence KKXX (X = any amino acid) has been shown to retrieve transmembrane proteins to the endoplasmic reticulum in mammalian cells as well as in yeast, when present at the C-terminus (Jackson *et al.*, 1990; Shin *et al.*, 1991; Gaynor *et al.*, 1994). Alg3p contains no potential N-glycosylation sites.

A search using the Alg3p sequence with the NCBI BLAST program (Altschul *et al.*, 1990) for homologous proteins revealed that the *l(2)not+* protein (EMBL accession no. X77820) from *Drosophila melanogaster* (U.Kurzig-Dumke, M.Kaymer, and D. Gundacker, in preparation) shares 34% amino acid identity with Alg3p (Cusick, 1994). In agreement with a functional homology, the ER-retrieval sequence at the C-terminus of Alg3p is also present in the *l(2)not+* amino acid sequence. Finally, the Alg3p sequence between residues 262 and 315 is 42% identical to the amino acid sequence encoded by two human cDNAs (Genbank R23327 and R76440) (Hillier *et al.*, 1995, unpublished).

Analysis of lipid-linked oligosaccharides

The *alg3–1* mutant has a defect in the biosynthesis of lipid-linked oligosaccharides (Huffaker and Robbins, 1983) because it accumulates lipid-linked $Man_5GlcNAc_2$. To test whether the isolated pALG3–1 can complement this defect, *alg3–1* cells carrying pALG3–1 were incubated with 2-[^{3}H]-mannose. Lipid-linked oligosaccharides were subsequently extracted. The oligosaccharides were released from the lipid by acid hydrolysis and analysed by HPLC (Figure 2). Wild-type cells synthesized lipid-linked $Glc_3Man_9GlcNAc_2$, whereas *alg3–1* mutant cells accumulated $Man_5GlcNAc_2$-PP-Dol (Figure 2, left panel). Transformation with pALG3–1 restored the ability of the cells to synthesize full length lipid-linked oligosaccharide (Figure 2, right panel). Thus, pALG3–1 complemented the *alg3–1* specific defect in the biosynthesis of dolichol-bound oligosaccharides. To test whether inactivation of the identified open reading frame results in the same phenotype as the *alg3–1* mutation, we generated haploid cells with a deletion in the *ALG3* locus. This deletion did not lead to a detectable growth phenotype under all conditions tested (data not shown). Lipid-linked oligosaccharides were analysed from Δ*alg3* cells carrying either the vector DNA or pALG3–1 (Figure 2). The disruption of the *ALG3* gene resulted in the same phenotype as the *alg3–1* mutation, that is, accumulation of $Man_5GlcNAc_2$-PP-Dol. As expected, transformation of Δ*alg3* cells with pALG3–1 restored the ability to synthesize $Glc_3Man_9GlcNAc_2$-PP-Dol (Figure 2).

Analysis of N-glycosylation of secretory proteins

The *alg3–1* mutation leads to an underglycosylation of secretory proteins (Huffaker and Robbins, 1983; Verostek *et al.*, 1993a,b; Zufferey *et al.*, 1995). Due to the leaky phenotype of the *alg3–1* mutation, complete $Glc_3Man_9GlcNAc_2$ is transferred to protein apart from incomplete $Man_5GlcNAc_2$ or $Glc_3Man_5GlcNAc_2$ (Verostek *et al.*, 1993a,b). Man_5-oligosaccharides can be distinguished from Man_9-oligosaccharides by their resistance to Endo-β-N-acetylglucosaminidase H (Endo H). The former is resistant to Endo H; the latter is sensitive to cleavage by Endo H (Huffaker and Robbins, 1983; Verostek *et al.*, 1991, 1993a,b; Zufferey *et al.*, 1995). Complementation of the *alg3–1* mutant phenotype by pALG3–1 should restore full glycosylation and Endo H sensitivity of sugars present on secretory proteins. To test this hypothesis, proteins were extracted from wild type (*wt*), *alg3–1* and Δ*alg3* cells, carrying either the vector plasmid or pALG3–1. Extracts were incubated with Endo H and analyzed by Western blotting using antibodies directed against Carboxypeptidase Y (CPY), Wbp1p and Gas1p (Figure 3). CPY is a vacuolar protein acquiring four N-linked sugars (Hasilik and Tanner, 1978), Wbp1p is a membrane-bound subunit of the oligosaccharyltransferase complex carrying two N-linked sug-

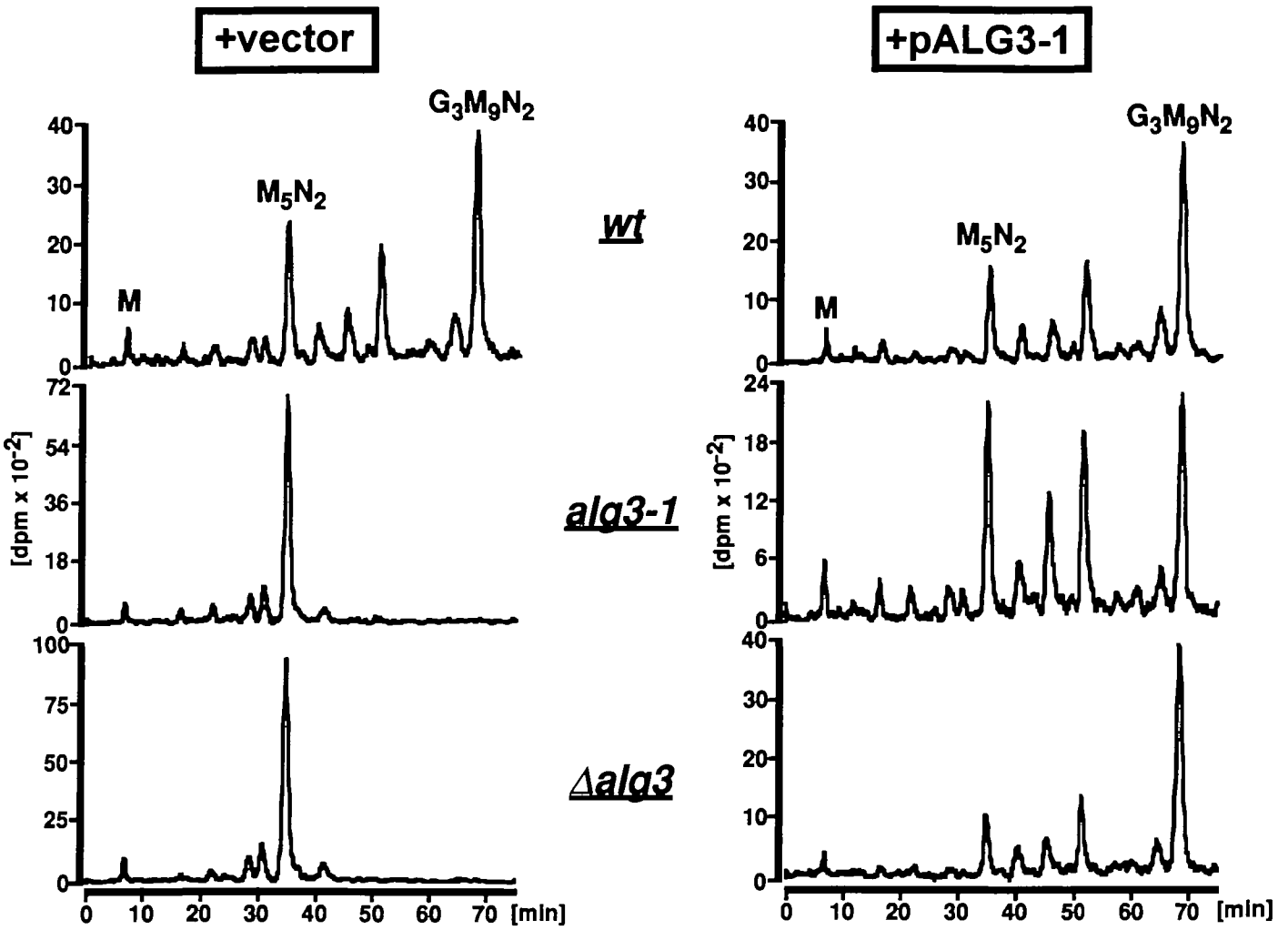

Fig. 2. Analysis of lipid-linked oligosaccharides from wild type (wt), *alg3–1* and Δ*alg3* cells carrying either the vector YEp325 (left panel) or pALG3–1 (right panel). Cells were labelled with 2-[^{3}H]-mannose, and lipid-linked oligosaccharides were extracted, hydrolyzed, and analyzed by HPLC using a Supelco LC-NH2 column as described previously (Zufferey *et al.*, 1995) with a water/acetonitrile gradient going from 30% to 50% water in 75 min. The flow rate was 1 ml/min, and the detection of radioactivity was performed with a FLOW-ONE detector (Packard, Meriden). M, Mannose; M_5N_2, $Man_5GlcNAc_2$; $G_3M_9N_2$, $Glc_3Man_9GlcNAc_2$.

ars (te Heesen *et al.*, 1992; Gaynor *et al.*, 1994) and Gas1p is a GPI-anchored protein which acquires approximately twelve N-linked chains as well as O-linked carbohydrates (Fankhauser and Conzelmann, 1991). In wild type cells, these proteins are fully glycosylated and the sugars were sensitive towards Endo H digestion (Figure 3, lanes 1,3 versus 2,4). In *alg3–1* and Δ*alg3* cells underglycosylation of proteins and Endo H resistant glycoforms were visible (Figure 3, lanes 5,9 versus lanes 6,10). In the case of *alg3–1*, some glycoforms, but not all of them, are sensitive to cleavage by Endo H (see CPY and Wbp1p in lanes 5,6), whereas in Δ*alg3* cells all glycoforms appear to be resistant (lanes 9,10). This confirmed the leaky phenotype of the *alg3–1* mutation (Huffaker and Robbins, 1983; Verostek *et al.*, 1993a,b; Zufferey *et al.*, 1995). The presence of pALG3–1 fully restored glycosylation and rendered all oligosaccharides sensitive towards Endo H digestion (Figure 3, lanes 7,8 and 11,12). We conclude that pALG3–1 complemented the *alg3–1* defect. The biosynthesis of the GPI-anchored protein Gas1p does not appear to be perturbed (Fankhauser and Conzelmann, 1991). Finally, we were interested in the nature of the *alg3–1* mutation. Sequencing of the *alg3–1* allele revealed a C to T missense mutation in codon 275 resulting in a change of an alanine to a valine.

Discussion

We have isolated the *ALG3* gene by complementation of the temperature-sensitive phenotype at 37°C of an *alg3–1 stt3–3* double mutant. The identity of the gene defined by the *alg3–1* mutation and the isolated locus is provided by three lines of evidence. First, the isolated plasmid pALG3–1 complemented the defect of the *alg3–1* mutant in the biosynthesis of lipid-linked oligosaccharides. Second, disruption of the isolated *ALG3* gene led to a block of lipid-linked oligosaccharide biosynthesis comparable with that of *alg3–1* (Figure 2). Furthermore, the inactivation of the *ALG3* gene function resulted in an apparently complete block of lipid-linked oligosaccharide biosynthesis as shown by strong underglycosylation and fully Endo H resistant secretory proteins (Figure 3). Finally, sequencing of the mutated *alg3–1* locus revealed an alteration of the open reading frame, changing alanine at position 275 to a valine. This relatively mild mutation would be in agreement

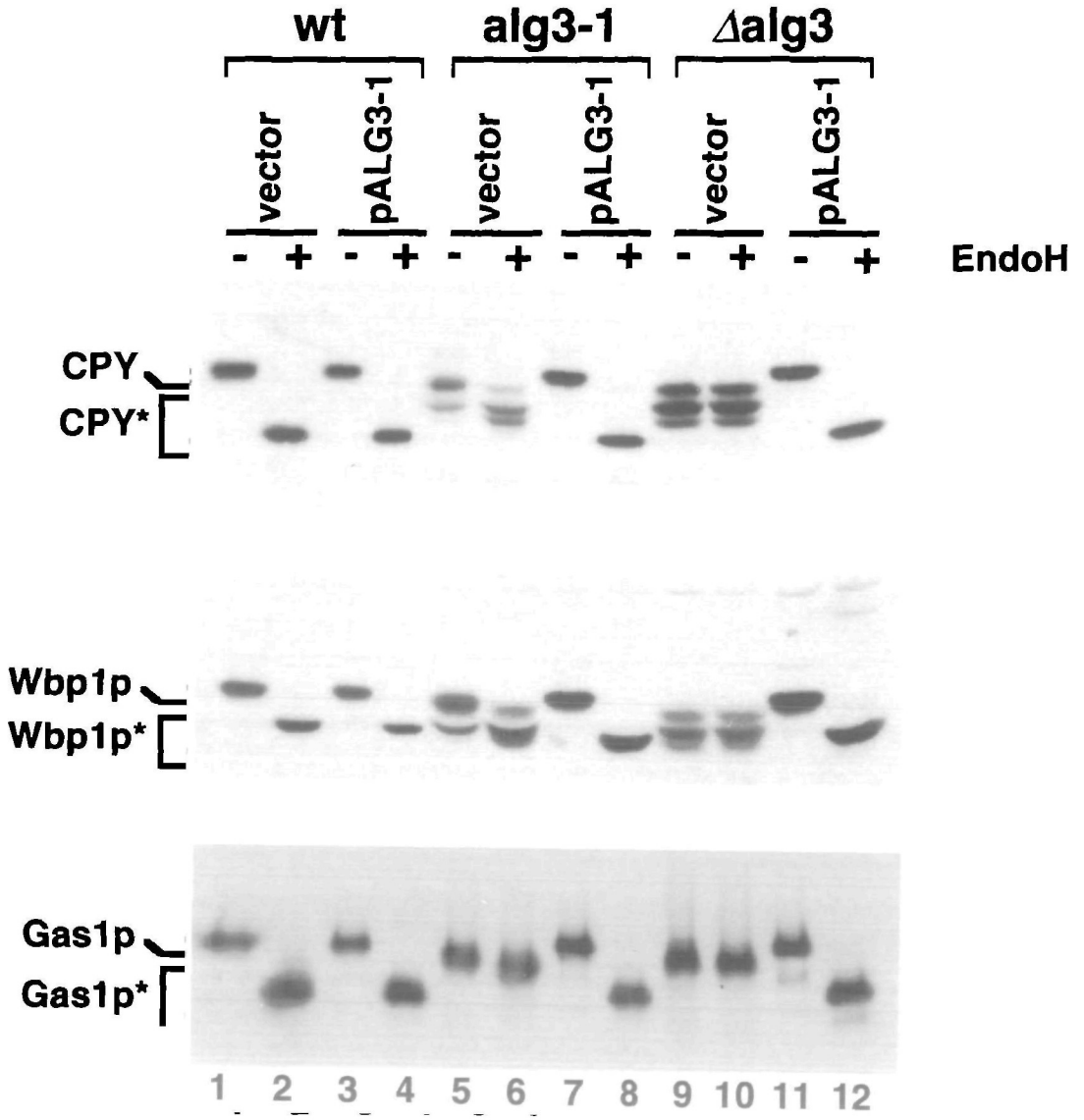

Fig. 3. Western blot analysis of secreted glycoproteins CPY, Wbp1p, and the GPI-achored protein Gas1p from either wild type cells (lanes 1–4), *alg3–1* mutant cells (lanes 5–8) or cells carrying a deletion in the *ALG3* open reading frame (Δ*alg3*) (lanes 9–12). CPY, Wbp1p, Gas1p: carry wild type N-glycosylation, CPY*, Wbp1p*, Gas1p*, underglycosylated forms. To determine the degree of N-glycosylation and resistance to Endo H, protein samples were digested with Endo H. Mutations in the *ALG3* gene led to underglycosylation and Endo H resistance of protein-bound carbohydrates These defects are complemented by the pALG3–1 plasmid, but not by the vector YEp352.

with the partial inactivation of the *ALG3* gene function observed in the *alg3–1* allele. The *ALG3* gene encodes a polypeptide of 458 amino acids, with a predicted molecular weight of 53 kDa. The phenotype of the *alg3–1* mutation, that is, accumulation of $Man_5GlcNAc_2$-PP-Dol is not completely diagnostic of a mannosyltransferase defect. A defect in the dolichol synthesis as in the CHO cell line Lec9 (polyprenol reductase) (Rosenwald and Krag, 1990; Rosenwald *et al.*, 1993) or in Dol-P-Man synthase would lead to a similar phenotype. Such mutation would, however, also affect two other Man-P-Dol requiring pathways, GPI-anchoring and O-glycosylation as has been shown for the yeast *dpm 1*-mutation (Orlean, 1990). Since both GPI-anchoring and O-linked glycosylation are essential processes (Hamburger *et al.*, 1995; Immervoll *et al.*, 1995) and the disruption of the *ALG3* gene does not lead to a growth defect, we conclude that Alg3p is not involved in these processes. This conclusion is supported by the finding of Conzelmann *et al.* (1991) that labeling of GPI-proteins with ^{3}H-myo-inositol is unaffected in *alg3–1* cells. In addition, O-mannosylation is not blocked in *alg3* cells (Orlean, 1994). The CHO mutant cell line Lec35 has similar phenotypes as the *alg3–1* mutation, in that it accumulates $Man_5GlcNAc_2$-PP-Dol and transfers Endo H resistant carbohydrates to protein (Lehrman and Zeng, 1989; Zeng and Lehrman, 1990). However, Lec35 cells are also defective in the biosynthesis of GPI-anchor (Camp *et al.*, 1993), arguing against Lec35 as a potential homologue of *alg3*. All data available so far places the function of Alg3p at a late step in the biosynthesis of lipid-linked oligosaccharides. It has been suggested that Alg3p functions as a Dol-P-Man-dependent mannosyltransferase, catalyzing the addition of the sixth mannose residue to $Man_5GlcNAc_2$-PP-Dol (Verostek *et al.*, 1991). Sharma *et al.* reported on the purification of a Dol-P-Man:$Man_5(GlcNAc)_2$-PP-Dol mannosyltransferase from pig aorta, which adds an α1,3 mannose from Dol-P-Man to $Man_5GlcNAc_2$-PP-Dol (Sharma *et al.*, 1990). The enzymatic activity cofractionated

with two proteins of 64.8 and 55 kDa; the latter would be in agreement with the predicted molecular weight of Alg3p. However, direct proof of whether Alg3p is the expected mannosyltransferase or an accessory protein has yet to be provided by a suitable *in vitro* system.

Alg3p shares 34% amino acid identity with the *l(2)not*$^+$ protein from *Drosophila melanogaster* (Kurzik-Dumke and Lohmann, 1995; U.Kurzig-Dumke, M.Kaymer, and D.Gundacker, in preparation) with the most conserved regions being hydrophilic domains of the Alg3 protein (Figure 1A, residues 68–104 and 151–175) (Cusick, 1994). The *l(2)not*$^+$ was found as a gene containing an intron that encodes a putative tumour suppressor gene (Kurzik-Dumke and Lohmann, 1995). The function of the *l(2)not*$^+$ protein is not known, but the similarity to the Alg3p suggested a functional homology between the two proteins. We have tried to determine whether the *Drosophila l(2)not+* can functionally replace the Alg3p in yeast, but have so far not been successful (U.Kurzik-Dumke and S.te Heesen, unpublished). In summary, the isolation of the yeast *ALG3* gene provides the tools to study a Dol-P-Man dependent mannosyltransferase of the endoplasmic reticulum on both the molecular and the biochemical level.

Materials and methods

Materials and strains

2-[^{3}H]-Mannose was from Amersham (555 GBq/mmol) (Zürich, Switzerland) or ICN Pharmaceuticals (Irvine, CA, USA) (925 GBq/mmol). Nitrocellulose BA-85 was from Schleicher & Schuell, Basel, Switzerland. *Saccharomyces cerevisiae* strains were SS328 (*MATα ade2–101 his3Δ200 lys2–801 ura3–52*), SS330 (*MATa ade2–101 his3Δ200 tyr1 ura3–52*) (Vijayraghavan *et al.*, 1989), YG170 (*MATα ade2–101 ade3 his3 alg3–1 stt3–3*) (Zufferey *et al.*, 1995), PRY90 (*MATα alg3–1 ade2–101 ura3–52*) was a gift from P.Robbins; YG247 (*MATα ΔALG3::HIS3 ade2 his3 ura3 tyr1*) (this study), YG248 (*MATa Δalg3::HIS3 ade2 his3 lys2 ura3*) (this study). Standard yeast media (Sherman, 1991) were used.

Construction of the Δalg3 strain

The construct used for the knock-out of *ALG3* ORF YBL0720 (Obermaier *et al.*, 1995) was linearized using SpeI and the linear DNA was electroporated into SS328. Resulting disruptants were backcrossed to strain SS330 and sporulated by standard techniques (Sherman and Hicke, 1991). YG247 and YG248 were segregants from this cross. The disruption of the *ALG3* gene was verified by PCR analysis according to Sathe *et al.* (1991) (data not shown).

Extraction and analysis of lipid-linked oligosaccharides

Cells were grown in minimal medium (2% glucose, 0.67% yeast nitrogen base, 0.5% casaminoacids, 20 mg/l adenine sulfate, 30 mg/ml tyrosine) at 25°C to a density of 4–8 × 10^6 cells/ml (optical density at 546 nm 0.4–0.8). Labeling, extraction, and HPLC analysis of lipid-linked oligosaccharides have previously been described (Zufferey *et al.*, 1995).

Protein extraction/Endo H treatment/Western analysis

Cells were grown in minimal medium (2% glucose, 0.67% yeast nitrogen base, 0.5% casaminoacids, 20 mg/l adenine sulfate, 30 mg/ml tyrosine) at 30°C to a density of 8–13 × 10^6 cells/ml; 3.7 × 10^8 cells were pelleted and suspended in 400 μl of 50 mM Tris·HCl (pH7.5), 1% SDS, protease inhibitors (Antipain 2 μg/ml, leupeptin 2 μg/ml, aprotinine 1 U/ml, chymostatin 2 μg/ml, pepstatin 2 μg/ml, PMSF 1 mM). Acid washed glass beads were added and the cells were lysed by 3 × 1 min vortexing, followed by heating for 5 min to 95°C. The samples were centrifuged 10 min. Ten microliters of the protein extract was digested with Endo H as described previously (Zufferey *et al.*, 1995), and proteins were separated by 7% SDS–PAGE. For Western blot analysis of CPY, Wbp1p and Gas1p, proteins were blotted onto nitrocellulose using a semidry blotter (Ken-En-Tec, Copenhagen, Denmark). Blocking of the membrane was performed for 1 h in 10% dry milk (w/v) in PBST (136 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.1% Tween 20 (v/v)). Incubation with antibodies at 1/4000 (α-CPY) or 1/2000 dilution for α-Wbp1p, (Gaynor *et al.*, 1994) and α-Gas1p (Fankhauser and Conzelmann, 1991) in 1% dry milk/PBST overnight at 4°C. Blots were washed 3 × 15 min in PBST and incubated in 1 μg/ml horseradish peroxidase conjugated to Protein A (Sigma) for 90 min at room temperature. Blots were washed 1 × 15 min and 4 × 5 min in PBST, followed by enhanced chemiluminescence (ECL)-detection system according to the supplier (Amersham, Zürich, Switzerland). Blots were stripped as described by the supplier (Amersham) and used for sequential reprobing with antibodies.

Sequence analysis of the alg3–1 allele

Sequencing of both strands of the *ALG3* open reading frame was performed as described previously (Obermaier *et al.*, 1995). To sequence *alg3–1*, three overlapping PCR products, covering the whole open reading frame of *alg3–1*, were generated from genomic DNA of the yeast strain PRY90. The PCR primers were removed with Pharmacia MicroSpin S-200 HR columns. The PCR products were then sequenced directly without subcloning

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Abbreviations

alg, mutant in asparagine-linked glycosylation, Dol, dolichol; Glc, glucose; GlcNAc, N-acetylglucosamine; Man, mannose; P, phosphate.

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