Carbohydrate deficient glycoprotein syndrome type IV: deficiency of dolichyl-P-Man:Man₅GlcNAc₂-PP-dolichyl mannosyltransferase

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Type IV of the carbohydrate deficient glycoprotein syndromes (CDGS) is characterized by microcephaly, severe epilepsy, minimal psychomotor development and partial deficiency of sialic acids in serum glycoproteins. Here we show that the molecular defect in the index patient is a missense mutation in the gene encoding the mannosyltransferase that transfers mannose from dolichyl-phosphate mannose on to the lipid-linked oligosaccharide (LLO) intermediate Man₅GlcNAc₂-PP-dolichol. The defect results in the accumulation of the LLO intermediate and, due to its leaky nature, a residual formation of full-length LLOs. N-glycosylation is abnormal because of the transfer of truncated oligosaccharides in addition to that of full-length oligosaccharides and because of the incomplete utilization of N-glycosylation sites. The mannosyltransferase is the structural and functional orthologue of the *Saccharomyces cerevisiae* ALG3 gene.

Keywords: ALG3 gene/carbohydrate deficient glycoprotein syndrome/dolichyl-P-Man:Man₅GlcNAc₂-PP-dolichyl mannosyltransferase

Introduction

The carbohydrate deficient glycoprotein syndromes (CDGS) comprise a clinically and biochemically heterogeneous group of multisystemic disorders that have in common the synthesis of glycoproteins with abnormal N-glycosylation. The molecular defects known so far affect the synthesis of mannose-6-phosphate (Niehues *et al.*, 1998), mannose-1-phosphate (van Schaftingen and Jaeken, 1995), the import of GDP-fucose into the Golgi (Lübke *et al.*, 1999), the synthesis of glucosylated forms of lipid-linked oligosaccharides (LLOs; Körner *et al.*, 1998a; Imbach *et al.*, 1999) or the processing of N-linked oligosaccharides (Jaeken *et al.*, 1994). An abnormal isoelectric focusing (IEF) pattern of sialylated serum glycoproteins serves as a diagnostic marker for CDGS. Based on the IEF pattern of serum transferrin, which normally carries two N-linked glycans of the complex type, four

subgroups of CDGS (I, II, III and IV) have been defined (Jaeken *et al.*, 1984, 1994; Stibler *et al.*, 1993, 1995). Clinically, CDGS type IV is characterized by the neonatal onset of a severe convulsive disease with almost no psychomotor development, and ophthalmological and skeletal abnormalities, including microcephaly. In contrast to the most common form of CDGS, type Ia, no liver dysfunction is observed. The IEF pattern of serum glycoproteins, including transferrin, α_1 -antitrypsin, antithrombin and thyroxine-binding globulin, demonstrated a partial deficiency of sialic acids, which was less pronounced than in CDGS types I and II (Stibler *et al.*, 1995).

Here we show that the abnormal N-glycosylation in CDGS type IV results from a defect in the assembly of full-length LLO in the endoplasmic reticulum (ER). Deficiency of the mannosyltransferase that transfers mannose from dolichyl-phosphate mannose (Dol-P-Man) on to Man₅GlcNAc₂-PP-Dol causes accumulation of the LLO intermediate, transfer of shortened oligosaccharides on to nascent glycoproteins and incomplete utilization of N-glycosylation sites in glycoproteins.

Results

Lack of an N-linked oligosaccharide in serum transferrin

The sialylation of serum transferrin is heterogeneous. The bulk of serum transferrin carries four sialic acid residues, but minor species with less or more sialic residues are observed. IEF of serum transferrin from CDGS type IV revealed the presence of a disialylated form (Figure 1, top), which migrates more quickly in SDS-PAGE and appears to be smaller in size by 2–3 kDa than control serum transferrin (Figure 1, bottom). Disialylated serum transferrin forms are also found in CDGS type I where transferrin lacks either one or both of its two N-linked glycans (Yamashita *et al.*, 1993) and in CDGS type II where transferrin carries two monosialylated N-glycans with a single branch (Jaeken *et al.*, 1994). The disialylated transferrin forms of CDGS type I and type IV behave similarly in SDS-PAGE (Figure 1, bottom), while the disialylated transferrin form of CDGS type II is only smaller in size by ~1 kDa (Figure 1, bottom). These data indicate that the disialylated serum transferrin in CDGS type IV is likely to lack one of the N-linked glycan chains, as in CDGS type I.

Shortened LLOs accumulate and are transferred to newly synthesized glycoproteins

To define the glycosylation defect, fibroblasts from controls and the patient were metabolically labelled for 30 min with 2-[³H]mannose. The LLOs were extracted and their glycan moieties were released by mild acid hydrolysis. After size fractionation by HPLC the majority of LLO-

Fig. 1. IEF pattern and SDS-PAGE of serum transferrin. Samples from control, CDGS type Ia, Ib, II, III and IV serum was analysed by IEF (upper panel) and SDS-PAGE (lower panel) followed by Western blotting and immunodetection of transferrin. 0, II and IV on the upper panel indicate transferrin forms with zero, two or four sialic acid residues. 0, 1 and 2 on the lower panel indicate transferrin forms with 0–2 oligosaccharide chains.

Fig. 2. LLOs in CDGS type IV. Fibroblasts from controls (A) and the patient (B) were metabolically labelled with 2-[^3H]mannose for 30 min. [^3H]oligosaccharides were released from LLOs by mild acid hydrolysis and size fractionated by HPLC. M₅ and G₃ refer to the position of GlcNAc₂Man₅ and GlcNAc₂Man₉Glc₃ standards.

derived oligosaccharides from control fibroblasts eluted at the position of a Glc₃Man₉GlcNAc₂ standard (Figure 2A), whereas the major oligosaccharide species from the

Fig. 3. Glycoprotein-derived oligosaccharides in CDGS type IV. Fibroblasts from controls (A) and the patient (B) were metabolically labelled with 2-[^3H]mannose for 30 min. [^3H]oligosaccharides were released from extracted glycoproteins by PNGase F digestion and size fractionated by HPLC. M₅, M₉ and G₁ refer to the position of GlcNAc₂Man₅, GlcNAc₂Man₉ and GlcNAc₂Man₉Glc₁ standards.

patient's fibroblasts eluted at the position of a Man₅GlcNAc₂ standard (Figure 2B). In four independent experiments Man₅GlcNAc₂ represented 60–80% of the LLO-derived oligosaccharides, the remainder corresponding to Man₉GlcNAc₂ oligosaccharides with up to three glucose residues.

N-glycans released by peptide-*N*-glycosidase F (PNGase F) from newly synthesized glycoproteins of control fibroblasts eluted at the position of Glc₁Man₉GlcNAc₂ and Man₉GlcNAc₂ standards, respectively (Figure 3A). N-glycans released from newly synthesized glycoproteins of CDGS type IV fibroblasts contained two additional species (Figure 3B). The first one eluted at the position of a Man₅GlcNAc₂ standard, whereas the second one contains one hexose unit more, thus corresponding to either Man₆GlcNAc₂ or Glc₁Man₅GlcNAc₂. The latter would be expected if glucosylated forms of a truncated Man₅GlcNAc₂ oligosaccharide are transferred on to nascent glycoproteins in CDGS type IV.

Deficiency of Dol-P-Man:Man₅GlcNAc₂-PP-Dol mannosyltransferase activity

The analysis of the lipid- and glycoprotein-derived oligosaccharides had suggested an impaired assembly of LLOs

Fig. 4. Activity of GDP-Man:Dol-P-mannosyltransferase (Dol-P-Man synthase). Microsomal extracts from control (●) and patient's fibroblasts (□) were incubated for the times indicated with GDP-[¹⁴C]mannose in the absence (lower panel) or in the presence (upper panel) of 5 µg of exogenously added Dol-P. Dol-P-Man was extracted and separated by TLC, Dol-P-[¹⁴C]mannose was quantified using a β-scanner.

at the level of Man₅GlcNAc₂-PP-Dol. The latter is assembled at the cytoplasmic side of the ER membrane and then translocated to the luminal side of the ER membrane, where four mannose and three glucose residues are added using Dol-P-Man and Dol-P-Glc as donors (Snider and Rogers, 1984). The accumulation of Man₅GlcNAc₂-PP-Dol in CDGS type IV fibroblasts points therefore to a deficiency of Dol-P-Man, a defect in the translocation of the Man₅GlcNAc₂-PP-Dol from the cytoplasmic to the luminal side of the ER membrane, or to a deficiency of the mannosyltransferase that transfers mannose from Dol-P-Man on to the Man₅GlcNAc₂-PP-Dol.

The activity of Dol-P-Man synthase was normal in the patient's fibroblasts, when the activity was measured in the presence or absence of exogenously added dolichol-phosphate (Figure 4A and B). The latter indicates that not only the enzyme activity itself is normal in CDGS type IV, but also the availability of dolichol-phosphate.

A defect in the translocation mechanism is unlikely since shortened oligosaccharides were found on the newly synthesized glycoproteins. In the case of impaired translocation, Man₅GlcNAc₂-PP-Dol would accumulate at the cytoplasmic side of the ER. The residual amount translocated on to the luminal side of the ER membranes should become converted to full-length Glc₃Man₉GlcNAc₂-PP-Dol. A translocation defect is therefore expected to result in the synthesis of glycoproteins that contain fewer but normally sized oligosaccharides.

Fig. 5. Dol-P-Man:Man₅GlcNAc₂-PP-Dol mannosyltransferase activity. Microsomal extracts from fibroblasts of controls (A and B) and the patient (C and D) were incubated for 30 min in the presence of [³H]Man₅GlcNAc₂-PP-Dol and Dol-P-[¹⁴C]mannose. After extraction of LLOs the oligosaccharides were released by mild acid hydrolysis and analysed by HPLC. The column fractions were examined separately for ¹⁴C (A and C) and ³H (B and D) radioactivity. The positions of GlcNAc₂Man₅₋₈ (M₅, M₆, M₇, M₈) and GlcNAc₂Man₉Glc₃ (G₃) standards are indicated.

In order to determine the activity of the Dol-P-Man:Man₅GlcNAc₂-PP-Dol mannosyltransferase, microsomal extracts were incubated with the donor Dol-P-[¹⁴C]mannose and the acceptor [³H]Man₅GlcNAc₂-PP-Dol. Microsomal extracts from control fibroblasts elongated the oligosaccharide of the [³H]Man₅GlcNAc₂-PP-Dol acceptor to ¹⁴C/³H-labelled products with the mobility of [³H]Man₆₋₈ GlcNAc₂ standards (Figure 5A and B) whereas the extracts derived from the patient's fibroblasts catalysed no detectable transfer of [¹⁴C]mannose on to the acceptor (Figure 5C and D). We conclude from these results that the elongation of Man₅GlcNAc₂-PP-Dol in CDGS type IV is due to a defect in the elongating mannosyltransferase.

A missense mutation in the human Not 56-like protein gene

In *Saccharomyces cerevisiae* the *ALG3* gene has been shown to be required for the activity of the Dol-P-Man:Man₅GlcNAc₂-PP-Dol mannosyltransferase and is suspected to encode the transferase itself or an accessory protein of the transferase (Aebi et al., 1996). Alg3p has 30% identity with the human Not (neighbour of tid) 56-like protein (Figure 6), the function of which is unknown. The cDNA sequence for the human Not 56-like protein (NCBI, Y09022) predicts a potential ER transmembrane protein of 438 amino acids with a C-terminal -KKXX retrieval signal. Sequence analysis of the cDNA encoding the Not 56-like protein in the patient revealed a missense mutation as the only deviation. A G→A transition of nucleotide 353 causes the substitution of Gly118 with aspartic acid. Sequencing of the genomic DNA showed that the patient was homozygous for the G353A mutation and that his parents were heterozygous (Figure 7).

Complementation of the defect in a Δalg3 strain of S.cerevisiae

To show the function of the human Not 56-like protein as a mannosyltransferase and the causality of the G353A

Fig. 6. Protein alignment and hydrophilicity plot of human Not 56-like protein and *S.cerevisiae* alg3p. Proteins were aligned using the CLUSTAL W (1.74) multiple sequence alignment program (EMBL, upper panel). Conserved residues are marked by asterisks, conserved substitutions by double points and semi-conserved substitutions by points. The arrow indicates Gly118 which is substituted with Asp in CDGS type IV (upper panel). The hydrophilicity plot was calculated by the method of Kyte–Doolittle with the PROTEAN program (DNA Star, lower panel). Areas above and below the axis indicate hydrophilic and hydrophobic sequences.

350 360

A G C C G A G G C A C T G A C

control

A G C C G A G A C A C T G A C

CDGS IV (J.E.)

A G C C G A G N C A C T G A C

mother

A G C C G A G N C A C T G A C

father

Ser	Arg	Gly Asp	Thr	Asp
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Fig. 7. Identification of 353G→A missense mutation encoding a Gly118Asp exchange in the human Not 56-like protein. The sequence alignment of the PCR-amplified genomic DNA fragment from a healthy control, the CDGS type IV patient homozygous for the mutation and the heterozygous parents.

corrective effect on the size of LLOs (Figure 8D), while the underglycosylation of carboxypeptidase Y was partially corrected (Figure 9, lane 4). It appears therefore that the glycosylation pattern of carboxypeptidase Y is a more sensitive parameter to indicate the correction of the metabolic defect in $\Delta alg3$ cells than the pattern of LLOs. The residual amount of full-length LLOs formed in $\Delta alg3$ cells expressing the G353A mutant of the human Not 56-like protein is apparently utilized rapidly for the synthesis of glycoproteins, thus keeping the steady-state level of Glc₃Man₆GlcNAc₇-PP-Dol low.

The defect in CDGS type IV affects the elongation of the Man₅GlcNAc₂-PP-Dol intermediate in the ER. The common precursor oligosaccharide Glc₃Man₉GlcNAc₂, which is transferred on to selected asparagine residues in nascent glycoproteins, is assembled on a dolichol-phosphate carrier. In a step-wise manner the first GlcNAc-1-phosphate, the following GlcNAc and the inner five mannose residues are transferred from the respective

Fig. 8. Analysis of LLOs in $\Delta alg3$ yeast transformed with the human *ALG3* orthologue. The yeast cells were metabolically labelled with 2- $[^3H]$ mannose for 30 min. $[^3H]$ oligosaccharides were released from LLOs and size fractionated by HPLC. (A) $\Delta alg3$, (B) $\Delta alg3$ transformed with yeast *ALG3*, (C) $\Delta alg3$ transformed with pNEV ManTwt (cDNA encoding the Not 56-like protein) and (D) $\Delta alg3$ transformed with pNEV ManTpat (cDNA encoding the Gly118Asp mutant of the Not 56-like protein).

Fig. 9. Complementation of *S. cerevisiae* $\Delta alg3$ mutant by the human *ALG3* orthologue. Wild-type yeast (lane 1), $\Delta alg3$ cells transformed with either vector plasmid (lane 2), pNEV-ManTwt (cDNA encoding the Not 56-like protein, lane 3) or pNEV-ManTpat (cDNA encoding the Gly118Asp mutant of the Not 56-like protein, lane 4), were labelled for 30 min with $[^{35}S]$ methionine. Carboxypeptidase Y was immunoprecipitated and analysed by SDS-PAGE. The position of the mature form of carboxypeptidase Y in wild-type yeast cells (mCPY) and in the $\Delta alg3$ cells (mCPY $\Delta alg3$) is indicated on the left. The carboxypeptidase Y forms in lanes 2 and 4 migrating more quickly than the mature forms represent incompletely glycosylated forms lacking one or two oligosaccharide chains. Carboxypeptidase Y with four N-glycans from the untransformed $\Delta alg3$ strain (lane 2) or from $\Delta alg3$ transfected with pNEV-ManTpat (lane 4) migrates more quickly than those from wild-type yeast (lane 1) or $\Delta alg3$ transfected with pNEV-ManTwt (lane 3), because in the former two strains truncated oligosaccharides are transferred on to carboxypeptidase Y. Molecular weight standards are indicated on the right.

nucleotide sugars on the cytoplasmic side of the ER membrane. The $\text{Man}_5\text{GlcNAc}_2\text{-PP-Dol}$ intermediate is then transferred on to the luminal side of the ER membrane and elongated to $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2\text{-PP-Dol}$ using Dol-P-Man and Dol-P-Glc as donors (Snider *et al.*, 1984). The mannosyltransferase activity that transfers mannose from

Dol-P-Man on to $\text{Man}_5\text{GlcNAc}_2\text{-PP-Dol}$ is missing in CDGS type IV.

Crucial for the identification of the affected gene was the earlier observation in *S. cerevisiae* that disruption of the *ALG3* gene results in a glycosylation defect closely resembling that in CDGS type IV (Aebi *et al.*, 1996). *ALG3* encodes a protein with multiple membrane-spanning helices and a C-terminal ER retention signal. It was suspected to be the Dol-P-Man: $\text{Man}_5\text{GlcNAc}_2\text{-PP-Dol}$ mannosyltransferase itself or an accessory protein of the transferase (Aebi *et al.*, 1996). Recent data from our laboratory demonstrate that Alg3p is indeed the mannosyltransferase itself (R.Knauer and L.Lehle, unpublished). The human Not 56-like protein shares with Alg3p 30% sequence homology, the C-terminal -KKXX ER-retrieval signal and multiple membrane-spanning helices. Complementation of the glycosylation defect in the $\Delta alg3$ strain by the human Not 56-like protein gene demonstrated that it is the orthologue of the *S. cerevisiae* *ALG3* gene and therefore encodes the Dol-P-Man: $\text{Man}_5\text{GlcNAc}_2\text{-PP-Dol}$ mannosyltransferase.

The CDGS type IV index patient is homozygous for a missense mutation in the Not 56-like protein gene, which apparently reduces, but does not abolish the mannosyltransferase activity. The residual activity is demonstrated by the residual formation of full-length LLOs and their utilization for N-glycosylation in the patient and in the yeast $\Delta alg3$ strain transfected with the Not 56-like protein cDNA carrying the missense mutation. The mutation causes the substitution of a glycine residue with aspartate in a putative cytoplasmic loop of the transferase. The neighbouring sequence is poorly conserved, which makes it difficult to decide with certainty whether the glycine residue is conserved between man and yeast or not.

In all CDGS types in which the assembly of LLO is affected, the defects are leaky (Powell *et al.*, 1994; Körner

et al., 1998a; Niehues *et al.*, 1998). This indicates that null mutations of the affected genes are lethal, suggesting that N-glycosylation is essential and that metabolic bypasses for the lost gene functions do not exist. Apart from the residual amount of full-length LLOs that are formed due to the leaky nature of the mutation in CDGS type IV, the accumulating $\text{Man}_5\text{GlcNAc}_2\text{-PP-Dol}$ is utilized for N-glycosylation. It is known that the $\text{Man}_5\text{GlcNAc}_2\text{-PP-Dol}$ intermediate can be glucosylated and that the $\text{Glc}_3\text{Man}_5\text{GlcNAc}_2$ oligosaccharides can be transferred by the oligosaccharyltransferase complex (Rearick *et al.*, 1981). The presence of $\text{Man}_5\text{GlcNAc}_2$ oligosaccharides in newly synthesized glycoproteins demonstrated the utilization of this alternative N-glycosylation pathway in CDGS type IV. The presence of serum transferrin forms that lack one of its two N-glycans, however, indicates that neither the residual mannosyltransferase activity nor the alternative N-glycosylation pathway utilizing truncated oligosaccharides can ensure in CDGS type IV the glycosylation of the asparagine residues that are normally utilized.

Materials and methods

Patient

The patient analysed here refers to case 1 in the original description of CDGS type IV (Stibler *et al.*, 1995). He is now 5 years of age suffering from tetraspastic paresis, a severe psychomotor handicap and multiple dysmorphisms including microcephaly, dysplastic ears, atrophy of the optic nerve and colomaba of the iris. The epilepsy is reasonably controlled by valproic acid.

Isoelectric focusing and SDS-PAGE of serum transferrin

Isoelectric focusing of serum transferrin was carried out as described previously (Niehues *et al.*, 1998).

Analysis of dolichol- and protein-linked oligosaccharides

Fibroblasts derived from controls and the patient were grown and metabolically labelled with $2[^3\text{H}]$ mannose. Dolichol- and protein-linked oligosaccharides were extracted and analysed by HPLC as described previously (Körner *et al.*, 1998b).

GDP-Man:Dol-P mannosyltransferase (Dol-P-Man synthase) assay

Fibroblasts were grown and extracted as described (Körner *et al.*, 1998b). The activity of Dol-P-Man synthase was determined from 20 μg of membrane extract in a total reaction volume of 70 μl as described previously (Lehle, 1980).

Dol-P-Man:Dol-PP-GlcNAc₂Man₅ mannosyltransferase assay

Dol-P- $[^{14}\text{C}]$ Man (45 000 c.p.m.) and $[^3\text{H}]$ Man₅GlcNAc₂-PP-Dol (50 000 c.p.m.) were dried under nitrogen. The mixture was dispersed in 20 mM NaH_2PO_4 -citrate buffer pH 6.5, containing 0.25% NP-40, 5 mM MnCl_2 , 1.5 mM phosphatidylcholine and microsomal membrane fraction (0.12 mg protein) in a final volume of 100 μl . Incubations were carried out at 25°C for 30 min and stopped by adjusting the mixtures to 20 mM HCl. Extraction of LLOs, release of oligosaccharides from LLOs by mild acid hydrolysis and analysis of the oligosaccharides by HPLC were as described (Knauer and Lehle, 1999a). Membrane fractions were isolated from fibroblasts and from yeast (Knauer and Lehle, 1994; Knauer and Lehle, 1999b) as described. Radioactive Dol-P- $[^{14}\text{C}]$ Man was enzymatically synthesized and purified (Lehle and Tanner, 1975; Lehle, 1980) using membranes from yeast wild-type strain X2180. $[^3\text{H}]$ Man₅GlcNAc₂-PP-Dol was isolated from Δalg3 metabolically labelled with $[^3\text{H}]$ mannose.

Mutation analysis

Total RNA was extracted from control and patient's fibroblasts using the RNeasy kit (Qiagen). First-strand cDNA was synthesized from 1 μg RNA with Superscript reverse transcriptase (Gibco-BRL) and the primer r2 (5'-GTAGACTCAGGTCCTGAGGGAAG-3'). In a first round of PCR the cDNA was amplified by the primers r2 and f10

(5'-TGGGCCCCACACAAGCGGCGCAC-3') using the Hot Start Taq polymerase kit (Qiagen) with a preincubation at 95°C for 15 min followed by 35 cycles with 1 min at 94°C, 1 min at 58°C and 3 min at 72°C. Further amplification was carried out with the nested primers r8 (5'-GGTCTCTGAGGGAAAGGGGTGGAC-3') and f9 (5'-CACAAGCGGCGCACCGTTAAG-3'). RT-PCR products were run on 1% agarose gels. The 1361 bp fragment was prepared with the Quiaquick PCR purification (Qiagen), subcloned into the pGEM-T Easy vector (Promega) and analysed by dye-determined cycle sequencing with the primers pUC M13 forward, pUC M13 reverse (Stratagene, Heidelberg), r6 (5'-GCCAGTTCACTGTCCAGTG-3') and f3 (5'-GCGTCATCAATGGTACCTATG-3') on an Applied Biosystems model 373A automated sequencer.

Genomic DNA was prepared from control and patient's fibroblasts as well as from peripheral blood leucocytes of the parents by standard procedures (Maniatis *et al.*, 1989). PCR was carried out as described above with the primers r6 and f3. The PCR products were run on a 1% agarose gel and the resulting 1850 bp fragments were prepared as described above. Sequencing was carried out with the primer f3.

Yeast genetics

The open reading frame of the mannosyltransferase from control and patient were isolated as *EcoRI* fragments from the plasmids pGEM-T-Easy-wt and pGEM-T-Easy-pat, respectively, and ligated into the *EcoRI*-cut NEV-E shuttle vector (Sauer and Stolz, 1994) under the control of the PMA1 promoter to give plasmids pNEV-ManTwt and pNEV-ManTpat, respectively. Plasmids were transformed into a Δalg3 yeast strain (*MAT α ade2 his3 ura3 tyr1 $\Delta\text{ALG3::HIS3}$*) using standard techniques (Gietz and Schiestl, 1991). For the construction of the Δalg3 mutant the *HIS3* cassette was inserted as a *PvuII-SmaI* 1892 bp fragment from pJJ217 (Jones and Prakash, 1990) into the blunted *SphI* sites of *ALG3* subcloned as a *SpeI* fragment into pGEM9Z. For disruption, the *ALG3::HIS3* construct was transformed as a linear 3250 bp *SpeI* fragment into strain SS330 (*MAT α ade2 his3 ura3 tyr1*). Disruption of *ALG3* was verified by showing the LLO defect in HIS prototrophic transformants.

In vivo labelling of LLO and carboxypeptidase Y

Yeast cells were grown at 30°C in YNBD (0.67% yeast nitrogen base, 2% glucose, supplemented with appropriate amino acids) to mid-log phase and labelled with $[^{35}\text{S}]$ methionine for 30 min. Cell lysis, extraction and analysis of LLO as well as immunoprecipitation of carboxypeptidase Y and analysis by SDS-PAGE on 8% gels followed by autoradiography were as described previously (Knauer and Lehle, 1999a).

DDBJ/EMBL/GenBank accession numbers

Human Not 56 (*Drosophila melanogaster*)-like protein (Y09022); putative α -(1-3)-mannosyltransferase from *S.cerevisiae* (Z35844).

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