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Review

Glycosylation defects corrected by the changes in GDPmannose level*°

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GDPMan is a key substrate in glycoprotein formation. This is especially true for lower eukaryotes where, in addition to the involvement in N-glycan biosynthesis and GPI-anchor formation, GDPMan takes part in the process which is unique for yeast and fungi i.e. O-mannosylation.

Several lines of evidence have been presented that the level of GDPMan affects the process occurring in the Golgi compartment i.e. the elongation of outer mannose chain of glycoproteins in *Saccharomyces cerevisiae*. Results from our laboratory indicate that the availability of GDPMan affects also the early steps of glycoprotein formation ascribed to the endoplasmic reticulum, i.e. assembly of the dolichol-linked oligosaccharide as well as mannosyl-phosphodolichol (MPD) formation.

The biochemical basis of carbohydrate deficient glycoprotein syndrome, a severe neurological disorder related to the GDPMan deficiency, is also discussed.

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Abbreviations: CDGS, carbohydrate deficient glycoprotein syndrome; DolP, DolPP- dolichylphosphate, diphosphate; ER, endoplasmic reticulum; GDPMan, GDPmannose; GlcNAc, *N*-acetylglucosamine; GPI, glycosylphosphatidylinositol; LLO, lipid linked oligosaccharide ($DolPPGlcNAc_2Man_9Glc_3$); MPD, dolichylphosphate mannose; MPG-transferase, GDP:α-D-mannose-1-phosphate guanyltransferase; NDP, nucleotide diphosphate; PMI, phosphomanno-isomerase; PMM, phosphomannomutase.

GDPmannose acts as a mannose donor in lipid and protein glycosylation. In the synthesis of N-linked glycans leading to the formation of DolPP-$GlcNAc_2Man_9Glc_3$ the oligosaccharide chain is linked to dolichyldiphosphate. All the mannose residues of the glycan originate from GDPMan, either directly or *via* dolichylphosphate, the lipid intermediate. It is believed (for reviews see Tanner & Lehle, 1987; Herscovics & Orlean, 1993) that the first five mannose residues are added directly from GDPMan at the cytosolic side of the endoplasmic reticulum (ER) and the subsequent four residues from dolichylphosphate mannose at the lumenal side of ER. Dolichylphosphate mannose (MPD) is synthesised from GDPMan and dolichylphosphate by MPD synthase (EC 2.4.1.83, compare Table 1). In yeast and filamentous fungi MPD is also a substrate for protein O-glycosylation, where it serves as a donor of the first mannose to be attached to hydroxyl groups of serine and threonine in the protein. The second and subsequent mannose residues are transferred directly from GDPMan (Tanner & Lehle, 1987; Herscovics & Orlean, 1993). MPD is also involved in the synthesis of the sugar part of glycosylphosphatidylinositol anchor in yeast and other eukaryotes (Herscovics & Orlean, 1993). Thus MPD synthase plays a central role in three different pathways in endoplasmic reticulum (Orlean, 1990).

Studies *in vitro* on the biosynthesis of N-linked glycans in retina (Kean, 1980) led to the conclusion that GDPMan, in addition to acting as a substrate for various mannosyltransferases, acts in an indirect manner to stimulate the biosynthesis of non-mannose-containing GlcNAc-lipids, a process which may participate in regulation of glycoprotein biosynthesis. A similar observation was also made for thyroid tissue (Ronin *et al.*, 1981). We have reported earlier (Kruszewska *et al.*, 1994) that in the filamentous fungus *Trichoderma reesei*, GDPMan stimulates mannosyl transfer from MPD to the hydroxyl groups of serine and threonine and thus, in turn, affects the rate of protein O-glycosylation.

POTENTIAL SOURCES OF GDPMan IN THE CELL

Biosynthesis of GDPMan and other sugar nucleotides, is a process occurring in the cytoplasm. The final reaction of sugar nucleotide assembly (Fig. 1) is preceded by the conversion of Man-6-P to Man-1-P catalysed by phosphomannomutase (EC 5.4.2.8). Several lines of evidence have been presented that in yeast the enzyme is coded by the *SEC53* gene, defective in the class B secretion mutant *sec53*, which seems to be blocked at an early stage in protein translocation into endoplasmic reticulum (Ferro-Novick *et al.*, 1984; Kepes & Schekman, 1988). Alleles of *SEC53* were also isolated as *ALG4* gene (Table 1) in a [^{3}H]-mannose suicide screen for glycosylation defective mutants (Huffaker & Robbins, 1982; 1983). A cDNA encoding human phosphomannomutase (PMM), a protein of 29 kDa that is in 55% identical and in 66% similar to yeast Sec53p, has been also isolated. Its expression in a temperature sensitive *sec53* yeast mutant confers growth at the restrictive temperature, strongly suggesting that this gene encodes functional PMM. When expressed in BHK cell, PMM is localised exclusively in the cytosol (Hansen *et al.*, 1997).

Mutants in PMM are blocked in N-glycosylation, O-mannosylation, glucosylphosphoinositol (GPI)-anchoring and in the formation of mannosylphosphoceramides (Huffaker & Robbins, 1983; Kepes & Sheckman, 1988; Conzelmann *et al.*, 1990; Orlean, 1990; Orlean *et al.*, 1991). This seems to be a pleiotropic effect due to a block in the synthesis of GDPMan. On the other hand, overexpression of the *Trichoderma mpg1* gene, encoding GTP:α-D-mannose-1-phosphate guanyltransferase (EC 2.7.7.13), catalysing the final reaction in GDPMan synthesis (compare Fig. 1,

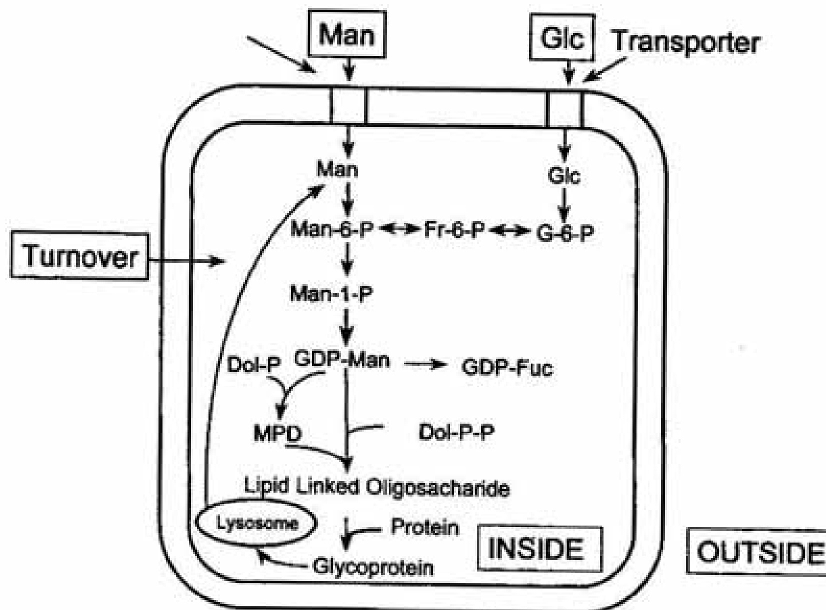

Figure 1. Cellular mechanism of GDPMan synthesis and turnover.

GDPMan is synthesised either from mannose or from glucose, which are translocated to the cell *via* specific transporters. Most of GDPMan takes part in several glycosylation reactions, localised in the ER lumen (synthesis of MPD and lipid linked oligosaccharide) and in the Golgi compartment (synthesis of the outer sugar chain of glycoproteins). In animal cells some of glycoproteins are transported to lysosomes, and degraded enriching the intra-cellular mannose pool. In the cytoplasm GDPMan can be metabolised to GDP-fucose (GDP-Fuc).

Table 1) did not restored viability of the thermosensitive *sec53* mutant. This suggest that the glycosylation defect might be due to the more complex changes in the cell than simply a lack of GDPMan.

Most of cellular Man-6-P might derive also by conversion of glucose (Fig. 1). The reaction is catalysed subsequently by phosphogluco- and phosphomannose isomerases (EC 5.3.1.9; EC 5.3.1.8-9) The latter is encoded by the *PMI40* gene (Table 1). A *pmi40* mutant was isolated as osmotically fragile mutant, whose phenotype could be rescued by addition of mannose to the culture medium (Payton *et al.*, 1991).

The final reaction in GDPMan synthesis (Fig. 1) is the transfer of mannose-1-phosphate to GTP which is catalysed by MPG-transferase (EC 2.7.7.13). This enzyme is also known as GDP-α-D-mannose pyrophosphorylase. Its occurrence has been reported in a number of tissues (Priess & Wood, 1964; Mendicino & Rao, 1975; Coates *et al.*, 1980). An attempt to purify the enzyme from porcine thyroid has been undertaken and a nearly 70000-fold purification was reported (Smoot & Serif, 1985). In the yeast *Saccharomyces cerevisiae* MPG-transferase is encoded by the *MPG1* gene, the complete sequence of which has been deposited in the yeast genome data base by Shultz and Sprague (accession No. P41940). Alleles of *MPG1* were isolated also as the *PSA1* (Benton *et al.*, 1996) a plasmid suppressor of the *alg1* mutation in the yeast asparagine N-glycosylation pathway (Herscovics & Orlean, 1993) and also as the *VIG9* gene (Hashimoto *et al.*, 1997) by the screen of the collection of vanadate-resistant mutants (Ballou *et al.*, 1991). All of these alleles (Table 1) encode the active enzyme. The *VIG9* gene is essential, encodes a 361 amino-acid protein homologous to NDP-hexose pyrophosphorylases, catalysing the formation of activated sugar nucleotides (Benton *et al.*, 1996).

Using a functional complementation approach we have recently cloned a cDNA fragment coding for MPG1-transferase in a filamentous fungus *T. reesei*. The gene was isolated based on its ability to suppress a temperature-sensitive defect of the yeast *S. cerevisiae* mutation in the *DPM1* gene encoding MPD synthase. The nucleotide sequence of the 1.6 kb long cDNA clone revealed an open reading frame (ORF) which encodes a protein of 364 amino acids. Sequence com-

Table 1. Enzymes involved in GDPMannose turnover

Enzyme	Encoding gene	Reaction
Phosphomannomutase Sec53p, Alg4p	*SEC 53, ALG 4*	Man-1-P ⇒ Man-6-P
Phosphomannoisomerase Pmip	*PMI 40*	Fr-6-P ⇒ Man-6-P
GTP:α-D-mannose-1 Phosphate guanyltransferase Mpg1p, Psa1p, Vig9p	*MPG1 PSA1, VIG9*	Man-1-P + GTP ⇒ GDPMan + P_i
Dolichyl-phosphate β-D-mannosyltransferase (MPD-synthase), Dpm1	*DPM1*	GDPMan + DolP ⇒ Dol-P-Man + GDP
Chitobiosyl diphosphodolichol β-mannosyltransferase, Alg1p	*ALG1*	$DolPPGlcNAc_2$ + GDPMan ⇒ $DolPPGlcNAc_2Man$ + GDP
Dimannosyl chitobiosyl diphosphodolichol α-mannosyltransferase, Alg2p	*ALG2*	$DolPPGlcNAc_2Man_2$ + GDPMan ⇒ $DolPPGlcNAc_2Man_3$ +GDP
Guanosine-diphosphate hydrolase (GDPase) Gda1p	*GDA1*	GDP + H_2O ⇒ GMP + PP_i
Phosphotransferase Guanylate kinase Guk1p	*GUK1*	GMP + ATP ⇒ GDP + ADP

parisons demonstrate its 70% identity with the *S. cerevisiae* guanyl transferase gene (*MPG1*) and 75% identity with the *Schizosaccharomyces pombe* homologue (Kruszewska *et al.*, 1998). Overexpression of the *S. cerevisiae MPG1* gene in the wild type yeast resulted in the 3-fold increase of GDPMan concentration (Shimma *et al.*, 1997). Similarly a significant increase of MPG-transferase was observed upon expression of the *Trichoderma mpg1* gene in the yeast *dpm1*-mutant (Kruszewska *et al.*, 1998) and the *VIG9* gene in the *vig9* mutant. These findings further substantiated the assumption that the *MPG1/VIG9* are the structural genes for MPG-transferase.

It has been reported earlier that the cellular level of GDPMan might be affected also by mutations in the genes encoding enzymes involved in GTP synthesis. One of these mutations involves impairment in the transport of GDPMan from the site of its synthesis, i.e., cytoplasm into the Golgi compartment where it serves as a mannose donor for the extension of the outer mannan chain. Import of GDPMan into the Golgi apparatus of *S. cerevisiae*, analogously as in the animal cells (Hirschberg & Snider, 1987) involves an antiport mechanism. GDPMan enters the lumen *via* a specific membrane-bound carrier protein and donates its mannose residue to the endogenous acceptor, releasing GDP, which is, in turn, hydrolysed to GMP, whose exit from the Golgi compartment is coupled to the further import of GDPMan. This model was supported by the further results from Hirshberg's laboratory when the Golgi GDPase, (EC 3.6.1.42) encoded by the *GDA* gene (Table 1), was identified as an essential component of this antiport mechanism (Abeijon *et al.*, 1992; Yanagisava *et al.*, 1990). Disruption of the *GDA* gene yielded viable haploids, although in the Golgi vesicles isolated from the *gda* mutant, the rate of GDPMan import was reduced to one fifth (Bernisone *et al.*, 1994).

More recently, a defect in GTP synthesis, caused by mutation in GMP-kinase (EC 2.7.4.8) encoded by the *GUK1* gene (Table 1), has been described (Shimma *et al.*, 1997). This is the cytoplasmic step responsible for the conversion of GMP to GTP which, in turn, affects the concentration of GDPMan (Fig. 2). The *guk1* mutant was found on screening of the collection of the yeast mutants that show higher cell wall porosity than do normal cells and are hypersensitive to antibiotics with a large molecular mass such as neomycin and geneticin (Shimma *et al.*, 1997).

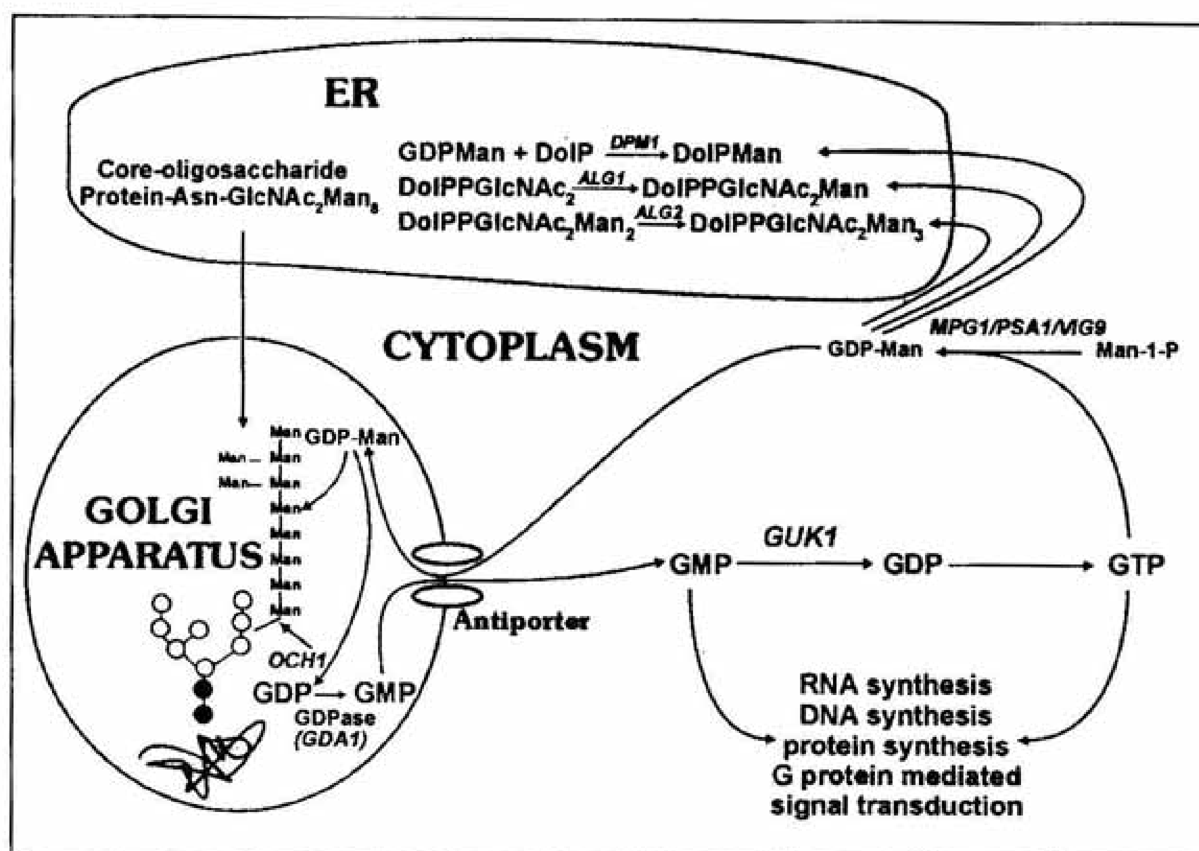

Figure 2. Glycosylation reactions affected by the intra-cellular level of GDPMan in the yeast cell.

Reactions involved in core oligosaccharide formation in the endoplasmic reticulum: Synthesis of DolPMan (MPD), reaction catalysed by the Dpm1-protein; elongation of the dolichol-linked oligosaccharide, catalysed by the Alg1- and Alg-2-proteins. In the Golgi compartment: addition of the outer sugar chain (α-1,6 linked mannose).

GLYCOSYLATION STEPS AFFECTED BY THE CHANGES IN GDPMan AVAILABILITY

Synthesis of dolichol-linked saccharides

Formation of dolicholphosphate mannose catalysed by MPD-synthase is a key reaction in protein glycosylation. The obligatory requirement of MPD-synthase, encoded by *DPM1* gene, for O-mannosylation, has been demonstrated previously in *S. cerevisiae* by finding that a temperature sensitive MPD-synthase mutant *dpm1-6* was completely blocked in O-mannosylation of the model protein chitinase (Orlean, 1990). Loss of *DPM1* expression has been shown to be lethal for the yeast cells (Orlean, 1990). MPD-synthase also participates in N-glycosylation of proteins as a donor of the last four mannosyl residues during the assembly of the dolichol-linked precursor oligosaccharide i.e. DolPPGlcNAc$_2$-Man$_9$Glc$_3$, and also is required for the biosynthesis of glycosylphosphatidylinositol membrane anchor. Recent, yet unpublished, results from our laboratory indicate, that in *S. cerevisiae* the availability of GDPMan which is synthesised in the cytoplasm affects the early steps of glycoprotein formation ascribed to the endoplasmic reticulum, i.e. assembly of the dolichol-linked oligosaccharide as well as MPD synthesis. Thus the yeast *S. cerevisiae dpm1-6* mutant which harbours thermosensitive allele of the *DPM1* (MPD-synthase encoding) gene on the plasmid was complemented by the genes encoding MPG-transferase from *T. reesei* and *S. cerevisiae.* The question arises however, why MPG transferase activity com-

plements the defect in MPD formation. The biochemical data on the synthesis of glycosyl derivatives of MPD *in vitro* indicate that the reaction is easily reversible and highly dependent on the concentration of sugar nucleotides and the product formed (Warren & Jeanloz, 1978). It is thus possible that a higher concentration of the substrate GDPMan which should result from overexpression of the *MPG1* gene allows the yeast Dpm1 mutant protein to function at restrictive temperature.

We have also compared the affinity of MPD-synthase towards GDPMan in the wild yeast extract and in the *dpm1-6* mutant transformed with the *MPG1* gene. The data obtained indicate increased affinity of MPD-synthase from the transformed *dpm1* mutant concomitant with severely impaired maximal velocity of the reaction. The above result could be explained by the pleiotropic effect of the *DPM1* gene mutation. Our results on the Dpm1-protein of the *Aspergillus nidulans* mutant, impaired in the activity of MPD-synthase as well as protein secretion, indicate also a 3–4-fold increase of the enzyme affinity towards GDPMan. This result supports the assumption that mutation in the *DPM1* gene, leading to the decreased MPD synthase activity, is concomitant with the increased affinity towards the substrate GDPMan. On the other hand, a possibility of interaction of the Dpm1 and Mpg1 proteins has to be also considered.

The yeast *MPG1* gene restored also the viability of the yeast N-linked glycosylation mutants *alg1* and *alg2* when cultivated at non-permissive temperature. Both the Alg1- and Alg2-proteins refer to mannosyltransferases (Table 1) responsible for the addition of the β-1,4 (Alg1-p) and α-1,6 (Alg2-p) linked mannose residues to the dolichol-linked oligosaccharide (DolPP-GlcNAc$_2$ and DolPP-GlcNAc$_2$Man$_2$). Suppression of the *alg1* mutation by overexpression of Mpg1 protein was reported also by Benton *et al.* (1996) and Shimma *et al.* (1997). On the other hand, as mentioned earlier *T. reesei mpg1* gene did not complement the temperature-sensitive *sec53-6* mutation of *S. cerevisiae* which affects the preceding cytoplasmic step in GDPMan formation i.e. conversion of Man-6-P to Man-1-P (Bernstein *et al.*, 1985). Thus at present we assume that the *MPG1* gene is able to suppress mutations of the proteins such as Dpm1-, Alg1- and Alg2-, involved in the steps which utilise GDPMan as a substrate.

Based on the results obtained for the mutated Dpm1p, where we observe up to tenfold decrease of the K_m value towards GDPMan in MPD formation, we hypothesize that the availability of GDPMan in the cell cytoplasm might affect the mannosyltransferases located in endoplasmic reticulum by changing their substrate affinity.

OUTER SUGAR CHAIN ELONGATION IN THE YEAST *SACCHAROMYCES CEREVISIAE*

Cloning of *S. cerevisiae* Golgi GDPase gene (*GDA1*) allowed to construct the *GDA1* disrupted strain (Abeijon *et al.*, 1993) which was viable and did not show *in vitro* the membrane bound GDPase activity. The *gda1* disruptant had a block in O-glycosylation of secreted chitinase. Chitinase produced by this strain contained 35% of the Man$_1$ species and the remaining 65% in the form of Man$_2$ (Abeijon *et al.*, 1993) whereas in the wild type cells Man$_1$ is almost completely absent and oligosaccharides containing O-linked Man$_{3-5}$ sugar residues are found (Herscovics & Orlean, 1993). Moreover the disruptant (the null mutant) showed only the core glycosylated form of carboxypeptidase Y, lacking mannose residues normally added in the Golgi apparatus. The glycosylation pattern of another N-glycoproteins i.e. invertase was also impaired and the level of mannosylphosphoceramides was drastically reduced (Abeijon *et al.*, 1993). All these results taken together strongly support the assumption that accumulation of GDP in the Golgi apparatus due to the *gda*

mutation perturbs the balance of the transporter-antiporter mechanism, and results in the decreased level of GDPMan in the Golgi and, in turn, in the altered mannosylation occurring in this compartment. Mannose outer chain elongation, which occurrs in the Golgi apparatus, is also impaired in the *guk1* mutant affected in the synthesis of GTP due to the defect in GMP-kinase (EC 2.7.4.8) (Fig. 2). Since *guk1* mutation results in a 75% decrease of cellular GDPMan it can be assumed that underglycosylation of protein is due to the shortage of the substrate for mannosyltransferase responsible for the α-1,6 Man outer sugar chain initiation (Shimma *et al.*, 1997). Similar defect was also observed in the *och1* mutants. Och1-p α-1,6 mannosyltransferase is a membrane protein localised in the early Golgi compartment (Herscovics & Orlean, 1993). The partial correction of the glycosylation defect observed in the *guk1* mutant might be achieved by the increase in GDPMan due to the overexpression of the *MPG1* gene. It is also possible that accumulation of GMP observed in the *guk1* mutant might decrease the anti-port activity of GDPMan and GMP through the Golgi membrane.

CARBOHYDRATE-DEFICIENT GLYCOPROTEIN SYNDROMES (CDGS)

These syndromes are a group of genetic disorders affecting mainly central nervous system. The diagnosis is mostly made by isoelectrofocusing of serum transferrin which shows an altered cathodal migration due to underglycosylation of the protein (Jaecken *et al.*, 1984; 1991; Yamashita *et al.*, 1993). Based on this property, two distinct major types of CDGS have been characterised. The CDGS type II has been described in two patients and was found to be caused by a deficiency of the Golgi oligosaccharide processing enzyme *N*-acetylglucosaminyl transferase II. The most common CDGS type I, resulting from the defect in the early glycosylation pathway, has been characterised by a partial or complete lack of entire glycan at the appropriate glycosylation site of protein (Jaecken *et al.*, 1984; 1991; Burda *et al.*, 1998). The defect was found in leukocytes, fibroblasts and liver tissue of the patients (de Koning *et al.*, 1998) and was correlated with severely reduced PMM activity (Hansen *et al.*, 1997). Another type of CDGS, Ib, is linked to PMI deficiency (Fig. 1). Clinical symptoms of CDGS Ib can be cured by mannose supplying therapy (Niehues *et al.*, 1998). The defect observed in the CDGS I was associated with the impairment of the synthesis of lipid-linked oligosaccharide ($DolPPGNAc_2Man_9Glc_3$).

It has to be mentioned that some cases of CDGS I are not directly linked to the GDPMan deficiency but relate to other defects in early (dolichol-dependent) steps of protein glycosylation. Recently biochemical data on four patients with CDGS-type I with normal PMM activity evidenced a specific deficiency in the assembly of dolichol-linked oligosaccharide resulting in the formation of $DolPPGNAc_2Man_9$ lacking three glucose residues (Burda *et al.*, 1998). Such truncated oligosaccharide still could take part in protein glycosylation, however, at least *in vitro* the yeast oligosaccharyltransferase had lower affinity to the non-glucosylated lipid-linked oligosaccharide (Tanner & Lehle, 1987). Moreover this glucosylation is most probably an important step in quality control of N-glycoprotein folding (Hammond & Hellenius, 1995).

Biochemical basis of the CDGS I symptoms were also found to be linked to the defect in dolichol synthesis. The dolichol-linked oligosaccharide levels in synchronised (the S phase) cultures of fibroblasts from these patients were severalfold lower than those in control cells although there was no differences in their sugar content. Analysis of the dolichol biosynthetic pathway indicated severe impairment of the final reaction in biosynthesis of dolichol i.e. conversion of dehy-

drodolichol to dolichol (Ohkura *et al.*, 1997). It should be mentioned that in higher eukaryotes, in contrast to bacteria and yeast, the α-unsaturated polyprenols are not utilized as substrates for the assembly of dolichol-linked oligosaccharides (Jankowski *et al.*, 1986).

CONCLUSIONS

The level of GDPMan synthesised in the cell cytoplams is probably one of the major factors affecting the early steps of glycoconjugates formation. It is noteworthy that mutations of the genes encoding cytoplasmic proteins, leading to lowering of the GMP/GDP level, might alter the structure of the outer sugar chain which is synthesised in the Golgi apparatus. The effect has been ascribed to the perturbed balance of transporter-antiporter mechanism responsible for GDPMan transport into the Golgi compartment. An impairment in GDPMan formation affects also the processes localised in the endoplasmic reticulum i.e. synthesis of MPD and the elongation of the dolichol linked oligosaccharide. Since no transporter activity for sugar nucleotides has been found in these membranes the question arises what is the mechanism of GDPMan translocation through ER membranes. We have observed an increased affinity of MPD-synthase towards GDPMan when the *MPG1* gene was overexpressed in the yeast MPD-synthase mutant. It is reasonable to consider therefore a possibility of direct interaction between the proteins involved in GDPMan and MPD formation. The cytoplasmic MPG1-protein could interact with Dpm1-protein in the ER-membranes and the latter may serve as a GDPMan transporter. This is further substantiated by very recent findings on the role of dolichol in translocation of substrates *via* ER membranes (Sato *et al.*, 1999).

Severe pathological changes observed in the patients with diagnosed CDGS, resulting from the defect in GDPMan synthesis, indicate that the problem is not only of theoretical interest but is also of clinical importance and therefore should be further investigated.

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