Abnormal synthesis of mannose 1-phosphate derived carbohydrates in carbohydrate-deficient glycoprotein syndrome type I fibroblasts with phosphomannomutase deficiency

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In fibroblasts from five patients with carbohydrate-deficient glycoprotein syndrome type I, the incorporation of [2-³H] mannose into mannose phosphates, GDP-mannose, GDP-fucose, dolichol-P-mannose, lipid-linked oligosaccharides, and glycoprotein fraction was determined. We observed a 3- to 5-fold reduction of incorporation of radioactivity into mannose 1-phosphate, GDP-mannose, GDP-fucose, dolichol-P-mannose, and nascent glycoproteins. The incorporation of radioactivity into mannose 6-phosphate was normal. The formation of lipid linked oligosaccharides was only slightly affected (≤20%), but their size was severely reduced, mostly containing five or fewer residues. As a consequence, truncated oligosaccharides were transferred to newly synthesized glycoproteins. The metabolic changes can be explained by a deficiency of phosphomannomutase activity, which was reduced to ≤10% of control.

Key words: phosphomannomutase deficiency/CDGS type I/ mannose metabolites

Introduction

Carbohydrate-deficient glycoprotein syndromes (CDGS) are a group of autosomal recessively transmitted disorders, in which abnormally glycosylated proteins are formed. The biochemical diagnosis of CDGS is based on the abnormal isoelectric focusing pattern of serum glycoproteins such as transferrin, which generally show higher isoelectric points (Jaeken and Carchon, 1993; Jaeken et al., 1993). In the more rare CDGS type II this is fully explained by a deficiency of the Golgi enzyme N-acetylgalactosaminyltransferase II (Jaeken et al., 1994; Tan et al., 1996). This enzyme is required for the synthesis of biantennary complex type oligosaccharides in glycoproteins. Its deficiency leads to the formation of undersialylated glycoproteins. The biochemical defects in CDGS type III (Stibler et al., 1993) and CDGS type IV (Stibler et al., 1995), which so far have both been described in two patients, are unknown.

In CDGS type I, the most common form of the CDGS, a higher isoelectric point of glycoproteins is observed that is due to incomplete usage of N-glycosylation sites, which normally carry sialylated complex type oligosaccharides (Wada et al., 1992; Yamashita et al., 1993). Incomplete usage of N-glycosylation sites points to a defect in the oligosaccharyl transferase or in the assembly of the lipid-linked oligosaccharide (LLO) precursor that serve as a donor for the oligosaccharides transferred onto nascent glycoproteins. While the activity of oligosaccharyl transferase was found to be normal (Knauer et al., 1994), a partial deficiency of dehydrodolichol reductase was observed in three CDGS type I of Japanese origin (Ohkura et al., 1997). The defect was accompanied by an accumulation of dehydrodolichol and decreased levels of dolichol and LLO and proposed to be the cause of CDGS type I. On the other hand, deficiency of phosphomannomutase (PMM) has recently been reported in fibroblasts, leukocytes, and liver from four CDGS typed patients. The intermediate PMM activity in parents of CDGS type I patients (van Schaftingen and Jaeken, 1995) and the identification of mutations in the PMM gene on chromosome 16p13 (Matthijs et al., 1997) strongly suggested that PMM deficiency is the primary cause of the disease.

The significance of the enzymatic abnormalities found in CDGS type I for the synthesis of glycoproteins has been difficult to evaluate due to conflicting data on the metabolic alterations in CDGS type I. Moreover, in most studies only a selected number of mannose metabolites and or enzyme activities have been determined, making it difficult to compare these studies. In three Japanese CDGS type I patients the levels of mannose 6-phosphate, mannose 1-phosphate and GDP-mannose were found to be normal in spite of a deficient PMM activity. Furthermore, the composition of the oligosaccharides in LLOs was normal in these patients (Ohkura et al., 1997). This is in contrast to other patients with CDGS type I, in which truncated oligosaccharides were observed in LLOs and nascent glycoproteins (Powell et al., 1994; Krasnewich et al., 1995; Panneerselvam and Freeze, 1996). While the observations in the Japanese patients would point to a secondary nature of the PMM deficiency, those in the other patients may indicate a decreased availability of GDP-mannose resulting from a deficiency of PMM.

While the present study was in progress, reports on truncated oligosaccharides in LLOs and glycoproteins and the deficiency of PMM and of dehydrodolichol reductase in CDGS type I appeared (Powell et al., 1994; Krasnewich et al., 1995; van Schaftingen and Jaeken, 1995; Panneerselvam and Freeze, 1996; Ohkura et al., 1997). For the five patients with CDGS type I, two of which were sibs, of which the fibroblasts were investigated, we have tried to obtain a comprehensive analysis of mannose derived metabolites. Part of these results have been reported as an abstract (Körner et al., 1995). In all fibroblasts we observed a profound deficiency of PMM, which was associated with a reduction in mannose 1-phosphate, GDP-mannose, GDP-fucose, dolichol phosphate mannose (Dol-P-Man), and the occurrence of truncated oligosaccharides in LLOs and nascent glycoproteins. Unexpectedly, however, the level of [³H] mannose 6-phosphate was normal and [³H] mannose labeled LLOs showed only a slight reduction up to 20% when compared to controls. The biochemical alterations in these patients can sufficiently be explained by a deficiency of PMM activity.
Table I. Incorporation of 3H-radioactivity into GDP-mannose and GDP-fucose

<table>
<thead>
<tr>
<th>Fibroblast line</th>
<th>3H-GDP-mannose (c.p.m./mg protein)</th>
<th>3H-GDP-fucose (c.p.m./mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O. K. (n = 2)</td>
<td>152.1 ± 15.3</td>
<td>99.5 ± 3.1</td>
</tr>
<tr>
<td>A. K.</td>
<td>166.7</td>
<td>103.6</td>
</tr>
<tr>
<td>CDGS type 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K. K. (n = 2)</td>
<td>41.5 ± 9.3</td>
<td>32.5 ± 9.1</td>
</tr>
<tr>
<td>G. M.</td>
<td>52.6</td>
<td>19.6</td>
</tr>
<tr>
<td>P. A.</td>
<td>31.7</td>
<td>11.0</td>
</tr>
</tbody>
</table>

Fibroblasts were metabolically labeled for 30 min with [2–3H] mannose. GDP-mannose and GDP-fucose were isolated from boiled cell extracts as described in Materials and methods.

Results

Synthesis of LLOs with truncated oligosaccharide moieties in CDGS type 1 fibroblasts

In an earlier study we could exclude a deficiency of the N-oligosaccharyltransferase as a cause for the incomplete usage of N-glycosylation sites in glycoproteins of CDGS type 1 fibroblasts (Knauer et al., 1994). We therefore analyzed the formation of LLOs, which serve as the glycosyl donor in N-glycosylation. Cells were metabolically labeled for 30 min with [2–3H] mannose and sequentially extracted with chloroform-methanol (3:2) and chloroform-methanol-H2O (10:10:3). In control fibroblasts LLOs were recovered exclusively in the chloroform-methanol extract. The LLOs of both extracts were subjected to mild acid hydrolysis and the released oligosaccharides were separated by RP-HPLC (Figure 1 and Table I). While the [3H] mannose radioactivity incorporated into LLOs was only slightly reduced (<20%) in two CDGS type 1 fibroblast lines, the structural composition of the oligosaccharides was strikingly different when compared to two control fibroblast lines. In the LLO fraction from controls, oligosaccharides with nine mannose and three glucose residues predominated, while truncated oligosaccharides with five or fewer mannose residues represent less than 10% of the [3H] oligosaccharides. In contrast in CDGS type 1 fibroblasts, oligosaccharides with five or fewer mannose residues were consistently found, their frequency varied considerably, extremes being 13% and 84%. This variation was observed also for each of the fibroblast lines when examined in independent experiments.

Truncated oligosaccharides are transferred onto nascent glycoproteins in CDGS type 1 fibroblasts

Next we examined whether CDGS type 1 fibroblasts transfer truncated oligosaccharides from LLO to nascent glycoproteins. Fibroblasts were metabolically labeled for 30 min with [2–3H] mannose and cells extracts were digested with the endoglycosidases PNGaseF and EndoH, respectively. The released oligosaccharides were subjected to size fractionation. The major oligosaccharide fraction released by PNGaseF from extracts of control fibroblasts had a size corresponding to an oligosaccharide standard containing two core N-acetylglucosamine and 9 mannose residues (Figure 2A). In addition, oligosaccharides with one hexose unit less (M8) or more (G1) were found. Among the oligosaccharides released by PNGaseF from extracts of CDGS type 1 fibroblasts truncated species with five to seven mannose residues were prominent and on a molar basis, assuming the same radioactive specificity, they amount to about 75% (Figure 2B). The size distribution of the oligosaccharides released by EndoH was comparable for control and CDGS type 1 fibroblasts (Figure 2C,D). Oligosaccharides with one core N-acetylglucosamine and nine mannose residues were the most abundant species. Altogether, these data show that truncated oligosaccharides are transferred onto nascent glycoproteins in CDGS type 1 fibroblasts. These are resistant to EndoH, indicating that they lack the α1,3 mannose residue linked to the α1,6 mannose of high mannose oligosaccharides (Kobata, 1979).

Truncated LLOs are suboptimal substrates for the oligosaccharyltransferase and should result in a decreased incorporation of [2–3H] mannose into nascent glycoproteins. Therefore, we compared the incorporation of [2–3H] mannose to that of [35S] methionine into TCA-precipitable material. After metabolic labeling for 30 min the relative incorporation of [2–3H] mannose was reduced in five CDGS type 1 fibroblasts lines to 28–49% of the incorporation of [35S] methionine.
Fig. 2. Size distribution of oligosaccharides released from nascent glycoproteins. Extracts of cell metabolically labeled with [2–3H] mannose for 30 min were digested with PNGaseF (A, B) or EndoH (C, D), and the released oligosaccharides were subjected to size fractionation. In (A) and (C) the oligosaccharides from a control (O.K.) are shown, in (B) and (D) those from a CDGS type 1 (K.K.) fibroblast line. M5, M9, and G3 refer to the elution of GlcNAc2Man5, GlcNAc2Man9 and GlcNAc2Man9Glc3 standards. Oligosaccharides with nine mannose or nine mannose plus one glucose residues released by EndoH elute at a slightly earlier position than the corresponding standard oligosaccharides, since the former carry only a single core N-acetylglucosamine residue.

Fig. 3. Incorporation of [2–3H] mannose and [35S] methionine into nascent proteins. Incorporation of [2–3H] mannose and [35S] methionine in TCA-insoluble material was determined after metabolic labeling of fibroblasts for 30 min. Shown is the ratio of [2–3H] mannose versus [35S] methionine incorporation into TCA insoluble material.

Deficiency of phosphomannomutase in CDGS type 1 is associated with decreased synthesis of mannose 1-phosphate, GDP-Man, GDP-Fuc and Dol-P-Man

After labeling fibroblasts for 30 min with [2–3H] mannose the amount of radioactivity in Dol-P-Man was decreased to 17–20% in three CDGS type 1 fibroblasts compared to controls (Figure 4). When membranes from CDGS type 1 and control fibroblasts were assayed for Dol-P-Man synthase activity, comparable values were measured. This holds true for assays following the transfer of mannose from GDP-[14C] mannose to endogenous or to exogenous and endogenous (not shown) dolicholphosphate.

The low incorporation of [2–3H] mannose into Dol-P-Man can neither be explained by a deficiency of Dol-P-Man synthase nor by a deficiency of dolicholphosphate. Synthesis of the latter has been reported to be normal in CDGS type 1 fibroblasts (Yasugi et al., 1994) and is also indicated by the normal Dol-P-Man synthase activity in the absence of exogenous dolicholphosphate (see above). We therefore examined the synthesis of GDP-mannose. After metabolic labeling for 30 min with [2–3H] mannose, incorporation of radioactivity into GDP-mannose and GDP-fucose was reduced to 11–33% of controls in three CDGS type 1 fibroblast lines (Table I).

While this study was in progress, deficiency of PMM was reported in fibroblasts from four patients with CDGS type 1 (van Schaftingen and Jaeken, 1995). Two of their patients (P.A. and P.S.) were also included in the present study. The deficiency of PMM activity could be confirmed in the fibroblasts of the five CDGS type 1 patients studied here using two different assays. The activities measured with an assay following the conversion of mannose 6-phosphate into mannose 1,6-bisphosphate in the presence of an excess of glucose 1,6-bisphosphate are shown in Table II. The residual PMM activity in CDGS type 1 fibroblasts was ≤10% of controls.

Table II. Phosphomannomutase activity in control and CDGS type 1 fibroblasts

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Phosphomannomutase activity (mU/mg protein) a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>O.K. (n = 6)</td>
<td>0.87 ± 0.086</td>
</tr>
<tr>
<td>M. J.</td>
<td>0.99</td>
</tr>
<tr>
<td>S. M.</td>
<td>1.01</td>
</tr>
<tr>
<td>A. K.</td>
<td>0.75</td>
</tr>
<tr>
<td>CDGS type 1</td>
<td></td>
</tr>
<tr>
<td>K. K. (n = 4)</td>
<td>0.12 ± 0.05</td>
</tr>
<tr>
<td>G. M.</td>
<td>0.02</td>
</tr>
<tr>
<td>P. A.</td>
<td>0.03</td>
</tr>
<tr>
<td>P. S.</td>
<td>0.04</td>
</tr>
<tr>
<td>W. N.</td>
<td>0.07</td>
</tr>
</tbody>
</table>

| a One unit of enzyme catalyzes the formation of 1 mmol of mannose-1,6-bisphosphate/min under standard conditions as described in Materials and methods. |

Type I CDG syndrome
This activity, which was consistently observed in all CDGS type 1 fibroblast lines to about 25% of controls, was below 10% of control. The residual PMM activity was profound deficiency of PMM. The residual PMM activity was measured by two independent assays was below 10% of control. This activity, which was consistently observed in all CDGS type 1 fibroblast lines, could originate from an unrelated phosphohexomutase. The deficiency of PMM led to a decrease in mannose 6-phosphate and metabolites such as GDP-mannose, Dol-P-Man and also of GDP-fucose, the synthesis of which depends on mannose 1-phosphate. One of the predictions for deficiency of PMM would be an accumulation of mannose 6-phosphate, which was not observed, however. This may indicate that phosphomannoisomerase maintains an equilibrium between fructose 6-phosphate and mannose 6-phosphate that prevents accumulation of mannose 6-phosphate.

### Table III. Incorporation of [2–3 H] mannose into mannose 6-phosphate and mannose 1-phosphate

<table>
<thead>
<tr>
<th>Cell line</th>
<th>[3 H]-Man-6-P (c.p.m./mg protein)</th>
<th>[3 H]-Man-1-P (c.p.m./mg protein)</th>
<th>RatioMan-1-P/Man-6-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O. K. (n = 3)</td>
<td>9974 ± 1600</td>
<td>1787 ± 560</td>
<td>0.179</td>
</tr>
<tr>
<td>S. M.</td>
<td>12819</td>
<td>3607</td>
<td>0.281</td>
</tr>
<tr>
<td>A. K. (n = 2)</td>
<td>9168 ± 2309</td>
<td>2318 ± 481</td>
<td>0.252</td>
</tr>
<tr>
<td>CDGS type 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K. K. (n = 3)</td>
<td>10003 ± 2734</td>
<td>512 ± 187</td>
<td>0.051</td>
</tr>
<tr>
<td>G. M.</td>
<td>18241</td>
<td>809</td>
<td>0.044</td>
</tr>
<tr>
<td>P. A. (n = 2)</td>
<td>7796 ± 1028</td>
<td>625 ± 81</td>
<td>0.080</td>
</tr>
</tbody>
</table>

Fibroblasts were metabolically labeled for 30 min with [2–3 H] mannose. Mannose 6-phosphate and mannose 1-phosphate were isolated from boiled cell extracts as described in Materials and methods.

In this as in other studies (Powell et al., 1994; Krasnewich et al., 1995; Panneerselvam and Freeze, 1996) a considerable variability was observed for the composition of oligosaccharides in LLOs from CDGS type 1 fibroblasts. This variability cannot be ascribed to variations in the genetic background, since it was observed for most of the cell lines when examined repeatedly. Apparently, the availability of GDP-mannose and Dol-P-Man for the synthesis of full length LLOs is borderline in CDGS type 1 fibroblasts. When it is limiting to the extent that LLOs with truncated oligosaccharides accumulate, the truncated oligosaccharides are transferred onto nascent glycoproteins.

The lower incorporation of [2–3 H] mannose into nascent glycoproteins can be explained in part by the transfer of truncated oligosaccharides, which on average contain five to six mannose residues compared to nine mannose residues in control fibroblasts. While this can explain a reduction of incorporation of [2–3 H] mannose to about two-thirds of control, the incorporation was reduced to about one-third. This implies that the specific activity of [2–3 H] mannose is lower in CDGS type 1 fibroblasts and that only a fraction of the N-glycosylation sites that are used in control fibroblasts are glycosylated in CDGS type 1 fibroblasts due to suboptimal, truncated glycosyl donor oligosaccharides. Either of the two possibilities is conceivable, and the latter has a precedent in the underglycosylation of transferrin in CDGS type 1 (Yamashita et al., 1993).

Since trimming reactions during oligosaccharide processing remove the glucose residues and the six outer of the nine mannose residues, the structural defect of the oligosaccharide moieties of nascent glycoproteins in CDGS type 1 fibroblasts will mostly be corrected in the mature glycoproteins that have passed the endoplasmic reticulum and the Golgi and carry complex type N-linked oligosaccharides. Mature glycoproteins, however, will still show the underglycosylation due to defective usage of oligosaccharides.
Materials and methods

Chemicals, enzymes, and isotopes
d-[2–3H]mannose (18 Ci/mmol), [35S]methionine, and GDP-D-JU-[14C] mannose (267 mCi/mmol) were from Amersham Buchler (Braunschweig). Recombinant endoglycosidase H (EndoH) from Streptomyces plicatus and N-glycosidase F (PNGase F) from Flavobacterium meningosepticum were from Boehringer (Mannheim). Dolicholphosphate was obtained from Sigma (Deisenhofen). [14C]Dol-P-Man, [2–3H]mannose-6-phosphate (Ohkura et al., 1997) and [2–3H]oligosaccharide standards of known size were prepared as described previously (Lehle, 1980).

Cell culture
Human primary fibroblast cultures established from skin biopsies were kindly provided by Prof. F. Hanefeld (Department of Pediatrics, University Hospital Göttingen, Göttingen, Germany) and Prof. J. Jaeken (Department of Pediatrics, University Hospital Gasthusberg, Leuven, Belgium). Cells were grown at 37°C in the presence of 5% CO2 on Dulbecco’s modified Eagles medium (DMEM, GibcoBRL) supplemented with 10% fetal calf serum (PANSYSTEMS) and passaged by trypsinization.

Metabolic labeling with [2–3H]mannose and [35S]methionine
Fibroblasts (6.6 × 10⁴/cm²) were plated onto culture dishes and grown for 60 h in DMEM. Cells were metabolically labeled for 30 min in MEM free of methionine and glucose reduced to 0.5 mM, supplemented with either 200 or 125 µCi [2–3H]mannose or 2 µCi [35S] methionine. Cells were then washed three times with ice-cold 10 mM phosphate-buffered saline pH 7.4 (PBS), scraped into 10 mM PBS/0.1% Triton X-100 or buffers as indicated and lysed by sonification. Aliquots were used for protein determination following the method of Lowry et al. (1951).

Determination of radioactivity incorporated into proteins
Aliquots of cell lysates were spotted onto Whatman 3MM paper filters and dried for 1 h. The filters were fixed for 10 min in 10% ice-cold TCA, boiled for 5 min in 5% TCA, and subsequently washed in 5% ice-cold TCA followed by a short wash in ethanol/diethyl ether (1:1). Radioactivity was determined by liquid scintillation counting.

Digestion with EndoH and PNGaseF
Cells metabolically labeled with [2–3H]mannose as above were suspended in water supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), lysed by sonification, and dialyzed overnight against water. An aliquot of the dialyzed material was used for protein determination. For EndoH treatment 20 µg of protein lysate was dried under vacuum; resuspended in 50 µl 50 mM 2-mercaptoethanol, 0.1% SDS; sonified; and heated for 5 min at 95°C. The sample was supplemented with 15 µl 0.5 M sodium citrate, pH 5.5, 5 µl 10% PMSF, 5 µl 0.5 µM EndoH, and 75 µl of water. After incubation for 12 h at 30°C the sample was heated for 5 min at 95°C. For PNGase F digestion 100 µg of protein was dried under vacuum, resuspended in 25 µl of 0.1 M 2-mercaptoethanol, 0.5% SDS and heated for 5 min at 95°C. After incubation in 25 µl 0.5 M Tris–HCl, pH 8.0, 10 µl 0.1 M 1,10-phenanthroline, 10 µl 10% Triton X-100, and 5 µl of PNGase F (250 µg/ml), the sample was incubated and heated as above. Released oligosaccharides were separated from protein bound oligosaccharides by elution with water from C18 Sep Pak cartridges (Waters), dried under vacuum, resuspended in 100 µl of water, and analyzed by HPLC (see below).

Metabolic labeling and extraction of Dol-P-Man and LLO
Cells were labeled with [2–3H]mannose as described, scraped into 2 ml of ice-cold methanol, and lysed by sonification. After addition of 4 ml chloroform, the material was again sonified, followed by centrifugation for 10 min at 5000 r.p.m. at 4°C. Supernatants were collected and the pellets extracted two more times with chloroform/methanol (3:2). The combined supernatants containing Dol-P-Man and LLOs of small size were dried under nitrogen, dissolved in 3 ml chloroform/methanol (3:2), washed, and analyzed by thin layer chromatography on Silica gel 60 aluminum sheets in a running buffer containing chloroform/methanol/water (65:25:4) as described previously (Lehle, 1980). The remaining pellet containing the large size LLOs were washed and extracted with chloroform/methanol/water (10:10:3) as described previously (Lehle, 1980). Corresponding aliquots of the chloroform/methanol and chloroform/methanol/H2O extracts were combined and dried under nitrogen and resuspended in 35 µl 1-propanol. To release the oligosaccharides by mild acid hydrolysis 500 µl 0.02N HCl were added followed by an incubation for 30 min at 100°C. The hydrolyzed material was dried under nitrogen, resuspended by sonification in 200µl H2O and cleared by centrifugation. The supernatant containing the released oligosaccharides was used for HPLC analysis (see below).

Size fractionation of oligosaccharides by HPLC
The separation of LLOs was performed on a Supelcosil LC-NH2 column (25 cm × 4.6 mm; 5 µm; Supelco) including a LC-NH2 (2 cm × 4.6 mm) precolumn. A linear gradient of acetonitril from 70% to 50% in H2O (total volume 75 ml) was applied at a flow rate of 1 ml/min. Eluate fractions were analyzed by liquid scintillation counting.
Determination of Dol-P-Man synthase activity

Cells grown as described above were harvested by scraping into 1 ml 10 mM PBS and collected by centrifugation at 1000 × g at 4°C. The cells were suspended in 500 μl PBS and passed 20 times through a 22 gauge needle. After centrifugation for 15 min at 100,000 × g the supernatant was discarded. The pellet was resuspended in 200 μl H₂O and Dol-P-Man synthase activity was measured as described previously (Lehle, 1980).

Preparation of mannose 6-phosphate, mannose 1-phosphate, GDP-mannose, and GDP-fucose

After labeling with [2–3H]mannose cells were harvested by scraping into 2 ml of ice-cold 50% methanol, 10 mM Tris, pH 7.4, 1 mM EDTA. The suspension was boiled for 3 min and sonified. After centrifugation the supernatant was collected. The pellet was extracted two more times. Combined supernatants were dried under N₂, suspended in methanol/water (1:1), and desalted by descending paper chromatography on Whatman no.1 paper in methanol/1 M ammonium acetate, pH 7.5 (5:2) for 16 h. The radioactivity was analyzed by flat screen scanning. Mannose phosphates were separated from nucleotide sugars (GDP-mannose, GDP-fucose) and free mannose. [3H]Mannose phosphates were eluted with water, dried by lyophilization and subjected to high voltage electrophoresis as 65 V/cm for 50 min on Whatman 3MM paper in a buffer containing 80 mM pyridine adjusted to pH 5.5 with acetic acid. [3H]Mannose-phosphates were eluted with water, dried by lyophilization, and subjected to descending paper chromatography on Whatman No.1 paper in butanol/acetic acid/water (3:3:2) for 16 h. [3H]Mannose-phosphates were eluted with water, dried by lyophilization and subjected to mild acid hydrolysis in 0.02 N HCl at 95°C for 30 min. [2–3H]Mannose released from [2–3H]mannose 1-phosphate and [2–3H]mannose 6-phosphate were separated by high voltage electrophoresis as above, eluted, and quantified by liquid scintillation counting.

The nucleotide sugars were eluted from the paper chromatogram (see above) and analyzed by high voltage electrophoresis, paper chromatography, and acid hydrolysis as the mannose phosphates except that acid hydrolysis products ([3H]mannose and [3H]fucose) were separated by thin layer chromatography in acetone/butanol/water (70:15:15).

Analysis of phosphomannomutase

Phosphomannomutase activity was determined by two different methods. The first one followed the protocol of Van Schaftingen and Jaeken (1995). Cells grown as described above, scraped into 20 mM HEPES/150 mM NaCl, and collected by centrifugation. Pellets were resuspended in homogenization buffer (20 mM HEPES, 25 mM KCl, 1 mM dithiothreitol, 10 μg/ml each of leupeptin and antipain). Homogenization was achieved by one freeze-thaw cycle followed by sonification. Phosphomannomutase activity was assayed in a reaction mixture containing 50 mM HEPES pH 7.1, 5 mM MgCl₂, 0.25 mM NADP, 0.1 mM mannose 1-phosphate, 1 μM mannose 1,6-bisphosphate, 10 μg/ml yeast glucose 6-phosphate dehydrogenase, 10 μg/ml phosphoglucomutase, 3.5 μg/ml phosphomannose isomerase, and varying concentrations of cell extract as described. All incubations were carried out at 23°C over a time period of 3 h with continuous recording of absorbance at 340 nm. In a two channel spectrophotometer a parallel assay without substrate was recorded.

In a second test, phosphomannomutase activity was determined by following the conversion of mannose 6-phosphate into mannose 1,6-bisphosphate in the presence of glucose 1,6-bisphosphate. The assay mixture contained 50 mM Tris–HCl, pH 7.5, 2 mM MgCl₂, 50,000 d.p.m. [2–3H]mannose 6-phosphate, 1 mM glucose 1,6-bisphosphate, and varying amounts of cell extract in a total volume of 25 μl. After incubation for 30 min at 37°C the supernatants were collected by centrifugation and [2–3H]mannose 6-phosphate was separated from [2–3H]mannose 1,6-bisphosphate by high voltage paper electrophoresis as described above.

Acknowledgments

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Abbreviations

CDGS, carbohydrate-deficient glycoprotein syndrome; LLO, lipid linked oligosaccharide; PMM, phosphomannomutase; EndoH, endoglycosidase H; PNGaseF, N-glycosidase F; Dol-P-Man, dolichol phosphate mannose.

References


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Type I CDG syndrome


