Mannose supplementation corrects GDP-mannose deficiency in cultured fibroblasts from some patients with Congenital Disorders of Glycosylation (CDG)

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Congenital Disorders of Glycosylation (CDG) are human deficiencies in glycoprotein biosynthesis. Previous studies showed that 1 mM mannose corrects defective protein N-glycosylation in cultured fibroblasts from some CDG patients. We hypothesized that these CDG cells have limited GDP-mannose (GDP-Man) and that exogenous mannose increases the GDP-Man levels. Using a well established method to measure GDP-Man, we found that normal fibroblasts had an average of 23.5 pmol GDP-Man/106 cells, whereas phosphomannomutase (PMM)-deficient fibroblasts had only 2.3–2.7 pmol/106 cells. Adding 1 mM mannose to the culture medium increased the GDP-Man level in PMM-deficient cells to approximately 15.5 pmol/106 cells, but had no significant effect on GDP-Man levels in normal fibroblasts. Similarly, mannose supplementation increased GDP-Man from 4.6 pmol/106 cells to 24.6 pmol/106 cells in phosphomannose isomerase (PMI)-deficient fibroblasts. Based on the specific activity of the GDP-[3H]Man pool present in [2-3H]mannose labeled cells, mannose supplementation also partially corrected the impaired synthesis of mannosphosphoryldolichol (Man-P-Dol) and Glcα3ManαGlcNAcβ2-P-P-Dol. These results confirm directly that deficiencies in PMM and PMI result in lowered cellular GDP-Man levels that are corrected by the addition of mannose. In contrast to these results, GDP-Man levels in fibroblasts from a CDG-Ie patient, who is deficient in Man-P-Dol synthase, were normal and unaffected by mannose supplementation even though mannose addition was found to correct abnormal lipid intermediate synthesis in another study (Kim et al. [2000] J. Clin. Invest., 105, 191–198). The mechanism by which mannose supplementation corrects abnormal protein N-glycosylation in Man-P-Dol synthase deficient cells is unknown, but this observation suggests that the regulation of Man-P-Dol synthesis and utilization may be more complex than is currently understood.

Key words: GDP-mannose/mannose/Congenital Disorders of Glycosylation (CDG)/fibroblast/phosphomannose isomerase

Introduction

Congenital Disorders of Glycosylation (CDG) are caused by defects in protein N-glycosylation (Figure 1; reviewed in Krasnewich and Gahl, 1997; Freeze, 1998, 1999). CDG is usually associated with mental and psychomotor retardation, but some forms cause coagulopathy, hypoglycemia, and liver fibrosis without neurologic involvement (Jaeken et al., 1991; Krasnewich and Gahl, 1997; Freeze, 1998). CDGs have different clinical presentations and biochemical features such as altered isoelectric focusing (IEF) patterns of serum transferrin (Tf) caused by glycosylation-dependent alterations in the number of sialic acid residues (Wada et al., 1992; Yamashita et al., 1993). CDG-I patients under-N-glycosylate glycoproteins due to an insufficient amount or incomplete synthesis of the dolichol-linked precursor oligosaccharide of the protein N-glycosylation pathway (Powell et al., 1994). The enzymatic steps in the assembly of the dolichol-linked oligosaccharide and the en bloc transfer of the precursor oligosaccharide to appropriate asparagine residues of nascent polypeptides in the ER have been reviewed (Kornfeld and Kornfeld, 1985; Waechter, 1989; Cummings, 1992).

Five known defects (Figure 1) alter the synthesis of the lipid intermediate and produce an altered Tf IEF pattern: phosphomannomutase (PMM2, CDG-Ia) (Van Schaftingen and Jaeken, 1995; Jaeken et al., 1997; Matthijs et al., 1997; Körner et al., 1998b), phosphomannose isomerase (PMI, CDG-Ib) (Niehues et al., 1998; Jaeken et al., 1998; De Koning et al., 1998), dolichyl-P-Glc:ManαGlcNAcβ2-P-P-Dol α1,3glucosyltransferase (CDG-Ic, Burda et al., 1998; Imbach et al., 1999; previously called Type V, Körner et al., 1998a), dolichyl-P-Man:ManαGlcNAcβ2-PP-dolichol mannosyltransferase (CDG-Ie, also called Type IV, Körner et al., 1999), and mannosyl-phosphoryldolichol (Man-P-Dol) synthase (CDG-Ie, Kim et al., 2000; Imbach et al., 2000).

Metabolic labeling of fibroblasts with [3H]mannose previously established that CDG-I patients abnormally assemble Glcα3ManαGlcNAcβ2-P-P-Dol, the oligosaccharide donor in the protein N-glycosylation pathway (Figure 1; reviewed by Freeze and Aebi, 1999; Powell et al., 1994; Krasnewich et al., 1995). Furthermore, the defects in Glcα3ManαGlcNAcβ2-P-P-Dol synthesis and reduced protein N-glycosylation could be reversed by the addition of mannose to the tissue culture medium (Panneerselvam and Freeze, 1996a). It has been assumed that insufficient synthesis of GDP-Man occurs in several types of patients, but the cellular levels of GDP-Man
have not been measured directly to confirm this tentative conclusion.

To test this hypothesis, we used a sensitive and reliable method (Rush and Waechter, 1995) to measure the levels of GDP-Man in normal and CDG-Ia and -Ib fibroblasts cultured in the presence and absence of exogenously supplied mannose. Metabolic labeling with [3H]mannose allowed us to calculate the specific activities of the GDP-[3H]Man pools and determine the amounts of Man-P-Dol and Glc\_\alpha\_Man\_GlcN\_\alpha\_2-P-P-Dol synthesized by the cells. These comparisons indicate that GDP-Man levels are dramatically lower in PMM- and PMI-deficient fibroblasts, and that adding 1 mM mannose to the tissue culture medium substantially corrects the GDP-Man deficiency and restores Man-P-Dol and Glc\_\alpha\_Man\_GlcN\_\alpha\_2-P-P-Dol synthesis to virtually normal levels. This study further shows that cellular GDP-Man levels are normal and unaffected by mannose supplementation in one Man-P-Dol synthase deficient fibroblast cell line (Kim et al., 2000).

**Results and discussion**

**Fibroblasts from PMM-deficient patients have reduced cellular levels of GDP-Man**

PMM-deficient patients are predicted to have reduced GDP-Man since they cannot make Man 1-P, the immediate precursor to GDP-Man (Van Schaftingen and Jaeken, 1995; Jaeken et al., 1997; Matthijs et al., 1997). To determine if cellular GDP-Man levels are altered in PMM-deficient fibroblasts, the amounts of GDP-Man in normal and affected fibroblasts were measured using a previously described method combining lectin-affinity chromatography on Con A-Sepharose at low pH and HPAEC on Partisil-10 SAX (Rush and Waechter, 1995). When the Con A-retarded fraction prepared from normal cells was chromatographed on Partisil-10 SAX, a prominent UV-absorbing compound was eluted in the same fractions as authentic GDP-Man (Figure 2, upper panel). However, GDP-Man is barely detectable in the corresponding fraction prepared from each PMM-deficient cell line. An example is shown Figure 2, lower panel. Identical recovery of the GDP-[14C]Man (internal standard) in both samples indicates that the absence of the UV\_254 GDP-Man peak in panel B is not due to poor recovery or to a chromatographic artifact. This experiment indicates that PMM-deficient fibroblasts have a ~90% reduction of GDP-Man. These analyses were done on duplicate samples with nearly identical results.

The GDP-Man peaks were integrated as rider peaks above a baseline as indicated by the dotted tracing in Figure 2. Although the GDP-Man peaks are small in the CDG samples, the Waters Baseline 810 Chromatography Workstation software reproducibly detects and integrates GDP-Man peaks at this attenuation. The reproducible baseline deviation seen is typical of analyses performed at this level of detection, and also occurs in control samples without cellular material. Note
also that the great majority of OD254 in the profile does not coincide with the GDP-[14C]Man internal standard.

Exogenous mannose, but not glucose, restores normal GDP-Man levels in PMM-deficient fibroblasts

Previous studies have shown that inclusion of 1 mM mannose in the tissue culture medium correctly restored the synthesis of truncated dolichol-linked intermediates and the under-N-glycosylation of glycoproteins observed in PMM-deficient fibroblasts (Panneerselvam and Freeze, 1996a). In order to determine if mannose supplementation also restored GDP-Man levels in PMM-deficient fibroblasts, cells from normal and affected individuals were cultured for 30 min in α-MEM containing 0.5 mM glucose in the presence and absence of 1 mM mannose. When fibroblast cultures from 4 unrelated PMM-deficient patients were incubated with 1 mM mannose, the GDP-Man levels increased from an average of 2.4 pmol/10⁶ cells (<10% of normal fibroblasts) to an average of 15.5 pmol/10⁶ cells (~75% of normal fibroblasts) (Table I). Mannose supplementation did not significantly affect the GDP-Man concentration in normal cells.

There are several possible explanations for the observation that exogenous mannose increases the size of the GDP-Man pool in PMM-deficient cells. The additional mannose may raise the cellular concentration of Man 6-P, consequently stimulating the synthesis of Man 1-P by the small amount of residual PMM2 activity (Pirard et al., 1999a). Alternatively, another isozyme, PMM1, could catalyze the reaction at higher concentrations of Man 6-P, although the presence of PMM1 has not yet been established in fibroblasts (Pirard et al., 1999b). Since CHO cells with only 5–10% of normal hexokinase activity maintain a 70% of normal GDP-Man pool (O’Reart et al., 1999), it is quite likely that hexokinase does not catalyze the rate-controlling step in GDP-Man biosynthesis. The possibility that GDP-Man can be synthesized by a hexokinase-independent route cannot yet be excluded.

To determine if the effect of mannose supplementation on cellular GDP-Man levels was specific for mannose, different amounts of glucose were added to control and PMM-deficient fibroblast cultures. As shown in Table II, GDP-Man in two

### Table I. Cellular levels of GDP-Man in normal and PMM-deficient fibroblasts

<table>
<thead>
<tr>
<th>PMM</th>
<th>Normal</th>
<th>PMMa</th>
<th>PMMb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity (nmol/min/mg)</td>
<td>No additions</td>
<td>1 mM mannose</td>
<td>No additions</td>
</tr>
<tr>
<td>Normal</td>
<td>2.68</td>
<td>22.3</td>
<td>19.5</td>
</tr>
<tr>
<td>PMM a</td>
<td>0.09</td>
<td>2.3</td>
<td>16.7</td>
</tr>
<tr>
<td>PMM b</td>
<td>0.16</td>
<td>1.9</td>
<td>15.8</td>
</tr>
<tr>
<td>PMM c</td>
<td>0.19</td>
<td>2.7</td>
<td>16.4</td>
</tr>
<tr>
<td>PMM d</td>
<td>0.10</td>
<td>2.7</td>
<td>13.0</td>
</tr>
</tbody>
</table>

Fibroblast cultures from 4 PMM-deficient CDG patients and one normal control were maintained as described previously (Panneerselvam et al., 1997b). The cells were cultured with α-MEM medium containing 0.5 mM glucose in the presence and absence of 1 mM mannose for 30 min at 37°C and the cellular levels of GDP-Man were determined as described in Materials and Methods.

PMM-deficient fibroblast cell lines was virtually undetectable when cultured in the presence of 0.5 mM glucose, whereas normal fibroblasts had 30.7 pmol/10⁶ cells under these culture conditions. After incubation for 30 min with 2.5 mM glucose, the GDP-Man concentration in the PMM-deficient cells was 9–11 pmol/10⁶ cells. However, when PMM-deficient fibroblasts were cultured in 0.5 mM glucose in the presence of 1 mM mannose, GDP-Man levels were restored to the normal range (25–37 pmol/10⁶ cells). This result indicates that mannose is more effective than glucose in correcting GDP-Man deficiency in PMM-deficient fibroblasts, although glucose, at a higher concentration, partially restores GDP-Man synthesis.

### Effects of supplemental mannose on GDP-Man levels in PMM-deficient fibroblasts

The effects of mannose supplementation on the GDP-Man levels of PMM-deficient fibroblasts were also evaluated (Table III). Previously, metabolic labeling of PMM-deficient fibroblasts with [3H]mannose had provided anomalous results (Niehues et al., 1998). Although the Tf IEF pattern clearly indicated protein “hypoglycosylation,” large amounts of [3H]mannose were incorporated into a variety of mannose containing glycoconjugates. Furthermore, in contrast to the Glc3Man9GlcNAc2-P-Dol intermediate found in PMM-deficient fibroblasts, the [3H]Man-labeled, dolichol-linked glycan from PMM-deficient cells, grown in the absence of mannose, eluted at the position of authentic Glc3Man9GlcNAc2 by gel filtration chromatography on Bio-Gel P-4 (data not shown).

To investigate further the metabolic defect in these cells and to assess the cellular levels of various mannose-containing intermediates, PMM-deficient fibroblasts were metabolically labeled with [3H]mannose for 48 h. The specific activity of the GDP-Man fraction was calculated and used to estimate the amounts of Man-P-Dol and Glc3Man9GlcNAc2-P-P-Dol synthesized by the mutant cells. PMM-deficient cells incubated in the absence of mannose (Table III) had reduced levels of GDP-Man (4.6 pmol/10⁶ cells, <20% of normal), but the specific activity (48,751 c.p.m./pmol) was 130-fold higher than normal cells (373 c.p.m./pmol). However, addition of 1 mM mannose to the tissue culture medium for 48 h restored normal cellular GDP-Man levels (24.6 pmol/10⁶ cells) in the PMM-deficient cells, consequently reducing the specific activity of the GDP-[3H]Man to 1050 d.p.m./pmol.

### Table II. Effect of supplemental glucose and mannose on cellular levels of GDP-Man in normal and PMM-deficient fibroblasts

<table>
<thead>
<tr>
<th>Additions</th>
<th>Glucose (mM)</th>
<th>Mannose (mM)</th>
<th>PMMa</th>
<th>PMMb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(pmol/10⁶ cells)</td>
<td>(pmol/10⁶ cells)</td>
<td>(pmol/10⁶ cells)</td>
<td>(pmol/10⁶ cells)</td>
</tr>
<tr>
<td>0.5</td>
<td>30.7</td>
<td>&lt;1</td>
<td>11.1</td>
<td>37.2</td>
</tr>
<tr>
<td>2.5</td>
<td>27.9</td>
<td>9.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>23.2</td>
<td>27</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fibroblasts from 2 PMM-deficient CDG patients and one normal control were cultured in α-MEM in the presence of the indicated amounts of glucose and mannose for 30 min and the cellular levels of GDP-Man were determined as described in Materials and Methods.
Man9GlcNAc2-P-P-Dol (49% of normal fibroblasts) were altering the total amount of cellular GDP-Man.

This surprising result indicates that mannose addition corrects truncated dolichol-linked oligosaccharide synthesis in Man-P-Dol synthase deficient fibroblasts without altering the total amount of cellular GDP-Man.

The results in Table IV show that a large amount of radioactivity was incorporated into Man-P-Dol and Glc0-3Man,GlcNAc2-P-P-Dol under both labeling conditions. In the absence of added mannose, significantly more [3H]mannose is incorporated into the Man-P-Dol and Glc0-3Man,GlcNAc2-P-P-Dol fractions in PMI-deficient fibroblasts than in the normal cells. However, using the specific radioactivity of the GDP-Man pool to determine the rate of GDP-Man synthesis in PMI-deficient fibroblasts cultured in the absence of added mannose (calculated from Table III), we estimate that the cellular levels of Man-P-Dol (~1.5% of normal) and Glc0-3Man,GlcNAc2-P-P-Dol (~6% of normal) are drastically reduced relative to normal fibroblasts (Table IV). When the PMI-deficient fibroblasts were cultured in the presence of 1 mM mannose, the synthesis of both Man-P-Dol (26% of normal fibroblasts) and Glc0-3Man,GlcNAc2-P-P-Dol (49% of normal fibroblasts) were significantly restored. Mannose supplementation did not affect the synthesis of Man-P-Dol or Glc0-3Man,GlcNAc2-P-P-Dol in normal cells.

These studies with PMI-deficient cells emphasize the importance of considering the specific activity of the GDP-Man pool rather than equating glycoconjugate synthesis with [3H]mannose incorporation (Rush and Waechter, 1995).

GDP-Man levels in Man-P-Dol synthase deficient fibroblasts

Fibroblasts from Man-P-Dol synthase-deficient CDG-Ie patients synthesize a truncated dolichol-linked intermediate (Imbach et al., 2000; Kim et al., 2000) which was corrected when the cells were incubated with exogenous mannose (Kim et al., 2000). To determine if mannose supplementation expanded the cellular GDP-Man pool, the GDP-Man levels of the Man-P-Dol synthase-deficient fibroblasts after metabolic labeling with [3H]mannose for 48 h in the presence and absence of 1 mM mannose were measured. As shown in Table V, the cellular GDP-Man level was not significantly different in Man-P-Dol-deficient cells compared to normal fibroblasts (see Table III) cultured in either the presence or absence of mannose. This surprising result indicates that mannose addition corrects truncated dolichol-linked oligosaccharide synthesis in Man-P-Dol synthase deficient fibroblasts without altering the total amount of cellular GDP-Man.

It is tempting to speculate that mannose addition may trigger an undescribed regulatory element in these CDG fibroblasts. Körner et al. (1998c) reported that long-term glucose starvation partially reverses the protein N-glycosylation defects observed in PMM-deficient fibroblasts. However, a number of laboratories have reported that tissue culture under conditions of glucose-starvation, or energy-depletion, results in the synthesis of truncated dolichol-linked oligosaccharides very similar to that seen in PMM-deficient CDG cells (Turco, 1980; Rearick et al., 1981; Gershman and Robbins, 1981; Datema and Schwarz, 1981). Recently, Doerrler and Lehrman (1999) have shown that induction of the unfolded protein response, by several independent means, reverses the synthesis of truncated dolichol-linked oligosaccharides observed following glucose-starvation in cultured human dermal fibroblasts. Prolonged glucose starvation induces synthesis of stress-related proteins such as GRP78 (BiP) and GRP94 that aid protein folding in the ER (Chapman et al., 1998). Analogous to the glucose regulated protein response, it is conceivable that the availability of mannose or one of its metabolites may regulate the expression of genes needed for protein N-glycosylation. The studies in this report suggest that additional, undescribed, regulatory elements in the assembly of the dolichol-linked intermediate of the protein N-glycosylation pathway may exist.

In conclusion, deficiencies in PMM and PMI reduce the total GDP-Man pool in fibroblasts from these patients and supplementing the culture medium with mannose effectively restores this depleted pool to normal levels. Glucose is significantly less effective in restoring normal GDP-Man levels. The results of this study further emphasize the importance of considering the specific activity of GDP-Man when calculating the rates of manno-olipid and protein N-glycosylation by metabolic labeling with [3H]mannose. It will be important to determine how cells select and utilize mannose or glucose for biosynthesis of GDP-Man in future studies. Finally, the observation that mannose supplementation apparently corrects a defect in Man-P-Dol synthase without affecting the size of the total GDP-Man pool suggests that there are aspects of the regulation of lipid intermediate biosynthesis that have yet to be elucidated.

Materials and methods

Materials

[2-3H]Mannose (15 Ci/mmol) and [U-14C]mannose (250 mCi/mmol) were obtained from American Radiolabelled Chemicals, Inc. (St. Louis, MO). Concanavalin A–Sepharose was purchased from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Partisil-10 SAX silica gel is a product of Whatman (Clifton, NJ). Econosafe scintillation counting cocktail was obtained from Research Products International Corp. (Elk Grove Village, IL). GDP-[1-14C]mannose was synthesized from [U-14C]mannose (Rush and Waechter, 1995). All other chemicals and reagents were obtained from standard commercial sources.

Cell lines and tissue culture

PMM-deficient fibroblasts were described previously (Panneerselvam et al., 1997b). The PMI-deficient fibroblast was obtained from Dr. Marc Patterson, Mayo Clinic, Rochester, MN. The Man-P-Dol synthase deficient cells from...
In Materials and methods without 1 mM mannose. Incorporation of [3H]mannose into GDP-Man, Man-P-Dol and Glc0-3Man9GlcNAc2-P-P-Dol was determined exactly as described previously except that the baseline for peak detection. GDP-Man concentrations were normalized for each determination using the recovery of the GDP-[U-14C]Man as an internal standard and determined the baseline for peak detection. GDP-Man concentrations were normalized for each determination using the recovery of the GDP-[U-14C]Man as an internal standard and were calculated by comparison with standard injections of known amounts of GDP-Man.

**Table IV.** Incorporation of [3H]mannose into Man-P-Dol and Glc0-3Man9GlcNAc2-P-P-Dol in normal and PMI-deficient fibroblasts

<table>
<thead>
<tr>
<th>Fibroblast line</th>
<th>Mannose in medium</th>
<th>[3H]Mannose incorporated into Man-P-Dol</th>
<th>Glc0-3Man9GlcNAc2-P-P-Dol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mM)</td>
<td>(dpm/10⁶ cells) (pmol/10⁶ cells)</td>
<td>(dpm/10⁶ cells) (pmol/10⁶ cells)</td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
<td>7288 (19.6)</td>
<td>23,530 (63.2)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>7096 (21.6)</td>
<td>16,647 (50.7)</td>
</tr>
<tr>
<td>PMIa</td>
<td>0</td>
<td>16,236 (0.3)</td>
<td>189,745 (3.9)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>5457 (5.2)</td>
<td>25,994 (24.7)</td>
</tr>
</tbody>
</table>

Normal and PMI-deficient fibroblast cultures were metabolically labeled with 50 μCi/ml [3H]mannose in α-MEM medium containing 5 mM glucose in the presence and absence of 1 mM mannose. Following incubation for 48 h at 37°C, the incorporation of [3H]mannose into Man-P-Dol and Glc0-3Man9GlcNAc2-P-P-Dol was determined as described in Materials and methods.

**Table V.** Incorporation of [3H]mannose into GDP-Man in Man-P-Dol synthase deficient fibroblasts

<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td></td>
<td>(pmol/10⁶ cells) (dpm/pmol)</td>
</tr>
<tr>
<td>None</td>
<td>26.8 (554)</td>
</tr>
<tr>
<td>1 mM</td>
<td>20 (411)</td>
</tr>
</tbody>
</table>

Dol-P-Man synthase-deficient fibroblast cultures were labeled metabolically with 50 μCi/ml [3H]mannose in α-MEM medium containing 5 mM glucose in the presence and absence of 1 mM mannose. Following incubation for 48 h at 37°C, the incorporation of [3H]mannose into GDP-Man was determined as described in Materials and methods.

the CDG-Ie patient were obtained from Dr. James Filiano, Dartmouth Hitchcock Medical Center. All cells were cultured in α-MEM medium (Gibco BRL) with 5 mM glucose and 10% fetal bovine serum, as described before (Panneerselvam et al., 1997).

Metabolic labeling of cultured fibroblasts with [3H]mannose and extraction of nucleotide sugars

Primary fibroblast cultures (100 mm dishes) were metabolically labeled for 0.5 to 48 h at 37°C by incubation with [3H]mannose (25–50 μCi/ml) in α-MEM, 10% FBS supplemented with or without 1 mM mannose. Incorporation of [3H]mannose into GDP-Man, Man-P-Dol and Glc0-3Man9GlcNAc2-P-P-Dol was determined exactly as described previously except that the cells were disrupted in methanol instead of ethanol, and the aqueous phase following partitioning of the CHCl3/CH3OH (2:1) fraction was retained and analyzed for GDP-Man as described below (Waechter et al., 1983; Rush and Waechter, 1995). The only isotopically labeled compound in the Man-P-Dol fraction was chromatographically identical to authentic Man-P-Dol as determined by thin layer chromatography on silica gel G TLC plates in CHCl3/CH3OH/H2O/NH4OH (65:35:4:1). The radiolabeled compound exhibited the characteristic sensitivity of Man-P-Dol to hydrolysis in mild acid (0.1 N HCl, 50% isopropanol, 50°C, 1h) and was resistant to alkaline hydrolysis (0.1 M KOH, toluene/methanol[1:1], 0°C, 30 min) (Waechter and Scher, 1981).

Assay of the incorporation of [3H]mannose into dolichol-linked oligosaccharides and N-linked glycoproteins

The amount of [3H]mannose incorporated into Glc0-3Man9GlcNAc2-P-P-Dol and glycoprotein in the delipidated residue, from the previous section, was determined by a multiple extraction procedure (Waechter et al., 1983). The radioactive products in the Glc0-3[H]Man9GlcNAc2-P-P-Dol fraction from the PMI-deficient fibroblasts were sensitive to mild acid hydrolysis (10 mM HCl, 80% tetrahydrofuran, 10 min, 100°C) (Lucas et al., 1975) which released a [3H]oligosaccharide that co-eluted with authentic Glc0-3Man9GlcNAc2 following gel filtration on Bio-Gel P-4 and by HPLC on an AX5 ion-exchange column. The delipidated residue was solubilized with 1ml of 1% SDS (3 min, 100°C) and analyzed for protein (Rodriguez-Vico et al., 1989) and for radioactivity. Incorporation of [3H]mannose into cellular products was normalized for cell number based on the amount of protein recovered in the delipidated membrane residue.

Partial purification of GDP-Man

The aqueous phases from the cellular extracts described above were dried under reduced pressure at 30°C, redissolved in 0.2 ml 15 mM ammonium phosphate (pH 3.5) containing 1 mM CaCl2, 1 mM MgCl2 and 1 mM MnCl2 (Con A buffer) and purified by chromatography on a Con A–Sepharose column (Rush and Waechter, 1995).

GDP-Man was purified further by anion exchange chromatography on a 0.5 × 20 cm column of Partisil-10 SAX silica gel using a Waters Baseline 810 Chromatography Workstation HPLC containing a Waters model U6K Universal Liquid Chromatograph Injector (equipped with a 10 ml injection loop), model 501 Solvent Delivery System and a Lambda-Max model 481 LC Spectrophotometer (Rush and Waechter, 1995). The UV absorbance peak associated with GDP-Man in the HPLC chromatogram was integrated using the Waters Base line 810 peak detection and integration software. GDP-Man integrations were rigorously and carefully reviewed from each HPLC determination to insure that the software appropriately determined the baseline for peak detection. GDP-Man concentrations were normalized for each determination using the recovery of the GDP-[U-14C]Man as an internal standard and were calculated by comparison with standard injections of known amounts of GDP-Man.
Acknowledgments
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Abbreviations
CDG, congenital disorders of glycosylation; PMM, phosphomannomutase; PMI, phosphomannose isomerase; GDP-Man, guanosine 5′-diphosphomannose; Man-P-Dol, mannosylphosphoryl dolichol; HPAEC, high performance anion-exchange chromatography.

References


