Analysis of Glycosylation in CDG-Ia Fibroblasts by Fluorophore-assisted Carbohydrate Electrophoresis

IMPLICATIONS FOR EXTRACELLULAR GLUCOSE AND INTRACELLULAR MANNOSE 6-PHOSPHATE*S

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Phosphomannomutase (PMM) deficiency causes congenital disorder of glycosylation (CDG)-Ia, a broad spectrum disorder with developmental and neurological abnormalities. PMM converts mannose 6-phosphate (M6P) to mannos-1-phosphate (M1P), a precursor of GDP-mannose used to make Glc₃Man₉GlcNAc₂-P-P-dolichol (lipid-linked oligosaccharide; LLO). LLO, in turn, is the donor substrate of oligosaccharyltransferase for protein N-linked glycosylation. Impairment of N-linked glycosylation in CDG-Ia blood are hypoglycosylated. Upon labeling with [³H]mannose, CDG-Ia fibroblasts have been widely reported to accumulate [³H]LLO intermediates. Since these are thought to be poor oligosaccharyltransferase substrates, LLO intermediate accumulation has been the prevailing explanation for hypoglycosylation in patients. However, this is discordant with sporadic reports of specific glycopolypeptides (detected with antibodies) from CDG-Ia fibroblasts being fully glycosylated. Here, fluorophore-assisted carbohydrate electrophoresis (FACE, a nonradioactive technique) was used to analyze steady-state LLO compositions in CDG-Ia fibroblasts. FACE revealed that low glucose conditions accounted for previous observations of accumulated [³H]LLO intermediates. Additional FACE experiments demonstrated abundant Glc₃Man₉GlcNAc₂-P-P-dolichol, without hypoglycosylation, in CDG-Ia fibroblasts grown with physiological glucose. This suggested a “missing link” to explain hypoglycosylation in CDG-Ia patients. Because of the possibility of its accumulation, the effects of M6P on glycosylation were explored in vitro. Surprisingly, M6P was a specific activator for cleavage of Glc₃Man₉GlcNAc₂-P-P-dolichol. This led to futile cycling of the LLO pathway, exacerbated by GFP-glycosyltransferase (FACE) experiments with G2M₉Gn₂-P-P-dolichol (lipid-linked oligosaccharide; LLO) added directly or via dolichol-P-mannose to LLOs, would be predicted to inhibit maturation of the oligosaccharide. Since 1994 (6, 8–12), studies on [³H]mannose-labeled LLOs in CDG-Ia fibroblasts have reported reduced quantities of Glc₃Man₉Gn₂-P-P-dolichol, and all but one (12) reported accumulation of immature LLOs (typically M₃-M₉-Gn₂-P-P-Dol). Deficient conversion of dehydrodolichol (polyprenol) to dolichol has also been reported in CDG-Ia fibroblasts and may contribute to LLO biosynthetic defects (12). Thus, the CDG-Ia phenotype has been explained by an inadequate supply of mature LLO coupled with over-abundance of immature LLOs that are poor OT substrates.

Most newly synthesized endoplasmic reticulum (ER) proteins are modified with Glc₃Man₉GlcNAc₂ (G₃M₉Gn₂) by luminal transfer from the mature lipid-linked oligosaccharide (LLO) G₃M₉Gn₂-P-P-dolichol (G₃M₉Gn₂-P-P-Dol) to asparaginyl residues in the context Asn-Xaa-Ser/Thr. The N-linked oligosaccharide is processed by ER glycosidases, resulting in high mannose-type intermediates that participate in folding of the glycoprotein (1, 2). Subsequent export to the Golgi apparatus allows additional modifications, forming complex-type structures, that can participate in the function of the glycoprotein.

The congenital disorders of glycosylation (CDG) are inherited disorders in which the synthesis of LLO (Type I) or the processing of the N-linked oligosaccharide (Type II) is defective. Twelve Type I subtypes (Ia–II) are known (3). In all cases of CDG-I, serum glycoproteins of hepatic origin (transferrin is typically used for clinical evaluation) are underglycosylated. Depending upon the CDG-I subtype, this hypoglycosylation can be due to either inadequate production of mature LLO (limiting the amount of donor substrate for the oligosaccharyltransferase (OT), which transfers oligosaccharide to protein) or production of structurally immature LLOs that are poor donor substrates for OT (4, 5) and can be caused by defects in mannos metabolism or the LLO assembly pathway.

CDG-Ia patients typically show symptoms by six months of age, including developmental and neurological abnormalities. The root cause of CDG-Ia (which accounts for most Type I cases) is a genetic deficiency of phosphomannomutase (PMM), encoded by the PMM2 gene, resulting in a diminished capacity to convert mannose 6-phosphate (M6P) to mannose 1-phosphate (M1P) (4, 5). PMM deficiency would thus be expected to hinder formation of M1P and its metabolite, GDP-mannose (GDP-M), as reported (6, 7). Reduced concentrations of GDP-M, which contributes mannosyl residues (directly or via dolichol-P-mannose) to LLOs, would be predicted to inhibit maturation of the oligosaccharide. Since 1994 (6, 8–12), studies on [³H]mannose-labeled LLOs in CDG-Ia fibroblasts have reported reduced quantities of G₃M₉Gn₂-P-P-Dol, and all but one (12) reported accumulation of immature LLOs (typically M₃-M₉-Gn₂-P-P-Dol). Deficient conversion of dehydrodolichol (polyprenol) to dolichol has also been reported in CDG-Ia fibroblasts and may contribute to LLO biosynthetic defects (12). Thus, the CDG-Ia phenotype has been explained by an inadequate supply of mature LLO coupled with over-abundance of immature LLOs that are poor OT substrates.

7-amino-1,3-naphthalenedisulfonic acid; CDG, congenital disorder(s) of glycosylation; Dol, dolichol; FACE, fluorophore-assisted carbohydrate electrophoresis; F6P, fructose 6-phosphate; GDP-M, GDP-mannose; G₂M₉Gn₂, Glc₃Man₉GlcNAc₂; G₆P, glucose 6-phosphate; LLO, lipid-linked oligosaccharide; M6P, mannose 6-phosphate; M1P, mannose 1-phosphate; SLO, streptolysin-O; PMM, phosphomannomutase; PMI, phosphomannose isomerase; HPLC, high pressure liquid chromatography; OT, oligosaccharyltransferase; FBS, fetal bovine serum.

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The second CDG-I subtype reported, Ib, is caused by a genetic deficiency of phosphomannomutase isomerase (PMI), with hindered formation of M6P from fructose 6-phosphate (F6P). Differing from CDG-Ia, CDG-Ib cells have mainly mature LLOs, although apparently in limited amounts (7, 13). The third form of CDG-I identified, Type Ic, is due to a defect in the specific glycosyltransferase that adds glucose to M6GDP-P-P-Dol. In CDG-Ic, hypoglycosylation presumably occurs because the accumulated M6GDP-P-P-Dol is a poor substrate for OT (14, 15).

Supporting the idea that PMM2 defects in CDG-Ia cause defective LLO synthesis, analyses of total [3H]mannose-labeled glycoprotein pools from cultured CDG-Ia fibroblasts have indicated hypoglycosylation (6, 8–11). However, paradoxically, the few studies examining specific N-linked glycoproteins from cultured CDG-Ia fibroblasts found no quantitative or qualitative glycosylation defects (16–18). Most notably, comparison of α1-antitrypsin from a CDG-Ia patient’s blood and fibroblasts found that only the blood form was hypoglycosylated (18). It is also a paradox that dietary mannose therapy has failed to improve protein glycosylation or symptoms in CDG-Ia patients (4), although LLO defects in cultured CDG-Ia fibroblasts were corrected by supplemental mannose (10), and dietary mannose was found that only the blood form was hypoglycosylated (18). It is also a paradox that dietary mannose therapy has failed to improve protein glycosylation or symptoms in CDG-Ia patients (4), although LLO defects in cultured CDG-Ia fibroblasts were corrected by supplemental mannose (10), and dietary mannose therapy is highly effective for CDG-Ib (13).

We noticed that reports of LLO abnormalities and hypoglycosylation in CDG-Ia fibroblasts were always associated with [3H]mannose pulse labeling of LLOs and glycoproteins (6, 8–12). These studies were done with culture media containing low glucose concentrations, typically 0–0.5 mM, below the physiological minimum of 4–5 mM. Such media are frequently used to increase labeling of cellular glycoconjugates with sugar precursors such as [3H]mannose and [3H]glucosamine and have long been considered suitable for labeling LLOs, since they can be used to detect synthesis of mature LLOs in normal human fibroblasts and cell lines such as CHO-K1. Analyses of CDG-Ia LLOs in physiological glucose have not been reported, because the efficiency of [3H]mannose labeling is so low. In contrast, experiments that reported normal glycosylation of specific glycoproteins used antibody-based methods and CDG-Ia fibroblasts cultured in media with physiological glucose (16–18).

This discrepancy might be explained if LLO synthesis in CDG-Ia fibroblasts was not seriously defective in physiological glucose. If so, the basis for the glycosylation deficiency in patients would require reevaluation. To address these points, we used a nonradioactive method, fluorescent-amine-assisted carbohydrate electrophoresis (FACE), to quantitatively analyze steady-state CDG-Ia LLO pools with media containing subphysiological and physiological concentrations of glucose (19, 20). Our FACE results demonstrated abundant Glc$_3$Man$_2$GlcNAc$_2$-P-P-dolichol, without hypoglycosylation, in CDG-Ia fibroblasts grown with physiological glucose and led us to identify a novel effect of M6P in vitro that might explain hypoglycosylation of proteins synthesized by CDG-Ia patients’ hepatocytes.

MATERIALS AND METHODS

**Cell Cultures and Media**—All cells were grown in a humidified 5% CO$_2$ atmosphere at 37 °C. When needed, low glucose media were prepared either by mixing complete and glucose-free formulations (see Fig. 2 and Table III) or by adding glucose to glucose-free media (Fig. 4). Normal and CDG-I dermal fibroblasts were obtained from the sources listed in Table I and were grown with 10% fetal bovine serum (Atlanta Biologicals) in either RPMI 1640 medium (11 mM glucose) or Dulbecco’s modified Eagle’s medium (5 mM glucose) (Invitrogen) as described (21). CHO-K1 cells and related mutant lines were grown in Ham’s F-12 medium with 2% fetal bovine serum and 8% calf serum. Freshly isolated rat hepatocytes (kindly provided by Dr. Guoxun Chen, Department of Molecular Genetics, University of Texas-Southwestern) were cultured in medium 199 with 10% fetal bovine serum on collagen-coated plates.

**FACE Analyses of Nonradioactive LLOs and N-Glycans from Intact Fibroblasts**—Fibroblasts were cultured until 90% confluent, harvested in methanol, and dried under N$_2$(g). LLOs were recovered in chloroform/methanol/water (10:10:3), hydrolyzed, modified with ANDS fluorophore, and analyzed by FACE exactly as described (19). For N-glycans, the residue remaining after lyophilization was dissolved by boiling with 20 µl of 2% SDS and then diluted 10-fold with 20 mM sodium phosphate buffer, pH 7.6. 10 µl was reserved for protein assay, and the remainder was mixed gently with 20 µl of 7.5% Nonidet P-40. 1 unit of N-glycanase (Calbiochem) was added, followed by incubation at 37 °C for 18 h. 660 µl of absolute ethanol was added, and the tube was placed on ice for 15 min. After centrifugation at 10,000 × g for 15 min, the supernatant was dried under vacuum. The residue was processed for labeling with ANDS as described (19, 20), either directly for total N-glycans or after sequential deamination with AG50-X8 and AG1-X8 (Bio-Rad) for neutral N-glycans. FACE profiling gels were prepared and run as for LLO glycans (19).

**HPLC Analyses of [3H]Mannose-labeled LLO Glycans and N-Linked Glycans from Intact Cells**—Cells were cultured and labeled as described previously (21) with RPMI 1640 medium containing 0.5 mM n-glucose, 10% dialyzed fetal bovine serum (Atlanta Biologicals, GA), and 40 µCi/ml [2-3H]mannose (10–20 mCi/mmol, Amersham Biosciences). In some cases, 10, 30, 100, 300, or 1000 µl unlabeled mannose were added. [3H]LLO glycans released from LLOs with mild acid, and [3H]labeled N-glycans released from protein with N-glycosidase F (Calbiochem), were analyzed by HPLC (22). The peak height of each [3H]glycan was normalized to mannose content prior to calculation of percentage abundance (21).

**FACE and HPLC Analyses of [3H]-Labeled LLO Glycans from Permeabilized Fibroblasts**—Fibroblasts were cultured until 90% confluent, refed with fresh medium for 1 h, permeabilized with SLO essentially as described for CHO-K1 cells (23), and incubated with 2 ml of reaction buffer (50 mM K-HEPES, pH 7.4, 78 mM KCl, 4 mM MgCl$_2$, 100 µM UDP-GlcNAc, 500 µM UDP-glucose, 2 mM 5′-AMP, 100 µg/ml castanospermine, 20 mM GDP-[3H]mannose, and different concentrations of unlabeled GDP-M as indicated). Cells were then incubated at 37 °C for 30 min. The buffer was removed, and cells were washed twice with ice-cold PBS and then harvested into methanol. The suspension was transferred to a 10-ml glass tube and dried under N$_2$(g). LLO [3H]glycans were recovered, detected by HPLC (23), or labeled with ANDS, mixed with ANDS-labeled mouse liver LLO glycan standards, and resolved with a FACE gel (19). Glycans were extracted from bands cut from the gel with 1 ml of water overnight and mixed with scintillation fluid, and tritium was detected by scintillation spectroscopy.

**FACE Analyses of Unlabeled LLO Glycans and Free Glycans from Permeabilized Cells**—Cells were cultured to 90% confluence, permeabilized with SLO (23), and incubated at 37 °C for 1 h with 2 ml of reaction buffer (see above, but without GDP-[3H]mannose and containing 20 µM unlabeled GDP-M). After removal of reaction buffer, the cells were washed twice with ice-cold PBS, and then harvested into methanol. After drying under N$_2$(g), sequential extractions were performed with chloroform/methanol (2:1) (discarded), with water (free glycans), and with chloroform/methanol/water (10:10:3) (LLOs). Free glycans were deionized with AG50-X8 and AG1-X8 resins. LLOs were cleared with weak acid, and LLO glycans were recovered (19). All glycans were labeled with ANDS and resolved with a FACE gel.

**In Vitro OT Assay**—Fibroblasts were permeabilized with SLO and labeled with GDP-[3H]mannose as described above, except 40 µM OT acceptor peptide or OT control peptide (24) was included. Cells were harvested and extracted as described above. The water extract was deionized with AG50-X8 and AG1-X8 to remove residual salt and GDP-[3H]mannose. Molecules with exposed reducing termini were modified with ANTS (19) and removed with a second AG1-X8 treatment. After drying, the glycopeptides were dissolved with 0.1 M sodium citrate buffer, pH 5.5, containing 0.1 units of endoglycosidase H and incubated at 37 °C for 18 h. Deionization with AG50-X8 and AG1-X8 was repeated. Endoglycosidase H-released glycans were modified with ANDS and analyzed in one of three ways: (i) a portion was counted directly to assess total [3H]Hglycan transferred from LLO to glycopeptide; (ii) a portion was treated with AG1-X8, and the bound (ANDS-modified) and unbound materials were counted to measure endoglycosidase H-sensitive and -resistant glycopeptide, respectively; or (iii) a portion was mixed with standards (endoglycosidase H-digested liver LLO glycans labeled with ANDS) and resolved by FACE (19), in which case bands containing individual glycans were cut from the gel, eluted with 1 ml of water, and counted. [3H]LLO glycans in the chloroform/methanol/water (10:10:3) fraction were analyzed with FACE gels in the manner described above for glycopeptides.
CDG-Ia Fibroblasts in Conventional Media Synthesize G3M9Gn2-P-P-Dol Efficiently—LLOs in four normal, five CDG-Ia, a CDG-Ib, and a CDG-Ic culture (Table I) were analyzed quantitatively by FACE after growth in conventional media. In Fig. 1, cells were cultured in RPMI 1640 medium containing 11 mM glucose and 10% undialyzed FBS. CDG-Ic cells accumulate M3Gn2-P-P-Dol by the [3H]mannose labeling technique (25). They gave a similar result with FACE (Fig. 1A) with mainly M3Gn2-P-P-Dol and smaller quantities of G3M9Gn2-P-P-Dol due to leakiness of the mutation. Also, as expected from [3H]mannose labeling, CDG-Ib (PMM-deficient) fibroblast LLOs were mainly M3Gn2-P-P-Dol (Fig. 1A). Prior studies suggested that CDG-Ib fibroblasts contain abnormally low amounts of M3Gn2-P-P-Dol (7, 13). By FACE, the amounts of G3M9Gn2-P-P-Dol detected in CDG-Ib fibroblasts were highly variable, from 100% (in Fig. 1A) to 20% of normal (data not shown). The basis for this variability was not determined, although the PMI activity was also quite variable (Supplemental Fig. 1).

Thus, it was anticipated that CDG-Ia (PMM-deficient) LLO glycan analyses by FACE would be mainly immature structures such as M2–5Gn2. However, with multiple independent cultures, LLO glycans from CDG-Ia fibroblast cultures grown with conventional RPMI 1640 were mainly G3M9Gn2- and were indistinguishable from normal (Fig. 1, A and B). This was verified by quantitative FACE analysis for duplicate cultures of three normal and four CDG-Ia fibroblasts (Table II). Due to this unanticipated result, we verified that our CDG-Ia fibroblasts were authentic, having expected decreases of PMM activity (26), with both a conventional enzyme-linked PMM assay and a novel FACE-based PMM assay (Supplemental Fig. 1).

In Subphysiological Glucose Both Normal and CDG-Ia Fibroblasts Synthesize LLOs Abnormally, but CDG-Ia Fibroblasts Are More Sensitive—To determine whether differences between the results of Fig. 1 and previous reports (6, 8–11) were due to the method of detection (FACE versus [3H]mannose labeling) or to the use of conventional media in place of low glucose media, LLOs were examined by FACE after culturing fibroblasts for 30 min. in different concentrations of glucose. Diazyized FBS was used to eliminate the serum as a source of hexose. An example is shown in Fig. 2A, and quantitative analyses are shown in Fig. 2B–E. LLOs from normal and CDG-Ia fibroblasts grown with RPMI 1640 (Fig. 2, A–C) containing 10 mM glucose are mainly G3M9Gn2-P-P-Dol, although a small amount of M3Gn2-P-P-Dol is apparent in CDG-Ia. Nearly equal representation of G3M9Gn2-P-P-Dol and M3Gn2-P-P-Dol occurs with 2.5 mM glucose for normal fibroblasts (Fig. 2B) and with 5 mM glucose for CDG-Ia (Fig. 2C). Similar results were obtained by comparing duplicate cultures of four normal and three CDG-Ia fibroblasts in a single experiment (Table III) and with Dulbecco’s modified Eagle’s medium (Fig. 2, D and E). These data reflect earlier

## RESULTS

### CDG-Ia Fibroblasts in Conventional Media Synthesize G3M9Gn2-P-P-Dol Efficiently

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<tr>
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### FACE Analysis of CDG-Ia

G3M9Gn2-P-P-Dol in normal and CDG-Ia fibroblasts, grown continuously with RPMI 1640 medium and 10% FBS, was measured by FACE (expressed as nmol/mg cellular protein) as in Fig. 2. The table is a composite of three sets of experiments.

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### Related experiments were done by [3H]mannose labeling

For [3H]mannose labeling efficient enough to detect glycans by HPLC, glucose was kept at 0.5 mM. The mannose concentration was then varied. Consistent with results presented above and reported earlier (7, 10), [3H]G3M9Gn2-P-P-Dol increased when fibroblasts were supplemented with mannose, but CDG-Ia fibroblasts required at least 10-fold higher [mannose] than did normal cells to produce a similar amount of [3H]G3M9Gn2-P-P-Dol (data not shown).

The minimal glucose concentration in blood is typically 4–5.
mm Glucose levels in CDG-Ia patients are usually in the normal range, although a rare case of hypoglycemia has been reported (27). CDG-Ia fibroblasts in physiological glucose make normal amounts of total M5Gn2-P-P-Dol plus G3M9Gn2-P-P-Dol (Fig. 3, B–E) and have G3M9Gn2-P-P-Dol readily available for transfer by OT (see below). However, in 5 mM glucose, they accumulate M5Gn2-P-P-Dol in amounts equal to G3M9Gn2-P-P-Dol (Fig. 2). If OT in CDG-Ia cells failed to use M5Gn2-P-P-Dol efficiently, due to selectivity for G3M9Gn2-P-P-Dol, this could cause hypoglycosylation. On the other hand, if OT recognizes both M5Gn2-P-P-Dol and G3M9Gn2-P-P-Dol, hypoglycosylation should not occur (although there may be other consequences of transferring M5Gn2). The net activity of OT in CDG-Ia cells with G3M9-P-P-Dol is normal (28), but selectivity of OT has not been examined. Below, the consequences of partial accumulation of M5Gn2-P-P-Dol in CDG-Ia cells in 5 mM glucose are examined in detail.

**OT in CDG-Ia Fibroblasts Is Selective for G3M9Gn2-P-P-Dol over M5Gn2-P-P-Dol in Vitro—Streptolysin-O (SLO) permeabilizes the plasma membrane, but not internal membrane systems** (29), and was used to develop an in vitro system programmed with GDP-[3H]mannose in which the selectivity of OT for [3H]G3M9Gn2-P-P-Dol over [3H]M5Gn2-P-P-Dol could be tested. The use of SLO eliminates LLO synthesis artifacts of microsomal membrane preparations (23, 24). Permeabilized cells were incubated with either 10 nM (Fig. 3, upper graph) or 200 nM (lower graph) GDP-[3H]mannose, resulting in different proportions of [3H]G3M9Gn2-P-P-Dol and [3H]M5Gn2-P-P-Dol. Reactions were done in the presence of a control OT nonacceptor peptide (Ac-Gln-Tyr-Thr-CONH2) to access LLO synthesis or with an acceptor peptide (Ac-Asn-Tyr-Thr-CONH2) to test the transfer selectivity of OT. The compositions of the [3H]LLOs were determined by HPLC and expressed as percentages of either G0–3M6–9Gn2-Dol or M1–5Gn2-Dol in the total pool after normalizing to mannose content. The compositions of peptide-associated glycans were assessed by digestion with endoglycosidase H, which distinguished G0–3M6–9Gn2-P-P-Dol peptides (sensitive) from M1–5Gn2-P-P-Dol peptides (resistant).

With control peptide, no differences were detectable between normal and CDG-Ia cells regarding the synthesis of [3H]LLOs (Fig. 3). Transfer of glycan to the control peptide was negligible (data not shown). Importantly, the selectivity of OT in normal and CDG-Ia fibroblasts was highly similar. In all cases, there was greater representation of G0–3M6–9Gn2, and lower representation of M1–5Gn2, in the N-glycan (acceptor peptide) pool compared with the LLO pool. Thus, N-linked glycoproteins produced by CDG-Ia fibroblasts in 5 mM glucose might be expected to be hypoglycosylated mainly with G3M9Gn2, even with a sizeable amount of M5Gn2-P-P-Dol.

**Failure of OT to Selectively Recognize G3M9Gn2-P-P-Dol in Intact Normal and CDG-Ia Fibroblasts—OT selectivity was then tested in intact cells, by pulse-labeling with 2.5 μM [3H]mannose in the presence of 0.5 mM glucose and different concentrations of unlabeled mannose. This resulted in [3H]LLO pools with various amounts of LLO intermediates and G3M9Gn2-P-P-Dol. As mentioned above, G3M9Gn2-P-P-Dol increased with [mannose], but CDG-Ia fibroblasts required relatively higher [mannose]. LLOs and N-glycans from the same dish were compared by HPLC. Typical results are shown in Fig. 4. The identities of the N-glycans as discussed previously (22, 30) made it possible to determine whether they were derived from intermediates, such as M5Gn2-P-P-Dol, or from G3M9Gn2-P-P-Dol. With 2.5 μM mannose, G3M9Gn2-P-P-Dol was barely detectable in normal fibroblasts (Fig. 4A), but it accounted for much of the N-glycan pool (Fig. 4B), consistent with selectivity by OT. With 12.5 μM mannose, there was more G3M9Gn2-P-P-Dol (Fig. 4C), but unexpectedly, the proportions of G3M9Gn2 in the LLO and N-glycan pools appeared similar (Fig. 4D), indicating a lack of selectivity by OT. Mannose concentrations in the 0.1–0.3 mM range were needed to produce enough G3M9Gn2-P-P-Dol to evaluate OT selectivity in CDG-Ia fibroblasts. As shown in Fig. 4, E–H, regardless of the proportion of G3M9Gn2-P-P-Dol, there did not appear to be selective transfer from this substrate to protein by OT.

To summarize, OT was selective for G3M9Gn2-P-P-Dol in vivo (Fig. 3) but not in intact fibroblasts (Fig. 4) unless very low concentrations of hexose (such as 2.5 μM mannose/0.5 mM glucose) were used. Throughout our experiments (Fig. 4 and data not shown) with intact CDG-Ia cells, selectivity of OT for G3M9Gn2-P-P-Dol was never observed. The effects of higher extracellular hexose concentrations are not understood but suggest that in media with 5 mM glucose, immature LLOs such as M5Gn2-P-P-Dol will be substrates for OT. Thus, in conventional media, CDG-Ia fibroblast glycoproteins would not be expected to be hypoglycosylated, as found in studies of specific glycoproteins (16–18). However, a significant fraction may be modified with truncated glycans that are not competent for protein folding (2), consistent with chronic ER stress in CDG-Ia fibroblasts grown in conventional media (21).

**Availability of G3M9Gn2-P-P-Dol for Glycosylation in CDG-Ia Fibroblasts—Because N-linked glycans derived from either G3M9Gn2-P-P-Dol or LLO intermediates such as M5Gn2-P-P-Dol can be processed by Golgi apparatus enzymes, there should be little difference between N-glycan pools from normal and CDG-Ia fibroblasts in conventional media. As shown in Supplemental Fig. 2, very similar FACE profiles for N-glycans...**
Mannose 6-Phosphate Stimulates Selective Cleavage of G\text{M}_{6}\text{Gn}_{9}\text{P-P-Dol, with Futile Cycling of the LLO Pathway—}In the absence of a clear explanation for hypoglycosylation of serum proteins in CDG-Ia patients, we considered that M6P could be a “missing link,” because it might accumulate in PMM-deficient hepatocytes. Thus, the possibility that M6P might interfere with LLO synthesis or stability was tested. This was done initially by GDP-[3H]mannose labeling with permeabilized fibroblasts (Fig. 6) and then by FACE analysis of permeabilized CHO-K1 cells and primary hepatocytes (Fig. 7).

Incubation with M6P, but no other sugar phosphates, increased the amount of water-soluble [3H]mannose-labeled material in a normal fibroblast system with 200 nM GDP-M (Fig. 6A) while having negligible effect on total radioactivity incorporated into LLO. Examinations of similarly prepared fractions by HPLC revealed that, in the absence of M6P, 200 nM GDP-M does not support efficient LLO extension, resulting in a modest amount of G\text{M}_{6}\text{Gn}_{9}\text{P-P-Dol mixed with LLO intermediates. M6P caused selective loss of G\text{M}_{6}\text{Gn}_{9}\text{P-P-Dol from the LLO pool, with insignificant effects on LLO intermediates (Fig. 6B, left). Loss of G\text{M}_{6}\text{Gn}_{9}\text{P-P-Dol coincided with appearance of free G\text{M}_{6}\text{Gn}_{9} in the water-soluble pool (Fig. 6B, right).}

Other hexose phosphates that are intermediates in mannose metabolism (M1P, G6P, glucose 1-phosphate, and F6P) had no effect (Fig. 6B). Thus, the cleavage was highly specific for G\text{M}_{6}\text{Gn}_{9}\text{P-P-Dol as the LLO and for M6P as the activating hexose-P. This suggests degradation by enzyme action rather than non-specific cleavage. With 400 nM GDP-M, extension of LLOs was efficient, and G\text{M}_{6}\text{Gn}_{9}\text{P-P-Dol was predominant (Fig. 6C). In this case, M6P did not affect the amount of G\text{M}_{6}\text{Gn}_{9}\text{P-P-Dol, but it still increased free G\text{M}_{6}\text{Gn}_{9}. Along with G\text{M}_{6}\text{Gn}_{9}, cleavage of G\text{M}_{6}\text{Gn}_{9}\text{P-P-Dol would be expected to yield Dol-P, which can be used for additional rounds of LLO synthesis, or either Dol-OH or Dol-P-P, which can be recycled to Dol-P (31). Thus, the increased (GDP-M) was able to compensate for futile cycling by driving additional complete rounds of G\text{M}_{6}\text{Gn}_{9}\text{P-P-Dol synthesis, without affecting G\text{M}_{6}\text{Gn}_{9}\text{P-P-Dol cleavage. Some G\text{M}_{6}\text{Gn}_{9}\text{P-P-Dol is cleaved even in the absence of M6P, a phenomenon often attributed to “transfer of the glycan to water” by OT. M6P (400 nM GDP-M) increased free G\text{M}_{6}\text{Gn}_{9} at all time points tested but did not affect LLO formation (Fig. 6D). It is highly unlikely that M6P itself was an acceptor, because “Glc\text{Man}_3\text{GlcNAc}_2\text{M6P” should not label with ANDS and should not comigrate with a G\text{M}_{6}\text{Gn}_{9}-ANDS standard. By a similar argument, it is unlikely that G\text{M}_{6}\text{Gn}_{9}-P was released instead of G\text{M}_{6}\text{Gn}_{9}.

Experiments in Fig. 6, A–D, used normal fibroblasts. Similar results were obtained with permeabilized CDG-Ia fibroblasts. With 200 nM GDP-M, M6P decreased G\text{M}_{6}\text{Gn}_{9}-P-Dol (Fig. 6E) and increased free G\text{M}_{6}\text{Gn}_{9} (Fig. 6F). Curiously, we consistently noticed that M6P caused appreciable accumulation of G\text{M}_{6}\text{Gn}_{9}-P-Dol in CDG-Ia cells (Fig. 6E), but not in normal cells (Fig. 6B). Since SLO treatment removes diffusible cytoplasmic components, this difference is most likely due to a membrane-associated property of CDG-Ia fibroblasts, possibly diminished conversion of polyoleryl to dolichol (12). The limited availability of dolichol may in some way impede mannosylation of G\text{M}_{6}\text{Gn}_{9}-P-Dol in CDG-Ia cells, and its resistance to cleavage by the action of M6P (Fig. 6B) would cause it to accumulate.

M6P Accelerates Cleavage of G\text{M}_{6}\text{Gn}_{9}-P-Dol Synthesized by CHO-K1 Lines and Primary Hepatocytes—The ability of M6P to specifically promote cleavage of G\text{M}_{6}\text{Gn}_{9}-P-Dol was
not limited to fibroblasts or GDP-\[^3\]H\]mannose labeling techniques. FACE analysis of SLO-permeabilized CHO-K1 cells demonstrated that M6P, but not M1P, increased the rate of release of G\(_{3}\)M\(_9\)Gn\(_2\) (Fig. 7A), with maximal release by 10–100 μM M6P (Fig. 7B). Comparison of parental CHO-K1 cells with a mutant line (Lec35.1), which synthesizes M5Gn2-P-P-Dol instead of G\(_{3}\)M\(_9\)Gn\(_2\)-P-P-Dol, and the same mutant line transfected (10A) to correct the mutant phenotype (23) showed that M6P caused cleavage of G\(_{3}\)M\(_9\)Gn\(_2\)-P-P-Dol but not M5Gn2-P-P-Dol (Fig. 7C), similar to its effects on fibroblast LLOs (Fig. 6B). M6P also stimulated release of G\(_{3}\)M\(_9\)Gn\(_2\) in permeabilized hepatocytes. Other hexose phosphates were not effective (Fig. 7D). As for CHO-K1, maximal release was achieved with 10–100 μM M6P (data not shown).

The preference for G\(_{3}\)M\(_9\)Gn\(_2\)-P-P-Dol as a substrate over M5Gn2-P-P-Dol in vitro is characteristic of both enhanced cleavage with M6P and glycan transfer to peptide, suggesting that OT catalyzes both activities. Although M6P caused cleavage of G\(_{3}\)M\(_9\)Gn\(_2\)-P-P-Dol in SLO-permeabilized CHO cells, it did not with enriched CHO-K1 microsomal preparations in the absence or presence of 0.1% Nonidet P-40 (data not shown). Thus, like the Lec35 mutant phenotype (23) and LLO synthesis inhibition by translation blockers (24), which are preserved in SLO-treated cells but not microsomes, the effect of M6P on G\(_{3}\)M\(_9\)Gn\(_2\)-P-P-Dol cleavage may be highly sensitive to physical perturbation. Unfortunately, this precluded testing M6P with solubilized or purified preparations of OT. A specific yeast OT inhibitor was evaluated, but this reagent was ineffective with OT in SLO-permeabilized CHO-K1 (data not shown). If M6P acted by enhancing the hydrolytic action of OT, OT acceptor peptide substrate should compete. As shown in Fig. 7E, OT acceptor peptide suppressed the M6P-stimulated cleavage of G\(_{3}\)M\(_9\)Gn\(_2\)-P-P-Dol, under conditions where G\(_{3}\)M\(_9\)Gn\(_2\)-Peptide was formed (data not shown).

**DISCUSSION**

**Choice of Analytical Method for LLO Analysis in Fibroblasts**—In contrast to previous analyses of \[^3\]H\]mannose-labeled CDG-Ia fibroblasts, which involved culture in low glucose and indicated robust LLO abnormalities and hypoglycosylation of protein, FACE analyses of CDG-Ia fibroblasts showed only moderate LLO abnormalities in 5 mM glucose (−50% accumulation of M\(_2\)Gn\(_2\)-P-P-Dol), which did not lead to obvious hypoglycosylation. Our results agree with previous studies of N-glycosylation in CDG-Ia fibroblasts with physiological glucose, which also failed to identify protein glycosylation abnormalities (16–18), and indicate that CDG-Ia dermal fibroblasts are not adequate models for clinical hypoglycosylation. Glucose concentrations (such as 2.5 mM in Fig. 2A) can be identified with which normal fibroblasts are relatively rich in G\(_{3}\)M\(_9\)Gn\(_2\)-Peptide. This may explain why investigators using the \[^3\]H\]mannose labeling technique were able to report the relative accumulation of truncated LLOs in CDG-Ia fibroblasts.

**Synergism between Elevated [M6P] and Diminished [GDP-M] in CDG-Ia**—Our results suggest a model involving both diminished M1P and increased M6P (Fig. 8). Due to PMM deficiency, M6P may be expected to accumulate in hepatocytes due to the absence of a functional ER-associated M6Pase system and then cause selective depletion of G\(_{3}\)M\(_9\)Gn\(_2\)-P-P-Dol with release of G\(_{3}\)M\(_9\)Gn\(_2\). In intact cells and in cells permeabilized with SLO, new rounds of LLO synthesis do not occur unless existing LLO is discharged (such as with an OT acceptor
M6P is a specific activator of G\textsubscript{M\textsubscript{6}P\textsubscript{Gn\textsubscript{2}}} release from the LLO pools of permeabilized fibroblasts labeled with GDP-[\textsuperscript{3}H]mannose. Experiments involved fibroblast N-1 (A–D) or CDG-Ia-1 (E and F) permeabilized with SLO and incubated with mixtures containing GDP-[\textsuperscript{3}H]mannose. A, tritium associated with the total LLO (black bars) and free glycans (white bars) pools after incubation for 30 min with 200 nM GDP-M, without hexose-P (CON), or with 50 \( \mu \)M hexose-P as indicated (averages of duplicates). B, HPLC of samples incubated as in A, for 30 min. Full scales for LLO and free glycan chromatograms are 50,000 and 300,000 cpm, respectively. C, as for B, except 400 nM GDP-M was used. Full scales for LLO and free glycan chromatograms are 50,000 and 70,000 cpm, respectively. D, as for A, except that 400 nM GDP-M was used, and the incubation time was varied. LLO and free glycans are plotted in the upper and lower graphs, respectively. Black symbols, control. White symbols, 50 \( \mu \)M M6P. E, incubations as for A, with 50 \( \mu \)M hexose phosphates. Shown are cpm incorporated into the LLO glycans G\textsubscript{M\textsubscript{6}P\textsubscript{Gn\textsubscript{2}}} (black bars) and M\textsubscript{Gn\textsubscript{2}} (white bars) resolved and isolated with FACE gels (averages of duplicates). F, as for E, but detecting free G\textsubscript{M\textsubscript{6}P\textsubscript{Gn\textsubscript{2}}} by HPLC (average of duplicates).
**FIG. 7.** FACE analyses of release of $\text{G}_3\text{M}_9\text{Gn}_2$ from the LLO pools of permeabilized CHO-K1 cells and primary hepatocytes. CHO-K1-derived cultures (A–C and E) and primary rat hepatocytes (D) were permeabilized with SLO. Water-soluble glycans were quantified by FACE. A, time-dependent release of $\text{G}_3\text{M}_9\text{Gn}_2$ (pmol/10$^7$ cells) from parental CHO-K1 cells incubated with 20 $\mu$M GDP-M in the absence of hexose-P (control, black circles) or with 50 $\mu$M M6P (white circles) or M1P (white squares). B, as for A, incubation for 60 min with varying concentrations of M6P. C, as for A (control (black bars) and 50 $\mu$M M6P (white bars)), with 60-min incubations for parental CHO-K1; the mutant line Lec35.1, which accumulates M$_5$Gn$_2$-P-P-Dol instead of G$_3$M$_9$Gn$_2$-P-P-Dol; or the 10A line, created by transfecting the Lec35.1 mutant with intact Lec35 cDNA, with restored production of G$_3$M$_9$Gn$_2$-P-P-Dol. The release of the predominant LLO glycan of each line was measured by FACE (average of duplicates). D, release of $\text{G}_3\text{M}_9\text{Gn}_2$ from primary hepatocytes incubated with 20 $\mu$M GDP-M for 60 min in the absence or presence of 100 $\mu$M hexose phosphate as indicated. Note that in A–D, the 20 $\mu$M GDP-M was 50 times that required to prevent M6P-dependent changes in the LLO pool (see Fig. 6, B and C). As a result, M6P treatment did not affect the LLO compositions in these experiments (data not shown). E, assays measured release of $\text{G}_3\text{M}_9\text{Gn}_2$ from permeabilized CHO-K1, as for B, in the absence or presence of 100 $\mu$M M6P and the indicated concentrations of OT acceptor peptide.

**FIG. 8.** Proposed metabolic consequences of PMM deficiency on the dolichol pathway in CDG-Ia hepatocytes. A, the normal pathway; B, the CDG-Ia pathway proposed around 1994 (8); C, the revised CDG-Ia pathway model based upon diminished GDP-M synthesis, M6P accumulation, and stimulation of OT by M6P to cleave G$_3$M$_9$Gn$_2$-P-P-Dol. Molecules expected to increase in concentration in CDG-Ia are indicated by larger red lettering, whereas those expected to decrease are indicated by smaller blue lettering. The thickness of the arrows emphasizes relative metabolic flux.
can then trigger the defects observed in CDG-Ia.

Incorporation of [⁴H]mannose into M6P occurs at a normal rate, whereas incorporation into M1P is 25% of normal, in CDG-Ia fibroblasts after a 30-min pulse in 0.5 mM glucose (6). If the use of low glucose did not invalidade these measurements, they would argue against M6P accumulation in CDG-Ia fibroblasts. By FACE monosaccharide analysis (data not shown), [M1P] in CDG-Ia fibroblasts was 44% of normal; [M6P] in normal and CDG-Ia was below the level of detection. However, extrapolation to hepatocytes may not be justified. In addition to having a more complex regulatory system for hexose metabolism, the endoplasmic reticulum in hepatocytes contains a highly selective G6P transporter that excludes M6P from the lumen, so that M6P is not hydrolyzed by luminal hexose 6-phosphatase. In contrast, the fibroblast endoplasmic reticulum contains less restrictive hexose phosphatase and hexose-P transporter activities that accommodate both G6P and glucose 1-phosphate and probably M6P (34).

CDG-Ia disease fails to respond to dietary mannose therapy, with no improvement of either clinical symptoms or protein glycosylation, whereas similar therapy is highly effective for CDG-Ib patients (35). If hypoglycosylation in CDG-Ia involves an inhibitory effect of M6P, approaches to lower intracellular concentrations of M6P and its precursors (such as F6P and G6P) should be considered.

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Analysis of Glycosylation in CDG-Ia Fibroblasts by Fluorophore-assisted Carbohydrate Electrophoresis: IMPLICATIONS FOR EXTRACELLULAR GLUCOSE AND INTRACELLULAR MANNOSE 6-PHOSPHATE
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