

# Increased Biosynthesis of Glycosphingolipids in Congenital Disorder of Glycosylation Ia (CDG-Ia) Fibroblasts

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## ABSTRACT

Congenital disorder of glycosylation Ia (CDG-Ia) is an autosomal recessive disease, characterized by the impaired biosynthesis of the N-linked oligosaccharide chains of proteins due to a deficiency of phosphomannomutase (PMM), the enzyme converting mannose-6-phosphate into mannose-1-phosphate. We investigated the consequences of the altered N-linked glycoprotein (GP) biosynthesis on the quantity and quality of glycosphingolipids (GSLs) in fibroblasts of CDG-Ia patients. First, we found that CDG-Ia fibroblasts contain an increased amount of total GSLs when compared with normal fibroblasts. Further, we assessed by metabolic labeling of CDG-Ia fibroblasts with radioactive sugar precursors, including galactose and N-acetylmannosamine, that a diminished biosynthesis of cellular GPs is antagonized by an increased biosynthesis of GSLs. An increased GSL biosynthesis was also observed by means of radio-labeled lipid precursors including sphingosine and lactosylceramide. Notably, also the degradation of GLSs is slowed down in CDG-Ia fibroblasts. Finally, when we labeled normal human fibroblasts and CHO cells with radioactive galactose in the presence and absence of deoxymannojirimycin (dMM), an inhibitor of N-glycan processing, we found that this cellular model mimics what occurs in CDG-Ia fibroblasts. Since an inverse relationship between GP expression and GSL content does exist, we assume that increased glycosphingolipid biosynthesis is secondary to protein hypoglycosylation. Al-

together, our data suggest that the cell metabolic machinery may be able to partially re-equilibrate protein hypoglycosylation with increased biosynthesis of glycosphingolipids, possibly to preserve the overall physico-chemical equilibrium of the outer layer of the plasma membrane. (*Pediatr Res* 52: 645–651, 2002)

### Abbreviations

**CDG-Ia**, congenital disorder of glycosylation Ia;  
**Cer**, ceramide  
**CHO**, Chinese hamster ovary  
**dMM**, deoxymannojirimycin  
**GC**, glucosylceramide  
**GP**, glycoprotein  
**GSL**, glycosphingolipid  
**LC**, lactosylceramide  
**Gb3**, globotrihexosylceramide  
**Gb4**, globotetrahexosylceramide  
**SM**, sphingomyelin;  
**PDMP**, *threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol  
**PMI**, phosphomannoisomerase  
**PMM**, phosphomannomutase  
**SD**, standard deviation

Glycoproteins (GPs) and glycosphingolipids (GSLs) represent two major classes of glycoconjugates present in mammalian cells. The glycosylation of both classes of molecules

shares some common features. Noteworthy, 1) the subcellular distribution of GP and GSL glycosylating enzymes is similar, with the Golgi apparatus being the compartment where the majority of glycosylations occurs (1–3); 2) some of the enzymes which glycosylate proteins and lipids are the same, *e.g.* those responsible for blood group antigenicity (4, 5); 3) the precursor pool of nucleotide-sugars of both GP and GSL glycosylation within the Golgi lumen is indistinguishable (6); 4) the GP and GSL transport through the Golgi complex displays biochemical and kinetical similarities (7); 5) a relevant portion of neosynthesized GPs and the totality of GSLs are delivered from the Golgi to the plasma membrane, the final site

Received October 15, 2001; Accepted May 22, 2002.

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This work was supported by institutional funding from the Institut National de la Santé et de la Recherche Médicale (INSERM), an INSERM-AFM research network grant (4MR29F) "Réseau de Recherche sur les CDG" and by grants from the Association Vaincre les Maladies Lysosomales.

R.G. was a visiting professor, supported by a Poste Orange INSERM; G.S. was recipient of a Fellowship from the University of Milan.

DOI: 10.1203/01.PDR.0000032383.67029.F7

of localization. The distribution of glycoconjugates, GPs and GSLs, on the plasma membrane of mammalian cells contributes to the organization of the glycocalyx, the complex of the oligosaccharide chains protruding from the membrane toward the extracellular environment. The glycocalyx maintains the electrical, physical and chemical equilibrium of the plasma membrane and is involved in prominent cell functions, including cell-cell interaction, adhesion, signal transduction.

In 1980, Jaeken *et al.* (8) described a hereditary multisystem disorder, biochemically characterized by defect in the biosynthesis of the oligosaccharide moiety of GPs and associated with psychomotor and mental retardation and abnormalities in blood coagulation. This disorder, formerly called carbohydrate deficient glycoprotein syndrome, CDGS, has been recently renamed congenital disorder of glycosylation, CDG (9) and represents the first genetic disease affecting the biosynthesis of N-linked GP glycochains. Several CDG forms are known and the enzymatic deficiencies have been already identified (reviewed in Ref 10). The most common form of the disease is called CDG-Ia. In CDG-Ia patients the primary defect is a deficiency of phosphomannomutase (PMM), the enzyme converting mannose-6-phosphate into mannose-1-phosphate (10). The gene involved, PMM2, has been cloned (11, 12).

We set out to use CDG-Ia as a model system to investigate the consequences of an altered GP biosynthesis on the content of GSLs. More specifically, we tested whether there is a relationship between lipid and protein glycosylation, since this may represent a compensatory mechanism, capable to preserve the overall physico-chemical equilibrium of the outer layer of the cell plasma membrane.

Here, we report that CDG-Ia fibroblasts contain a higher level of GSLs, compared with normal fibroblasts. In addition, by metabolic studies with radioactive precursors, we observed a diminished biosynthesis of cellular GPs, concomitant with an increased biosynthesis of GSLs. Similarly, increased GSL biosynthesis was observed with radioactive lipid precursors, together with diminished GSL degradation. Finally, we used normal human fibroblasts and CHO cells labeled with radioactive galactose in the presence and absence of dMM, an inhibitor of N-glycan maturation (13) and found that there is an inverse relationship between GP expression and GSL content.

## METHODS

**Cells and cell cultures.** Human fibroblasts were obtained from upper arm skin biopsies of five PMM deficient CDG-Ia patients (three males, 2, 3, and 6 years old and two females, 4 and 6 years old) and of five sex- and age-matched controls,

with disorders known not to express the transferrin abnormality of any form of CDG-Ia. All CDG-Ia patients displayed the major abnormalities typical of this disease, namely growth and mental retardation, hepatopathy, cerebellar hypoplasia/atrophy and ataxia, polyneuropathy, and dysmorphic features and displayed serum transferrin multiple band pattern (14, 15). CDG-Ia fibroblasts were characterized by defective mannose uptake and defective PMM activity ( $<1.2$  nmoles/min/mg protein). Table 1 summarizes the mutations involved in PMM2 gene, when published (16, 17), and both phosphomannomutase (PMM) and phosphomannoisomerase (PMI) activities of CDG-Ia patient fibroblasts.

Fibroblast cultures, established from skin punch biopsies, were grown in DMEM, supplemented with 10% FCS and serially passaged by trypsinization. Cell labeling was performed on confluent cells. All the experiments were performed at a PDL (population doubling levels) value ranging from 7 to 15, starting from the first *in vitro* passage. Chinese Hamster Ovary (CHO) cells were cultured in Ham's F12 medium, supplemented with 10% FCS and serially passaged by trypsinization.

**Radiolabeled precursors and metabolic labeling.** D-[1- $^{14}$ C]Galactose (specific radioactivity: 58 mCi/mmol) and N-acetyl-[6- $^3$ H]mannosamine (specific radioactivity: 39.1 Ci/mmol) were from Amersham (Cologno Monzese, Italy) and NEN (Boston, MA, U.S.A.), respectively. [3- $^3$ H]Sphingosine (specific radioactivity: 1.25 Ci/mmol), ([6- $^3$ H]gal)lactosylceramide (specific radioactivity: 0.95 Ci/mmol) and ([3- $^3$ H]sph)lactosylceramide (specific radioactivity: 1.05 Ci/mmol) (20) were prepared in our laboratory (18–20).  $10^5$  cells were pulse-labeled in 1 mL serum-free medium, at final concentration of 1  $\mu$ Ci/mL for galactose and sphingosine and 5  $\mu$ Ci/mL for lactosylceramides and N-acetylmannosamine. A pulse protocol (48 h) was used for sphingosine, lactosylceramides and N-acetylmannosamine labeling. A pulse-chase protocol (6 h pulse, 12 h chase) was used for galactose labeling. The chase period was initiated by substituting the pulse medium with 1 mL serum-free medium. In all cases, at the end of the incubation, cells were rinsed three times with ice-cold saline, harvested with a rubber policeman, pelleted, frozen and stored at  $-20^\circ\text{C}$ , before lipid extraction or protein determination (21). In the case of lactosylceramide, cell labeling was also stopped after the pulse period (6 h), and cell treated as before, to measure the initial radioactivity incorporation.

**dMM treatment.** Normal human fibroblasts and CHO cells were pretreated with 1 mM dMM for 48 h followed by 6 h

**Table 1.** Mutations involved in PMM2 gene and enzyme activities of the CDG-Ia patient fibroblasts used in this study

Patient	PMM	PMI	Mutation 1	Mutation 2	Reference
1	<0.5	14	L32R	nd	np
2	1.0	20.3	C241S	F157S	16
3	<0.5	24.3	T266S	R141H	17
4	<0.5	16.1	V129M	R141H	17
5	1.2	21.7	nd	nd	n.p

PMM, phosphomannomutase; PMI, phosphomannoisomerase; nd, not determined; np, not published. PMM and PMI activities are expressed as nmoles/min/mg protein. Control fibroblasts had normal PMM (ranging from 2.7 to 4.3 nmoles/min/mg protein) and PMI (ranging from 18.3 to 24.3 nmoles/min/mg protein) activities.

incubation with both 1 mM dMM and 1  $\mu\text{Ci/mL}$  D-[1- $^{14}\text{C}$ ]galactose and finally 12 h incubation with regular medium.

**PDMP treatment.** Normal human skin fibroblasts were pre-treated with 40  $\mu\text{M}$  PDMP for 24 h followed by a 6 h incubation with both 40  $\mu\text{M}$  PDMP and 1  $\mu\text{Ci/mL}$  D-[1- $^{14}\text{C}$ ]galactose and finally 12 h incubation with regular medium. Optimal PDMP concentration was determined in preliminary experiments to minimize cytotoxicity.

**Lipid extraction and analysis.** To determine the endogenous GSLs content, lipids were extracted by the tetrahydrofuran/phosphate buffer method (22), starting from  $4 \times 75 \text{ cm}^2$  dishes, for each sample. The organic phase, containing neutral GSLs, was analyzed by HPTLC, using chloroform/methanol/formic acid/water, 65/25/8.9/1.1, by vol, as the eluting solvent system. Visualization was performed with a 10%  $\text{CuSO}_4$ /8% phosphoric acid aqueous spraying solution. The aqueous phase, containing gangliosides, was analyzed by HPTLC, using chloroform/methanol/0.2% aqueous  $\text{CaCl}_2$ , 6/4/1, by vol, as the eluting solvent system. Visualization was performed with a *p*-dimethylaminobenzaldehyde spray reagent (23). Quantification of the endogenous GSL pattern was performed by scanning densitometry, using commercial standards as reference compounds. To analyze radioactive GSLs and ceramide, lipids were extracted by the chloroform/methanol/pyridine/water method (24). HPTLC separation was performed using chloroform/methanol/water, 60/35/7, by vol, as the eluting solvent system, except in sphingosine labeling experiments, where a chloroform/methanol/formic acid/water, 65/25/6/4, by vol, solvent system was used, to separate sphingomyelin and GM3. Radioactive lipids were visualized by fluorography and identified by comparison with radiolabeled standards available in our laboratory or with commercial nonradioactive standards. The spots corresponding to known lipids were scraped from the plate and counted by liquid scintillation.

**Radioactivity incorporation into cell protein.** Cell pellets were delipidized with chloroform/methanol/water, 10/10/3, by vol. The residue after  $900 \times g$  centrifugation, 15 min, contain-

ing radiolabeled proteins, was dissolved in 200  $\mu\text{L}$  solouene at  $60^\circ\text{C}$  for 1 h and counted by liquid scintillation.

## RESULTS

**Endogenous GSLs content in CDG-Ia fibroblasts versus normal fibroblasts.** To investigate the consequences of an altered GP biosynthesis on the content of GSLs, we tested whether there is a relationship between lipid and protein glycosylation, by measuring the total endogenous GSL content in CDG-Ia fibroblasts *versus* normal fibroblasts. As reported in Table 2, we established that the qualitative GSL pattern, including the neutral GSLs GC, LC, Gb3, Gb4, and the gangliosides GM3, GM2, GD3, GM1, and GD1a (Gangliosides are designated according to Svennerholm L (1964), *J Lipid Res*, 5, 145–155.), was identical between CDG-Ia and normal fibroblasts. In contrast, the quantitative composition between normal and CDG-Ia fibroblast was quite different, even in the presence of identical protein and DNA content. More specifically, the content of some species of GSLs (Gb3, GM2, GD3, and GD1a) was significantly ( $p < 0.05$ ) increased in CDG-Ia fibroblasts *versus* normal controls. GM2 and GD3 gangliosides were the most markedly increased.

**Biosynthesis of glycoconjugates (GPs and GSLs) in CDG-Ia fibroblasts after labeling with sugar precursors.** Next we tested whether there is a relationship between the GP and GSL glycosylation. To gain information on the *de-novo* glycosylation of both lipid and protein moieties, we labeled normal and CDG-Ia fibroblasts with [ $^{14}\text{C}$ ]galactose, and found: 1) similar qualitative patterns for radioactive GSLs and gangliosides in CDG-Ia and normal fibroblasts (Fig. 1A); 2) an increase of approximately 19% radioactivity incorporation in CDG-Ia fibroblasts (Fig. 1B) [ $155,600 \pm 19,980$  (mean value  $\pm$  SD); 125,900–179,800 (range); 153,600 (median) dpm/mg protein] *versus* control fibroblasts [ $131,100 \pm 7,310$  (mean value  $\pm$  SD); 120,100–139,500 (range); 133,100 (median) dpm/mg protein,  $p < 0.05$ ]; 3) a distribution of this increased

**Table 2.** Neutral GSL and ganglioside endogenous pattern of CDG-Ia and control fibroblasts

Cell population	GC	LC	Gb3	Gb4	GM3	GM2	GD3	GD1a
Controls								
1	2.45	3.45	4.70	1.86	2.36	0.05	0.52	0.11
2	2.40	3.76	4.64	1.46	2.43	0.05	0.57	0.09
3	2.59	3.20	4.30	1.98	2.12	0.03	0.32	0.12
4	2.38	3.25	4.98	1.97	2.54	0.08	0.35	0.09
5	2.64	3.45	4.25	1.76	2.24	0.04	0.43	0.10
Mean	2.49	3.42	4.57	1.81	2.34	0.05	0.44	0.10
SD	0.12	0.22	0.30	0.21	0.16	0.02	0.11	0.01
CDG-Ia								
1	2.70	3.72	4.91	2.21	2.57	0.09	0.79	0.11
2	2.57	3.65	5.32	1.90	2.35	0.11	0.71	0.13
3	2.81	3.90	5.11	1.79	2.64	0.07	0.59	0.14
4	2.34	3.54	5.09	2.15	2.43	0.07	0.62	0.11
5	2.89	3.59	4.78	2.19	2.31	0.13	0.69	0.12
Mean	2.66	3.68	5.04	2.05	2.46	0.09	0.68	0.12
SD	0.22	0.14	0.21	0.19	0.14	0.03	0.08	0.01
<i>p</i>	ns	ns	<0.05	ns	ns	<0.05	<0.05	<0.05

Neutral GSLs and gangliosides were separated by HPTLC and quantified by scanning densitometry, using known amounts of spotted commercial standard as reference. Data in the table are expressed as nmol/mg protein. ns, not significant.



radioactivity in all lipids, the most remarkable changes concerning GC, Gb3, GD3 and GM2 (Fig. 1C); 4) a reduced incorporation of [ $^{14}$ C]galactose into the cell protein glycochains in CDG-Ia fibroblasts (Fig. 1B). This accounted for about 28% [242,300  $\pm$  27,410 (mean value  $\pm$  SD); 201,000–

276,800 (range); 245,700 (median) dpm/mg protein, in control fibroblasts *versus* 188,900  $\pm$  16,010 (mean value  $\pm$  SD); 165,400–204,200 (range); 188,200 (median) dpm/mg protein in CDG-Ia ones,  $p < 0.05$ ].

To evaluate the sialylation rates of gangliosides and GPs, we labeled cells with N-acetylmannosamine, and observed that 1) the incorporated radioactivity was about 31% higher in CDG-Ia [44,200  $\pm$  5,280 (mean value  $\pm$  SD); 37,800–52,000 (range); 43,100 (median) dpm/mg protein] than in control [33,700  $\pm$  4,070 (mean value  $\pm$  SD); 28,300–39,000 (range); 34,600 (median) dpm/mg protein,  $p < 0.05$ ] specimens, thus indicating that sialylation is significantly augmented (Fig. 1B); 2) significant changes concern the disialylated gangliosides GD3 and GD1a (Fig. 1D), that are approximately 2-fold more radioactive in CDG-Ia with respect to control cells; 3) radioactivity incorporation into cell GPs was dramatically impaired (45%) [168,100  $\pm$  21,350 (mean value  $\pm$  SD); 138,600–197,200 (range); 165,900 (median) dpm/mg protein in CDG-Ia *versus* 299,800  $\pm$  36,550 (mean value  $\pm$  SD); 246,900–340,000 (range); 296,900 (median) dpm/mg protein in controls,  $p < 0.05$ ] (Fig. 1B).

In conclusion, these data show that there is an inverse relationship between GP and GSL biosynthesis in CDG-Ia fibroblasts.

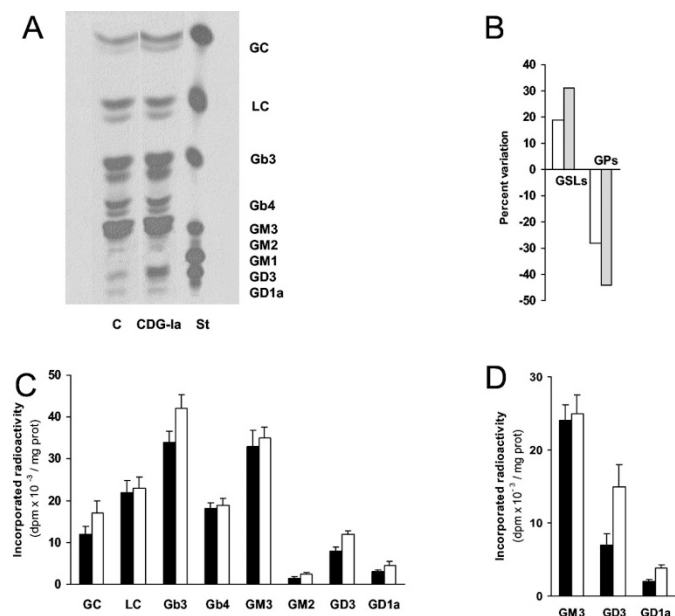
**Biosynthesis of glycosylated and nonglycosylated sphingolipids in CDG-Ia fibroblasts after labeling with lipid precursors.** To test whether the previous data could be related to the rate of incorporation of radiolabeled sugars into the nucleotide-sugar pool that may be different between CDG-Ia and normal fibroblasts (25), we metabolically labeled cells with nonsugar precursors, including ([ $^3$ H]gal)lactosylceramide and [ $^3$ H]sphingosine.

Exogenous lactosylceramide can be used *in vivo* and in intact cells as a substrate for the biosynthesis of more glycosylated GSLs (26). As can be seen in Fig. 2A, CDG-Ia fibroblasts incorporated 15–34% more radioactivity in Gb3, Gb4, and GM3, when compared with normal control fibroblasts.

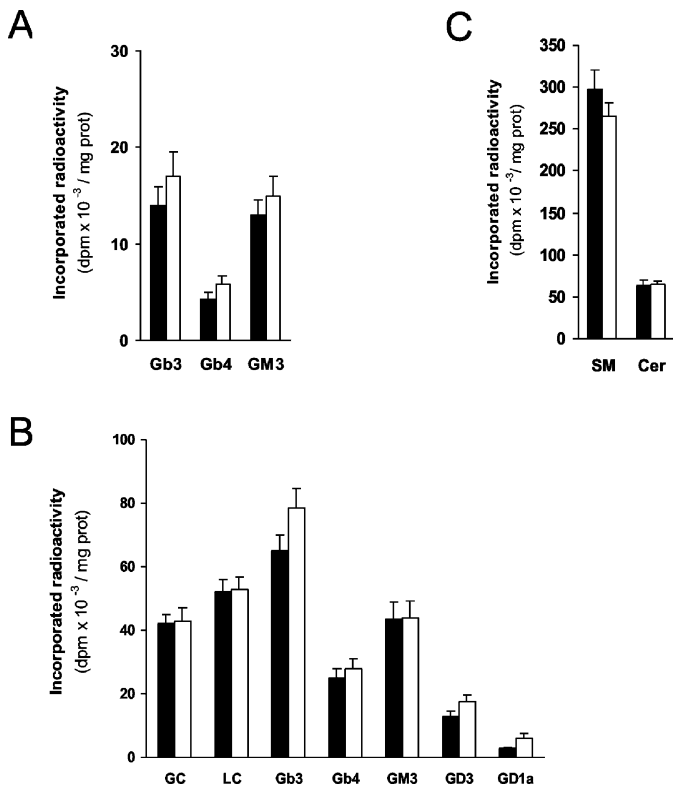
To gain information on the *de-novo* biosynthesis of the sphingolipid backbone in CDG-Ia fibroblasts, we labeled these cells with [ $^3$ H]sphingosine and observed that the radioactivity incorporation in Gb3, GD3 and GD1a was remarkably increased, over the normal fibroblasts (Fig. 2B). Noteworthy, the incorporation of radioactivity into nonglycosylated sphingolipids, ceramide and SM, showed that ceramide biosynthesis remained unmodified, whereas SM biosynthesis was slightly but significantly reduced (Fig. 2C).

Altogether these data suggest that the nucleotide-sugar pool, when not used for GP biosynthesis, may be available to lipid glycosylation.

**GSL degradation in CDG-Ia fibroblasts after labeling with ([ $^3$ H]sph)lactosylceramide.** To assess on the lysosomal GSLs degradation rates in CDG-Ia fibroblasts, we labeled cells with ([ $^3$ H]sph)lactosylceramide. We used this molecule, since the labeling on the sphingoid base is conserved in degradation products originated from lactosylceramide itself (19). We detected a consistent impairment of glycohydrolases-assisted deglycosylation in CDG-Ia fibroblasts. Particularly, glucosylceramide and ceramide were 25% to 30% lower than in normal



**Figure 1.** Biosynthesis of glycoconjugates (GPs and GSLs) in CDG-Ia fibroblasts after labeling with sugar precursors. (A) TLC separation of radioactive GSLs in control and CDG-Ia fibroblasts, after [ $^{14}$ C]galactose labeling. Radioactive GSLs, after extraction, were separated by HPTLC and visualized by fluorography. Eluting solvent system: chloroform/methanol/water, 60/35/7, by vol. Lane C: GSLs of fibroblasts from control 4; lane CDG-Ia: GSLs of fibroblasts from patient 2; lane St: reference radioactive GSLs. (B) Percent variation of radioactivity incorporation into GPs and GSLs after metabolic labeling with sugar precursors in CDG-Ia, referred to normal control fibroblasts. Results are the mean values of five experiments. Empty bars, [ $^{14}$ C]galactose labeling; full bars: N-acetyl-[ $^3$ H]mannosamine labeling. (C) Quantitative determination of radioactivity incorporation into individual GSLs after [ $^{14}$ C]galactose labeling. After visualization by fluorography, individual spots were scraped off the plate and counted by liquid scintillation. Results are the mean values  $\pm$  SD of individual experiments of five control and five CDG-Ia fibroblasts. Full bars: control fibroblasts; empty bars: CDG-Ia fibroblasts. Further statistical data (all expressed as dpm  $\times 10^{-3}$ /mg protein): Control fibroblasts, range values: GC, 10.2–14.4; LC, 17.3–24.3; Gb3, 30.3–37.2; Gb4, 15.1–19.2; GM3, 28.3–36.9; GM2, 1.2–1.8; GD3, 7.1–10.3; GD1a, 2.0–4.7; medians: GC, 12.1; LC, 22.0; Gb3, 32.9; Gb4, 16.8; GM3, 34.5; GM2, 1.6; GD3, 8.1; GD1a, 3.1. CDG-Ia fibroblasts, range values: GC, 13.4–21.4; LC, 18.2–25.2; Gb3, 38.4–46.8; Gb4, 16.3–20.1; GM3, 30.4–37.3; GM2, 2.3–2.9; GD3, 10.3–13.5; GD1a, 3.2–5.6; medians: GC, 16.9; LC, 22.8; Gb3, 42.6; Gb4, 18.0; GM3, 35.7; GM2, 2.4; GD3, 12.0; GD1a, 4.1. Significance, CDG-Ia *vs* controls: GC, Gb3, GD3 and GM2  $p < 0.05$ ; others: not significant. (D) Quantitative determination of radioactivity incorporation into individual GSLs after N-acetyl-[ $^3$ H]mannosamine labeling. Radioactive gangliosides, after extraction, were separated by HPTLC and visualized by fluorography. Individual spots were scraped off the plate and counted by liquid scintillation. Results are the mean values  $\pm$  SD of individual experiments of five control and five CDG-Ia fibroblasts. Full bars: control fibroblasts; empty bars: CDG-Ia fibroblasts. Further statistical data (all expressed as dpm  $\times 10^{-3}$ /mg protein): Control fibroblasts, range values: GM3, 21.2–27.0; GD3, 5.0–8.9; GD1a, 1.5–2.9; medians: GM3, 24.1; GD3, 7.8; GD1a, 2.1. CDG-Ia fibroblasts, range values: GM3, 22.3–27.9; GD3, 13.5–21.2; GD1a, 2.8–4.2; medians: GM3, 25.6; GD3, 16.1; GD1a, 3.6. Significance, CDG-Ia *vs* controls: GD3 and GD1a,  $p < 0.05$ ; GM3, not significant.

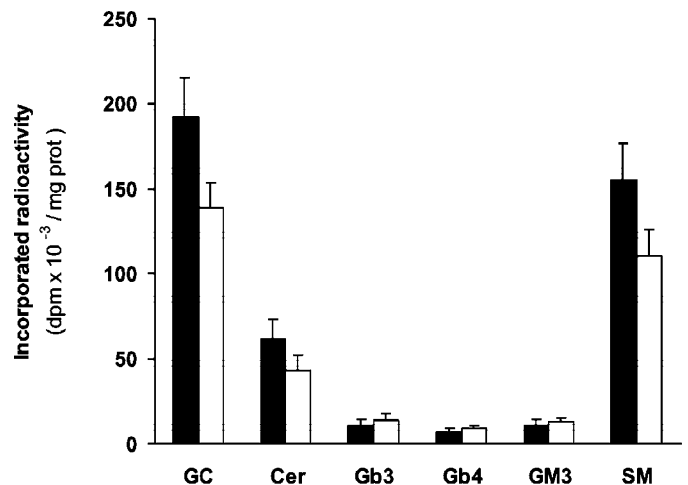


**Figure 2.** Biosynthesis of glycosylated and nonglycosylated sphingolipid in CDG-Ia fibroblasts after labeling with lipid precursors. (A) Quantitative determination of radioactivity incorporation into individual radioactive GSLs in control and CDG-Ia fibroblasts, after [ $^3\text{H}$ ]galactosylceramide labeling. Radioactive GSLs, after extraction, were separated by HPTLC and visualized by fluorography. Individual spots were scraped off the plate and counted by liquid scintillation. Results are the mean values  $\pm$  SD of individual experiments of five control and five CDG-Ia fibroblasts. Full bars: control fibroblasts; empty bars: CDG-Ia fibroblasts. Further statistical data (all expressed as  $\text{dpm} \times 10^{-3}/\text{mg protein}$ ): Control fibroblasts, range values: Gb3, 10.6–15.7; Gb4, 3.6–5.1; GM3, 11.6–17.0; medians: Gb3, 13.5; Gb4, 4.1; GM3, 13.5. CDG-Ia fibroblasts, range values: Gb3, 14.2–19.3; Gb4, 4.9–7.2; GM3, 12.6–18.1; medians: Gb3, 17.9; Gb4, 6.1; GM3, 14.7. Significance, CDG-Ia vs controls: Gb3 and Gb4,  $p < 0.05$ . GM3, not significant. (B) Quantitative determination of radioactivity incorporation in individual radioactive GSLs in control and CDG-Ia fibroblasts after [ $^3\text{H}$ ]sphingosine labeling. Radioactive GSLs, after extraction, were separated by HPTLC and visualized by fluorography. Individual spots were scraped off the plate and counted by liquid scintillation. Results are the mean values  $\pm$  SD of individual experiments of five control and five CDG-Ia fibroblasts. Full bars: control fibroblasts; empty bars: CDG-Ia fibroblasts. Further statistical data (all expressed as  $\text{dpm} \times 10^{-3}/\text{mg protein}$ ): Control fibroblasts, range values: GC, 35.4–44.6; LC, 47.2–56.1; Gb3, 57.2–69.8; Gb4, 20.4–28.1; GM3, 36.1–49.2; GD3, 12.1–16.9; GD1a, 3.1–3.9; medians: GC, 42.5; LC, 51.9; Gb3, 64.3; Gb4, 23.5; GM3, 42.2; GD3, 13.5; GD1a, 3.3. CDG-Ia fibroblasts, range values: GC, 38.1–47.7; LC, 49.2–56.0; Gb3, 70.4–86.2; Gb4, 22.9–31.1; GM3, 36.5–50.3; GD3, 14.2–19.3; GD1a, 4.2–7.5; medians: GC, 44.6; LC, 53.9; Gb3, 79.2; Gb4, 28.2; GM3, 44.1; GD3, 13.5; GD1a, 6.4. Significance, CDG-Ia vs controls: Gb3, GD3 and GD1a  $p < 0.05$ ; others, not significant. GM2 was not distinguishable from GM3. (C) Quantitative determination of radioactivity incorporation in individual radioactive sphingomyelin and ceramide in control and CDG-Ia fibroblasts after [ $^3\text{H}$ ]sphingosine labeling. Radioactive sphingolipids, after extraction, were separated by HPTLC and visualized by fluorography. Individual spots were scraped off the plate and counted by liquid scintillation. Results are the mean values  $\pm$  SD of individual experiments of five control and five CDG-Ia fibroblasts. Full bars: control fibroblasts; empty bars: CDG-Ia fibroblasts. Further statistical data (all expressed as  $\text{dpm} \times 10^{-3}/\text{mg protein}$ ): Control fibroblasts, range values: SM, 281.3–320.0; Cer, 52.3–71.9; medians, SM, 289.4; Cer, 64.6. CDG-Ia fibroblasts, range values: SM, 222.3–270.3; Cer, 54.4–71.1; medians, SM, 243.3; Cer, 66.6. Significance, CDG-Ia vs controls: SM  $p < 0.05$ ; Cer, not significant.

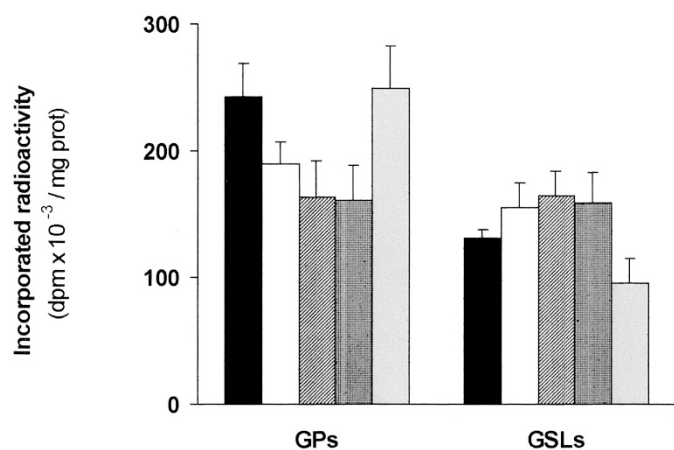
fibroblasts (Fig. 3), even in the presence of similar incorporation of radioactivity after the pulse period between control [ $412,700 \pm 47,660$  (mean value  $\pm$  SD);  $356,800$ – $487,700$  (range);  $409,600$  (median)  $\text{dpm}/\text{mg protein}$ ] and CDG-Ia [ $420,800 \pm 49,990$  (mean value  $\pm$  SD);  $347,100$ – $486,600$  (range);  $426,000$  (median)  $\text{dpm}/\text{mg protein}$ ] fibroblasts. We conclude that the GSLs catabolism is decreased in CDG-Ia cells. This is supported by the finding that CDG-Ia fibroblasts displayed 30% less SM than normal controls, since the biosynthesis of SM occurs *via* a recycling route of the free sphingoid base released in the lysosomes (19).

**Biosynthesis of glycoconjugates (GPs and GLS) in normal human fibroblasts and CHO cells after dMM and PDMP treatment.** To test whether sphingolipid glycosylation is secondary to protein hypoglycosylation, we labeled normal human fibroblasts and CHO cells with radioactive galactose in the presence and absence of deoximannojirimycin (dMM), an inhibitor of N-glycan processing. As shown in Fig. 4, we observed a 33% decrease of GP biosynthesis and a concomitant 25% increase of GSL biosynthesis in dMM treated *versus* untreated normal human fibroblasts. The GSL molecular species undergoing significant increase were GC (35%), Gb3 (18%), GM2 (57%), and GD3 (23%). Differences in other species were not significant.

To assess whether a reciprocal effect does occur, normal human fibroblasts were also labeled with radioactive galactose in the presence and absence of PDMP, an inhibitor of glucosylceramide synthase, the first enzyme in the multi-step glycosylation of GSLs. Concurrent to a 30% inhibition of sphingolipid glycosylation, no significant increase of protein glycosylation



**Figure 3.** GSL degradation in CDG-Ia fibroblasts after labeling with [ $^3\text{H}$ ]sphingolactosylceramide. Radioactive sphingolipids, after extraction, were separated by HPTLC and visualized by fluorography. Individual spots were scraped off the plate and counted by liquid scintillation. Results are the mean values  $\pm$  SD of individual experiments of five control and five CDG-Ia fibroblasts. Full bars: control fibroblasts; empty bars: CDG-Ia fibroblasts. Further statistical data (all expressed as  $\text{dpm} \times 10^{-3}/\text{mg protein}$ ): Control fibroblasts, range values: GC, 170.3–226.3; Cer, 50.4–75.9; Gb3, 6.2–17.2; Gb4, 4.8–11.7; GM3, 5.5–15.6; SM, 140.3–185.8; medians: GC, 194.4; Cer, 59.9; Gb3, 11.4; Gb4, 6.0; GM3, 9.9; SM, 162.2. CDG-Ia fibroblasts, range values: GC, 128.3–168.2; Cer, 33.4–57.8; Gb3, 9.2–19.2; Gb4, 5.7–12.7; GM3, 8.2–15.6; SM, 103.5–140.6; medians: GC, 145.4; Cer, 41.7; Gb3, 13.0; Gb4, 8.5; GM3, 12.7; SM, 119.9. Significance, CDG-Ia vs controls: GC, Cer, SM,  $p < 0.05$ ; others, not significant.



**Figure 4.** Effect of dMM and PDMP treatments on radioactivity incorporation into GSLs and GPs after [<sup>14</sup>C]galactose labeling. Incorporated radioactivity was measured by liquid scintillation. Results are the mean values  $\pm$  SD of five individual experiments. ■ untreated normal human fibroblasts; □ CDG-Ia fibroblasts; ▨ dMM-treated normal human fibroblasts; ▩ dMM-treated CHO cells; ▧ PDMP-treated normal human fibroblasts. Further statistical data (all expressed as dpm  $\times$  10<sup>-3</sup>/mg protein). Untreated normal human fibroblasts and CDG-Ia fibroblasts, see text. dMM-treated normal human fibroblasts; GPs, range values: 139.5–206.8; median: 169.8; GSLs, range values, 136.3–180.8; median, 167.7. dMM-treated CHO cells; GPs, range values: 140.1–209.1; median: 162.2; GSLs, range values, 140.2–194.2; median, 167.0. PDMP-treated normal human fibroblasts; GPs, range values: 216.1–301.8; median: 239.5; GSLs, range values, 72.5–120.5; median: 93.2. Significance: GPs, CDG-Ia, dMM-treated normal human fibroblasts and dMM-treated CHO cells *vs* untreated normal human fibroblasts,  $p < 0.05$ , PDMP-treated normal human fibroblasts *vs* untreated normal human fibroblasts, not significant; GSLs, CDG-Ia and all treated fibroblasts *vs* untreated normal human fibroblasts:  $p < 0.05$ .

occurs in PDMP treated fibroblasts (Fig. 4). In conclusion, these data suggest that increase of GSLs is secondary to protein hypoglycosylation and that the reciprocal phenomenon does not occur.

## DISCUSSION

The most relevant finding of the present work is an increased GSL content in CDG-Ia fibroblasts, compared with normal matched fibroblasts, apparent from the quantitation of endogenous GSLs, under strictly controlled experimental conditions. This was necessary since the endogenous pattern of GSLs and gangliosides in human fibroblasts is dramatically dependent on various factors, including cell density (27), tissue of origin (28) and individual variability (29). To minimize any bias due to these factors, we performed all the experiments with fibroblasts from patients matched with normal individuals of the same age of the patient ones and at the same passage in culture. We found that the endogenous profile of GSLs was in agreement with previously published profiles (29–31) and included the most represented species GC, LC, Gb3, Gb4, GM3, GD3 and the less abundant species GD1a and GM2. The increase of total GSL content in CDG-Ia patients is, to our knowledge, the first evidence of a compensatory mechanism aimed to re-equilibrate disturbed protein glycosylation by increased GSL biosynthesis.

Our supposition was corroborated by a series of labeling studies. We showed that increased GLSs content is not conse-

quent to a single step, as demonstrated by lack of specificity of incorporation, when different (sugar or lipid) radioactive precursors were used.

Most notably, in parallel with GSL increase, there was a marked reduction of protein glycosylation in CDG-Ia fibroblasts *versus* control cells. This was evident with galactose labeling and even more evident with N-acetylmannosamine labeling. This result demonstrates that nonsecreted GP biosynthesis is affected in CDG-Ia patients, in addition to the known secreted GP biosynthesis defect (8). The impairment of protein glycosylation in general, and sialylation in particular, corroborates a recent observation (32), reporting a reduced surface expression of sialoglycans on B lymphocytes from CDG-Ia patients. We speculate that the combined impairment of galactose and N-acetylmannosamine incorporation into GPs could be due to a diminished availability of core structures and/or a delayed GP transport along the secretory pathway (33, 34).

A diminished GP biosynthesis is concurrent with an increased incorporation of the same sugar precursors into GSLs. This demonstrates the occurrence of inverse relationship between GP and GSL contents in CDG-Ia fibroblasts. The Golgi apparatus is likely to contribute in supporting this phenomenon.

By the results obtained with lipid precursors, one attractive evidence was that SM biosynthesis is slightly depressed, thus suggesting that the ceramide precursor pool is preferentially used for glycosylating purposes, in CDG-Ia.

As far as the GSL degradation was concerned, we demonstrated that it is impaired in CDG-Ia *versus* control fibroblasts. This finding is in agreement with the evidence (35) that the activity of lysosomal enzymes is decreased in CDG-Ia cells.

We finally observed a higher GSL content in normal human and murine fibroblasts, treated with dMM, an inhibitor of GP biosynthesis. The metabolic change of dMM-treated fibroblasts resembles that occurring in CDG-Ia fibroblasts, suggesting that the increase of GSLs is actually depending on protein hypoglycosylation. Since no reciprocal effect was observed on protein glycosylation after PDMP inhibition of GSL biosynthesis, we presume different regulatory mechanisms for protein and lipid glycosylation.

Altogether, this paper shows that the increased sphingolipid biosynthesis is secondary to protein hypoglycosylation in CDG-Ia fibroblasts. This may represent a cellular process devised to preserve the homeostasis of the plasma membrane constituents.

**Acknowledgments.** The authors thank Dr. S. Moore and Dr. N. Sacchi for critical reading of the manuscript, Dr. V. Cormier-Daire for providing CDG-Ia fibroblasts, Dr. G. Durand for useful discussion, and Dr. M. Laburthe for access to facilities during the course of the present work.

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