A Partial Deficiency of Dehydrodolichol Reduction Is a Cause of Carbohydrate-deficient Glycoprotein Syndrome Type I*

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Carbohydrate-deficient glycoprotein (CDG) syndrome type I is a congenital disorder that involves the underglycosylation of N-glycosylated glycoproteins (Yamashita, K., Ideo, H., Ohkura, T., Fukushima, K., Yuasa, I., Ohno, K., and Takeshita, K. (1993) J. Biol. Chem. 268, 5783–5789). In an effort to further elucidate the biochemical basis of CDG syndrome type I in our patients, we investigated the defect in the multi-step pathway for biosynthesis of lipid-linked oligosaccharides (LLO) by the metabolic labeling method using [3H]glucosamine, [3H]mannose, and [3H]mevalonate. The LLO levels in synchronized cultures of fibroblasts from these patients were severalfold lower than those in control fibroblasts in the S phase, and the oligosaccharides released from LLO showed the same structural composition, Glc1–3Man9GlcNAc2–Glcnac-GlcNAc, in the case of both the patients and controls. The amount of [3H]mannose incorporated into mannose 6-phosphate, mannose 1-phosphate, and GDP-mannose was greater in fibroblasts from these patients than in the control fibroblasts in the G1 period, although the ratios of these acidic mannose derivatives as indicated by the relative levels of radioactive activity were the same for the two types of fibroblasts. Furthermore, upon metabolic labeling with [3H]mevalonate, the level of [3H]dehydrodolichol in fibroblasts from these patients increased in the S phase, and the levels of [3H]dolichol and [3H]dolichol-PP oligosaccharides concomitantly decreased, although the chain length distribution of the respective dolichols and dehydrodolichols was the same in the two types of fibroblasts. These results indicate that the conversion of dehydrodolichol to dolichol is partially defective in our patients and that the resulting loss of dolichol leads directly to underglycosylation.

It has recently been determined that carbohydrate-deficient glycoprotein (CDG) syndrome type I is an autosomal recessive endoplasmic reticulum disorder (1–3). In 1980, Jaeken et al. (4) first reported this syndrome as encountered in 2-year-old twin girls with neuroimpairment, cerebellar atrophy, hepatomegaly, abnormal subcutaneous fat deposition, and skeletal abnormalities. Both patients had a number of biochemical abnormalities mainly affecting glycoproteins. Later, it was reported that sialic acid-deficient serum and cerebrospinal fluid transferrins are features of this newly recognized genetic syndrome (5), and the finding of pathologic transferrin heterogeneity led to the demonstration of a partial deficiency of the sialyl-N-acetyllactosamine group in serum transferrins (6, 7). More recently, it was elucidated that CDG syndrome type I is a partial asparagine-N-linked sugar chain transfer deficiency in endoplasmic reticulum (8–10). However, the fundamental defect remains unclear, and at least two models, defective mannose uptake (11) and defective conversion of mannose 6-phosphate to mannose 1-phosphate (12), have been suggested. In preliminary experiments by the metabolic labeling method using [3H]mannose, fibroblasts from our patients with CDG syndrome type I did not accumulate any smaller sized [3H]mannose-labeled oligosaccharide moieties in LLO as compared with those from patients in the previously reported families (11, 13, 14), although most of the clinical features and the clinical biochemical features in our patients (15) are the same as those of other groups’ families (1, 2). In order to elucidate the primary defect responsible for CDG syndrome type I in our patients, the biosynthetic pathway from mevalonate to Glc1–3Man9GlcNAc2–PP dolichol in the fibroblasts was investigated by metabolic labeling with [3H]glucosamine, [3H]mannose, and [3H]mevalonate.

Synchronized cultures of the CDG syndrome type I and control fibroblasts were used for the metabolic labeling studies, because we found, using synchronized cultures of rat 3Y1 cells, that the biosynthesis of LLO sharply increases in the S phase and decreases in the G1, G2, and M periods, concomitantly with dehydrodolichyl diposphosphate synthase activity.2 In this paper we report that the biosynthesis of LLO sharply increases in the S phase, and the LLO levels in CDG syndrome type I fibroblasts are severalfold lower than those in control fibroblasts. The oligosaccharides released from LLO in both types of fibroblasts have the same structure, Glc1–3Man9GlcNAc2–PP dolichol, which may result in random failure of glycosylation of the available Asn-X-Ser/Thr sites. Furthermore, since the biosynthetic pathway for dolichol is proposed to be mevalonate → isopentenyl diposphate → geranyl diposphate → farnesyldiphtat → dehydrodolichyl diposphate → dehydrodolichyl monophosphate → dehydrodolichol → dolichol (16, 17), the biosynthetic pathway for dolichol in relation to LLO in CDG syndrome type I fibroblasts was also investigated by means of metabolic labeling with [3H]mevalonate.

EXPERIMENTAL PROCEDURES

Chemicals and Enzymes—[2-3H]Mannose (17.6 Ci/mM), [1,6-3H]glucosamine-HCl (54.1 Ci/mM), GDP-[14C]mannose (286 mCi/mM), [3H]dehydrodolichol, [3H]mannose, and [3H]mevalonate.

2 Fukushima, K., Ohkura, T., and Yamashita, K. (1997) J. Biochem. (Tokyo), in press.
mmol), and [5-3H]mevalonate triethylammonium salt (40 Ci/mmol) were purchased from DuPont NEN. [6-3H]Thymidine (26.0 Ci/mmol) was obtained from Amersham Life Sciences, Inc. Diethylenethiouyl cellulose (DE52) was from Whatman Biosystems Ltd. The Sep-Pak Vac C18 cartridges and µPorasil silica column (3.9 × 150 mm) were purchased from Waters. A sample of the column was labeled with [3H]mevalonate triethylammonium (4.6 × 250 mm), was from Chemco Scientific Co., Ltd. (Osaka, Japan). Acid phosphatase from potato was from Sigma. Dehydrodrol- dolichol and dolichol were generous gifts from Kuranay Co. (Japan). Bio-Gel P-4 (extra fine) and AG-50W-X2 (100–200 mesh) were purchased from Bio-Rad. Concanavalin A-Sepharose was from Pharmacia Biotech Inc. Yeast hexokinase was obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan). Methyl-α-mannoside, mannose 1-phosphate, glucose 1,6-bisphosphate, and dolichyl phosphate were from Sigma. All other chemicals were of analytical grade.

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Clinical Features and Clinical Biochemical Descriptions of the CDG Syndrome Type I Patients—The clinical features of three patients, A.H., Y.M., and M.M., with CDG syndrome type I, whose established fibroblasts were used.

- The activities of lysosomal a–b-hexosaminidases and a–L-fucosidase in the sera of these patients were several-fold higher than those in normal controls. These clinical features and clinical biochemical features in our patients (3, 15) are the same as those of other reported groups (1, 2), except one of the characteristic features designated as “fat pads on the buttocks” was not noticed in our patients.

- Synchronized cultures were obtained by the density-arrested culture method (19). In brief, cells were inoculated into 90-mm plastic dishes and confluent. The confluent cells were washed with fresh medium and then cultured for an additional 3 days. Then, the density-arrested cells were stimulated by replating in fresh medium at a density of 1 × 105 cells per 90-mm dish. At regular intervals, [3H]thymidine (1 µCi/ml) was added to the medium, and the cells were labeled for 2 or 3 h. After the cells had been lysed in 5% sodium dodecyl sulfate, 5% trichloroacetic acid, and 10% trichloroacetic acid-precipitable macromolecules (i.e., DNA) were trapped on a glass filter (Whatman GF/C). The size of the cell population entering the S phase was determined based on the synchronous increase in [3H]thymidine incorporation. The size of the cell population entering the M period was determined by counting mitotic cells among total cells.

- The DNA profiles of various serum glycoproteins were higher than those in normal controls. Both the activities and antigen levels of plasma antithrombin III, protein C, and protein S were decreased to nearly half normal levels; however, the activities of lysosomal a–b-hexosaminidases and a–L-fucosidase was congenital, the atrophy progressed for several years after birth, the polyneuropathy appeared in childhood, and the growth retardation became apparent at late childhood. During infancy, internal strabismus was noticed. The biochemical description is as follows: the pI values of various serum glycoproteins were higher than those in normal controls. Both the activities and antigen levels of plasma antithrombin III, protein C, and protein S were decreased to nearly half normal levels; however, the activities of lysosomal a–b-hexosaminidases and a–L-fucosidase.

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Analysis of Metabolically Labeled [3H]mannose 6-phosphate—Synchronized cultures of fibroblasts (1 × 105 cells per dish) were pulse-labeled with 0.25 mCi of [3H]mannose in glucose-free medium at the respective indicated times for 20 min, because the rate of incorporation of [3H]mannose into LLO was constant for at least 20 min. After pulse labeling, the medium in the dishes was immediately replaced with 7 ml of chilled 50% methanol in 10 mM Tris-HCl buffer (pH 8.2) containing 1 mM EDTA, and then the cells were scraped off. After 3000 dpm of GDP-[3H]mannose was added to the tubes as an internal standard, the mixtures were boiled for 2 min, sonicated five times for 5–15 s, and then centrifuged. The supernatants were dried, and the residues were subjected to paper chromatography with 95% methanol, 1 mM ammonium acetate (pH 7.5), and then the cell homogenate (5–10 µg of protein) of subconfluent CDG syndrome type I or control fibroblasts was incubated at 37°C for 30 min. After boiling for 2 min, the tritium-labeled mannose 1,6-bisphosphate was separated from [3H]mannose 6-phosphate by paper electrophoresis at pH 5.4. The radioactivity of [3H]mannose 1,6-bisphosphate, which was diluted with approximately 105 times more glucose 1,6-bisphosphate, was measured as follows. A 1/104 of the [3H]mannose 1,6-bisphosphate synthesized should be converted to [3H]mannose 1-phosphate.

- [3H]mannose 1-phosphate, and GDP-[3H]mannose—Synchronized cultures of fibroblasts (1 × 105 cells per dish) were pulse-labeled with 0.25 mCi of [3H]mannose in glucose-free medium at the respective indicated times for 20 min, because the rate of incorporation of [3H]mannose into LLO was constant for at least 20 min. After pulse labeling, the medium in the dishes was immediately replaced with 7 ml of chilled 50% methanol in 10 mM Tris-HCl buffer (pH 8.2) containing 1 mM EDTA, and then the cells were scraped off. After 3000 dpm of GDP-[3H]mannose was added to the tubes as an internal standard, the mixtures were boiled for 2 min, sonicated five times for 5–15 s, and then centrifuged. The supernatants were dried, and the residues were subjected to paper chromatography with 95% methanol, 1 mM ammonium acetate (pH 7.5), and then the cell homogenate (5–10 µg of protein) of subconfluent CDG syndrome type I or control fibroblasts was incubated at 37°C for 30 min, since mannose 1-phosphate is acid-labile and converted to free mannose, and mannose 6-phosphate is resistant (26).

- Extraction and Analysis of Polyprorenols Metabolically Labeled with [3H]mevalonate—Synchronized cultures of CDG syndrome type I and control fibroblasts were obtained by the density-arrested culture method. The density-arrested cells were replated in fresh medium at the density of 1 × 105 cells per 90-mm dish, and five dishes were used at each point. Synchronization was checked by measuring the incorporation of [3H]mevalonate into DNA and by determining the frequency of mitotic cells among total cells of monolayers in the mitotic phase. The synchronized cultures were incubated with [3H]mevalonate (25 µCi/ml) for 27, 30, 36, 42, and 48 h. After the cells had been washed with phosphate-buffered saline and scraped, they were centrifuged. The packed cells were homogenized and extracted three times with 10 volumes of 1-butanol saturated with water (27). The 1-butanol extracts were washed with water saturated with 1-butanol, dried, and then applied to a DE52 column (bed volume, 1 ml), which had been equilibrated with 1-butanol/methanol (9:1). The pass-through fractions were mixed with 0.1 µg of each dehydrodolichol and dolichol as internal standards and then dried at 100°C. The residue was dissolved in chloroform and then applied to a Sep-Pak C18 column (Waters). After washing with methanol, the column was eluted with hexane, and the eluate was analyzed by HPLC. HPLC was performed using a µPorasil silica column (3.9 × 150 mm) with 3% ethyl ether, 0.2% acetic acid in hexane as the eluent at a flow rate of 0.6 ml/min (28). Internal standards were detected at 210 nm, and 0.6-ml fractions were collected. Dehydrodolichol...
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RESULTS

Cell Cycle-dependent Incorporation of [3H]Glucosamine into Lipid-linked Oligosaccharides—The defect in the pathway of LLO synthesis responsible for CDG syndrome type I was investigated by metabolic labeling with [3H]glucosamine, which is a precursor in LLO synthesis. Fibroblasts with similar population doubling levels (5–15) and similar proliferation ability (5–15) and similar proliferation ability showed similar results (data not shown).

The released [3H]polyprenols were extracted with n-hexane and then analyzed on a Bio-Gel P-4 column chromatography, although these polyprenols are not separated according to their chain lengths. The chain lengths of dolichol and dehydrodolichol are clearly separated by this silica column chromatography, although these polyprenols are not separated according to their chain lengths. The elution positions of glucose oligomers (numbers indicate the glucose units). Black triangles indicate control and ○ indicates patient A.H. Patients Y.M. and M.M. showed similar results (data not shown).

Extraction and Analysis of LLO Metabolically Labeled with [3H]Glucosamine—LLO from cells metabolically labeled with [3H]glucosamine was extracted as described under "Extraction of LLO Metabolically Labeled with [3H]Glucosamine," and [3H]polyprenol was released from LLO by hydrolysis with 0.5 ml of n-propyl alcohol, 1 ml of 0.01 N HCl at 100°C for 25 min, and then digested with potato acid phosphatase. The released [3H]polyprenols were extracted with n-hexane and then analyzed on a silica gel column (3.9 × 150 mm) with 3% diethyl ether, 0.2% acetic acid in hexane as the eluant at a flow rate of 0.6 ml/min.

Sugar Analysis of LLO—LLO from the patients and controls was hydrolyzed with 0.01 M sodium acetate buffer (pH 5.0), 0.1% Triton X-100, 0.2 mg of acid phosphatase, 40% methanol, 0.1% HCl at 100°C for 25 min, and then digested with potato acid phosphatase. The released [3H]polyprenols were extracted with n-hexane and then analyzed on a silica gel column (3.9 × 150 mm) with 3% diethyl ether, 0.2% acetic acid in hexane as the eluant at a flow rate of 0.6 ml/min.

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GlcNAc-GlcNAc-P-P-dolichol are functioning as normal, and some earlier step in the LLO synthetic pathway may be partially deficient in our CDG syndrome type I patients. Results similar to those shown in Fig. 2 were obtained upon metabolic pulse labeling with [3H]mannose, and no smaller sized oligosaccharides were released from LLO (data not shown). These phenomena are clearly different from findings previously reported for other groups’ patients. Panneerselvam and Freeze (11), and Krasnewich et al. (14) previously reported that the initial velocities of [3H]mannose uptake were 2–3-fold less in CDG syndrome type I cells compared with controls (11), and the size of the lipid-linked oligosaccharide precursor is much smaller than in controls (11, 13, 14). These results suggest the possibility that there could be multiple causes of CDG syndrome type I.

Phosphomannomutase Activity in CDG Syndrome Type I and Control Fibroblasts—Our findings indicate that CDG syndrome type I in our patients is caused by a partial deficiency of an enzyme in the earliest steps of LLO biosynthesis. However, Van Schaftingen and Jaeken (12) recently proposed that this syndrome is caused by a phosphomannomutase deficiency. Thus, we examined whether the fibroblasts in our patients, A.H., Y.M., and M.M., display decreased phosphomannomutase activity, as seen in the case of CDG syndrome type I patients of European families. Phosphomannomutase (Enz) mediates the following reactions: mannose 6-phosphate $\rightarrow$ Enz-P $\leftarrow$ Enz + mannose 1,6-bisphosphate $\leftarrow$ Enz-P + mannose 1-phosphate. The linearity of the rate of product (mannose 1,6-bisphosphate) formation was maintained until approximately 80% of the substrates had been consumed in our enzyme assay system. Because an excess amount of glucose 1,6-bisphosphate at concentrations up to 1 mM did not inhibit the phosphomannomutase activity, both as a catalyst of this enzyme, we measured the amount of the synthesized radioactive intermediate, i.e. [3H]mannose 1,6-bisphosphate, which was diluted with at least a 104-fold excess amount of glucose 1,6-bisphosphate as the phosphomannomutase activity. The mean value of phosphomannomutase activity in fibroblasts from five control subjects was 6.02 ± 1.05 pmol/min/mg protein and in the fibroblasts from our three patients the value was 2.71 pmol/min/mg protein (A.H.), 1.08 pmol/min/mg protein (M.M.), and 1.50 pmol/min/mg protein (Y.M.). The phosphomannomutase activity in CDG syndrome type I fibroblasts was 18–45% that in the control fibroblasts. Similar data were obtained for CDG syndrome type I fibroblasts in the study by Jaeken et al. (41). When fibroblasts that had been frozen and thawed were used as the enzyme source, the phosphomannomutase activity in control fibroblasts remained rather constant, whereas the fibroblasts from our patients decreased to several percent that in the controls. These results indicate that the phosphomannomutase activity in the fibroblasts of these patients is more easily inactivated than that in control fibroblasts. If the conversion of mannose 6-phosphate to mannose 1-phosphate is deficient, CDG syndrome type I fibroblasts metabolically labeled with [3H]glucosamine should accumulate [3H]-labeled GlcNAcβ1→4GlcNAc-P-P-dolichol in the lipid fraction. However, this compound could not be detected in either the control or CDG syndrome type I fibroblasts (Fig. 2B, dotted line). These results showed that decreased phosphomannomutase activity is not a cause of CDG syndrome type I, at least in our patients. But there still remains the possibility that the conversion of mannose 6-phosphate to mannose 1-phosphate is mediated by phosphoglucosomutase.

Therefore, we measured the radioactivity of [3H]mannose 6-phosphate, [3H]mannose 1-phosphate and GDP-[3H]mannose as described under “Experimental Procedures.” After synchronization and stimulation, CDG syndrome type I and control fibroblasts (1 × 106 cells per dish) were pulse-labeled with [3H]mannose (0.25 mCi/ml) in glucose-free medium for 20 min at the indicated times; the labeling medium in the dishes was immediately replaced with chilled 50% methanol in 10 mM Tris-HCl buffer (pH 8.2), containing 1 mM EDTA, and then the cells were scraped off and boiled for 2 min. Such immediate inactivation is indispensable for obtaining reproducible results, since these acidic [3H]mannose derivatives are immediately transferred to other compounds.

The levels of [3H]mannose 6-phosphate, [3H]mannose 1-phosphate, and GDP-[3H]mannose in synchronized control fibroblasts were constant throughout from the G1 to the S phase (Fig. 3, B–D, ○). In contrast, the levels of these acidic [3H]mannose derivatives in CDG syndrome type I fibroblasts varied depending on the cell cycle and were constantly higher than in controls in the G1 period (Fig. 3, B–D, □). The ratios of [3H]mannose 6-phosphate to [3H]mannose 1-phosphate in the CDG syndrome type I fibroblasts (Fig. 3E, ○) and control fibroblasts (Fig. 3E, □) were equal, and the equilibrium was shifted toward mannose 6-phosphate rather than mannose 1-phosphate in the ratio of approximately 4:1. A similar equilibrium has been reported for the phosphomannomutase of rabbit brain (23) and that of plant (32). These results suggest that even though phosphomannomutase activity in vitro is decreased, in intact cells this enzyme activity is sufficient for a normal rate of conversion of mannose 6-phosphate to mannose 1-phosphate in CDG syndrome type I. The higher levels of GDP-[3H]mannose in CDG syndrome type I fibroblasts may indicate that the synthesized GDP-mannose is not effectively used as a substrate by dolichylphosphomannose synthase and LLO: α- and β-mannosyltransferases involved in the biosynthesis of LLO. In fact, when [3H]mannose-labeled LLO obtained from the precipitates by extraction with CMW was analyzed, the levels in CDG syndrome type I and control fibroblasts were highly increased in the S phase; however, the levels of fibroblasts from patient A.H. were severalfold lower than the levels in control fibroblasts, and the smaller sized lipid-linked oligosaccharide precursor was not detected, similar to the data in Fig. 2 (data not shown). These results also support the view that the site of the defect in biosynthesis of LLO in these patients is a step earlier than the synthesis of GlcNAc-P-P-polyprenol.

Incorporation of [3H]Mevalonate into Polyprenols—In order to examine this possibility directly, we incubated synchronized CDG syndrome type I fibroblasts and control fibroblasts, from three persons in each instance (similar population doubling levels, 5–15, 1 × 106 cells per dish), with [3H]mevalonate (25 µCi/ml) for 30 h after inoculation (i.e. in the S phase). After

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3 Phosphomannomutase deficiency is the major cause of carbohydrate-deficient glycoprotein syndrome type I (41).
incubation, lipidic molecules were extracted with saturated 1-butanol as described under “Experimental Procedures,” and the evaporated extracts were first fractionated on a DE52 column, which had been equilibrated in 1-butanol/methanol (9:1), to separate neutral lipids from anionic lipids. The DE52 pass-through neutral lipids were further purified on a Sep-Pak C18 column. Most of the radioactivity was found in ubiquinone, cholesterol, cholesteryl ester, and polyprenols. The polyprenols derived from [3H]mevalonate were pooled and analyzed by high-performance liquid chromatography (HPLC) on a silica column, which can easily separate dolichols and dolichol, and polyprenols of LLO in synchronized CDG syndrome type I and control fibroblasts were hydrolyzed with 0.01 M HCl (1:2) at 100 °C for 25 min and then digested with potato α-amylase and amyloglucosidase.

Results indicated that the CDG syndrome type I fibroblasts partially lack the enzymatic activity, which reduces the terminal isoprene unit of dehydrodolichol.

The level of DE52-bound acidic polyprenols was below one-tenth that of neutral polyprenols, and all of the acidic polyprenols were converted to neutral polyprenols by phosphatase digestion; however, further analysis could not be performed.

Incorporation of [3H]Mevalonate into LLO—Because it has been reported that the oligosaccharides of dehydrodolichyl pyrophosphate oligosaccharides are not efficiently transferred to nascent polypeptides (33), polyprenols of LLO were analyzed by metabolically labeling with [3H]mevalonate. After metabolically labeling with [3H]mevalonate under the same conditions as described above, dehydrodolichol, dolichol phosphate, dolichol phosphate mono-saccharides, and dolichol pyrophosphate mono- and di-saccharides were removed by extraction with chloroform/methanol (2:1), and [3H]-LLO were sequentially extracted with chloroform/methanol/water (10:10:3) from the residue. The [3H]-LLO derived from CDG syndrome type I and control fibroblasts were hydrolyzed with n-propyl alcohol, 0.01 M HCl (1:2) at 100 °C for 25 min and then digested with potato acidic phosphatase. The released [3H]polyprenols were extracted with n-hexane, and then analyzed on a silica gel column. All of the polyprenols derived from both the CDG syndrome type I fibroblasts and control fibroblasts were exclusively dolichols (Fig. 4, G and H). These results indicated that dolichol is preferentially utilized in vivo for the glycosylation reaction in human fibroblasts.

The Chain Length Distributions of Polyprenols in CDG Syndrome Type I and Control Fibroblasts—In order to elucidate whether the chain length distributions of dehydrodolichol and dolichol in CDG syndrome type I and control fibroblasts are different, dehydrodolichol and dolichol from one patient (Fig. 4D) and a control (Fig. 4A), respectively, were analyzed on a reverse-phase column, which easily separates polyprenoids of different chain lengths. As shown in Fig. 5, the chain length distribution of dehydrodolichols and dolichols in fibroblasts from the patient was equal to that in the control in every preparation from 17 to 20 isoprene units, with the major labeled species corresponding to 18 and 19 isoprene units. These results suggest that the amount of dehydrodolichyl diphos-
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FIG. 5. The chain length distributions of polyprenols in CDG syndrome type I and control fibroblasts. Arrows at the top indicate the elution positions of polyprenols (numbers indicate the methyl(ene) units). The dehydrodolichols and dolichols in Fig. 4A (control) and Fig. 4D (CDG syndrome type I), respectively, were pooled and then analyzed on a Chemcopak Nucleosil 7C18 column. A, the elution pattern of dolichol in Fig. 4A; B, that of dehydrodolichol in Fig. 4A; C, that of dolichol in Fig. 4D; D, and that of dehydrodolichol in Fig. 4D.

Reduction of Dehydrodolichol to Dolichol—We reported in the preceding part that the biosynthesis of LLO is dependent on the cell cycle and that a lower level of LLO synthesis in CDG syndrome type I fibroblasts can be clearly observed in the S phase. In order to elucidate whether the slower conversion of dehydrodolichol to dolichol in the fibroblasts from these patients depends on the cell cycle, synchronized CDG syndrome type I (patient A.H.) and control fibroblasts (similar population doubling levels, 11–15; 1 × 10^6 cells per dish) were continuously labeled with [3H]mevalonate (25 μCi/ml) for 27, 30, 36, 42, and 48 h. After the respective incubation times, polyisoprenoids were extracted from the dishes and analyzed by HPLC on a silica column. Although radioactivity incorporated into dehydrodolichol and dolichol of CDG syndrome type I and control fibroblasts continuously increased with incubation time in the presence of [3H]mevalonate, as shown in Fig. 6, B and C, the amounts of radioactivity incorporated into dehydrodolichol (Fig. 6B) and dolichol (Fig. 6C) in the fibroblasts from the patients and the controls were different. The ratio of [3H]dolichol (patient/control) (Fig. 6C, ●●) was constantly lower than 1.0, and the ratio of [3H]dehydrodolichol (patient/control) (Fig. 6B, ●●) was constantly higher than 1.0. Also, the levels of [3H]dehydrodolichol were extremely elevated in the S phase as compared with those in the G2 and M periods. These results indicate that the dehydrodolichol reductase in CDG syndrome type I fibroblasts is partially deficient, and the effectively synthesized dehydrodolichol might be accumulated in the S phase. Furthermore, the amount of radioactivity incorporated into the dolichol moiety of LLO in CDG syndrome type I fibroblasts was severalfold lower than that in control fibroblasts (Fig. 6D). These results are in accordance with the ratio of [3H]glucosamine-labeled LLO in patient and control fibroblasts (see Fig. 1).

DISCUSSION

Metabolic labeling with [3H]glucosamine or [3H]mannose showed that the levels of LLO in synchronized CDG syndrome type I fibroblasts were severalfold lower than those in control fibroblasts, although the sizes of oligosaccharides released from these LLO showed the same composition corresponding to Glc1–3Manα1–3GlcNAc2 in the two types of fibroblasts. In contrast, the amount of [3H]mannose incorporated into mannose-6-P, mannose-1-P, and GDP-mannose in CDG syndrome type I fibroblasts was greater than that in control fibroblasts, and the ratio of mannose-6-P to mannose-1-P was lower than that in control fibroblasts. These results indicate that even though phosphomannomutase activity in vitro is decreased, in intact cells this activity is sufficient for a normal rate of conversion of mannose 6-phosphate to mannose 1-phosphate, and the sequentially synthesized GDP-mannose was not effectively used for LLO synthesis in our patients with CDG syndrome type I.

As seen upon [3H]mevalonate labeling of synchronized skin
**Partial Deficiency of Dehydrodolichol Reduction in CDG Syndrome**

**Fig. 7.** The behavior of intermediates in relation to the biosynthetic pathway for LLO in CDG syndrome type I fibroblasts upon metabolic labeling with [3H]glucosamine, [3H]mannose, or [3H]mevalonate, and the possible primary defect site. →, increase; ←, decrease; ←/→, partial defect site.

fibroblasts from CDG syndrome type I patients and controls, [3H]dehydrodolichol was accumulated in the S phase in the fibroblasts from these patients, and the amounts of [3H]dolichol and [3H]LLO were lower than those in control fibroblasts, although the chain length distributions of the dolichol and dehydrodolichol were equal in every preparation. On the basis of these results, the behavior (→, ←) and possible primary defect site (←/→) in the LLO biosynthetic pathway responsible for CDG syndrome type I in our patients are summarized in Fig. 7.

Considering that dehydrodolichyl diphosphate synthase activity increases in the S phase and decreases in the G1, G2, and M periods, substantial quantities of dehydrodolichol should be synthesized in the S phase. At the same time, because a lot of membrane-bound glycoproteins are also synthesized in the S phase, an abnormality of dolichol synthesis could be clearly detected in the S phase.

The biosynthesis of LLO is dependent on the cell cycle, and the cellular level of LLO is at least 10 times higher in the S phase than in the G1 and G2 periods (see Fig. 1). Also, the level of LLO gradually decreases with an increase in PDL (data not shown). Accordingly, in order to obtain reproducible results, it was important that cells with similar population doubling levels and with similar proliferative potential should be tightly synchronized for metabolic studies. Furthermore, the medium used for metabolic labeling with [3H]glucosamine or [3H]mannose was immediately replaced with 50% methanol in buffer containing EDTA, and the harvested cells were then treated with chloroform or boiled at 100 °C for 2 min. Since metabolically labeled LLO, mannose 1-phosphate, mannose 6-phosphate, and GDP-mannose, which are intermediates of N-linked oligosaccharide synthesis, are immediately transferred to the corresponding polypeptides within several minutes, such immediate inactivation of enzymes in the metabolically labeled fibroblasts was also important for obtaining reproducible results.

It is known that several mutants of yeast that are devoid of certain enzymes required for the complete formation of LLO accumulate small-sized LLO, as follows (34): alg 1 (β1-4-mannosyltransferase deficiency), alg 2 (α1-3-mannosyltransferase deficiency), alg 3 (dolichylphosphomannose synthase deficiency?), alg 5 (dolichylphosphoglucose synthase deficiency), and alg 6 (α1-3-glucose transferase deficiency) accumulate GlcNAc2PP-dolichol, Man2GlcNAc2PP-dolichol, Man3GlcNAc2PP-dolichol, and Man4GlcNAc2PP-dolichol, respectively. Because no accumulation of small-sized LLO was observed in our CDG syndrome type I patients, it appears that none of the glycosyltransferases responsible for LLO biosynthesis was missing.

It has been reported that Chinese hamster ovary cells of the Leec 9 recessive complementation group contain a lower amount of dolichol, and mutants biosynthesize underglycosylated glycoproteins (29, 35) similar to those described in our CDG syndrome type I cases (8). One of the mutants, F2A8 cells accumulated 10 times more polypropens than the wild type, 99% of the polypropens being dehydrodolichol and the polypropens of LLO being exclusively dehydrodolichol (36), showing that F2A8 cells completely lack dehydrodolichol reductase activity. On the other hand, the conversion of dehydrodolichol to dolichol in our CDG syndrome type I patients was not blocked, but was slower, since the accumulation of dehydrodolichol was not so high.

When the biosynthesis of dolichol is partially deficient, not only glycoproteins containing N-linked sugar chains but also glycosphatidylinositol (GPI)-anchored glycoproteins should be partially underglycosylated, because dolichylphosphomannose is used as a substrate for glycan formation of the GPI anchor (37). In preliminary experiments, normal serum alkaline phosphatases were found to be exclusively linked to glycans derived from the GPI anchor; however, a portion of the alkaline phosphatases in the sera of patients with CDG syndrome type I were devoid of the glycan portion derived from the GPI anchor. These results also support the view that a partial deficiency of dehydrodolichol reduction is the primary cause of CDG syndrome type I in our patients.

Two models have already been suggested for the fundamental defect in CDG syndrome type I as follows: Panneerselvam and Freeze (11) proposed defective mannose uptake and Van Schaftingen and Jaeken (12) proposed a phosphomannomutase deficiency. In our patients, we suggest that the abnormal mannose metabolism in CDG syndrome type I is not the actual problem but that conversion of dehydrodolichol to dolichol is partially defective. The resulting loss of dolichol may lead directly to underglycosylation. Judging from the results so far described, there might be multiple causes of CDG syndrome type I.

It has been observed that the abundance of various glycoproteins decreases in CDG syndrome type I. For example, 1) partially underglycosylated glycoproteins tend to be precipitable (9), resulting in their immediate clearance from the blood-
stream. 2) Intracellular lysosomal enzymes in CDG syndrome type I decrease, as in the case of I-cell disease, because a trafficking signal for lysosomes might be absent. 3) mRNA expression of decorin is reduced in CDG syndrome type I fibroblasts, which might be due to the decrease in cytokine receptors on the cell surface (38). The decrease in phosphomannomutase in our patients might be due to a mechanism similar to that in the case of other glycoproteins so far described. Such decreases in various enzymes and glycoproteins in CDG syndrome type I should be taken into account when we search for the defect in this disease.

Yasugi et al. (39) previously proposed that CDG syndrome type I is not due to a deficiency of the enzyme responsible for the biosynthesis of dolichyl phosphate, because total dolichols, including free dolichol and esterified dolichol, in skin fibroblasts and sera from patients with CDG syndrome type I and normal controls were the same. However, it was previously reported regarding the intracellular distribution of dolichols in pig liver that 76.7% of the free dolichol was located in the mitochondria, while 52.7% of the esterified dolichol was in the cytoplasm. 2) Intracellular lysosomal enzymes in CDG syndrome type I decrease, as in the case of I-cell disease, because a trafficking signal for lysosomes might be absent.

Because dehydrodolichol reductase activity in cultured cells has not yet been measured, we are now improving the microassay system for this enzyme activity, and we intend to use it to compare CDG syndrome type I and control fibroblasts in the near future.

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