Molecular Cloning and Expression of GDP-\(\beta\)-mannose-4,6-dehydratase, a Key Enzyme for Fucose Metabolism Defective in Lec13 Cells*

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Subsets of mammalian cell surface oligosaccharides contain specific fucosylated moieties expressed in lineage- and/or temporal-specific patterns. The functional significance of these fucosylated structures is incompletely defined, although there is evidence that subsets of them, represented by the sialyl Le\(^\alpha\) determinant, are important participants in leukocyte adhesion and trafficking processes. Genetic deletion of these fucosylated structures in the mouse has been a powerful tool to address functional questions about fucosylated glycans. However, successful use of such approaches can be problematic, given the substantial redundancy in the mammalian α1,3-fucosyltransferase and α1,2-fucosyltransferase gene families. To circumvent this problem, we have chosen to clone the genetic locus encoding a mammalian GDP-\(\beta\)-mannose-4,6-dehydratase (GMD). This enzyme generates GDP-\(\beta\)-mannose-4-keto-6-D-deoxymannose from GDP-mannose, which is then converted by the FX protein (GDP-4-keto-6-D-deoxymannose epimerase/GDP-4-keto-6-L-galactose reductase) to GDP-\(\alpha\)-fucose. GMD is thus imperative for the synthesis of all fucosylated oligosaccharides. An expression cloning approach and the GMD-deficient CHO host cell line Lec13 were used to generate a population of cDNA molecules enriched in GMD cDNAs. This enriched plasmid population was then screened using a human expressed sequence tag (EST AA065072) with sequence similarity to an Arabidopsis thaliana GMD cDNA. This approach, together with 5′-rapid amplification of cDNA ends, yielded a human cDNA that complements the fucosylation defect in the Lec13 cell line. Northern blot analyses indicate that the GMD transcript is absent in Lec13 cells, confirming the genetic deficiency of this locus in these cells. By contrast, the transcript encoding the FX protein, which forms GDP-\(\alpha\)-fucose from the ketosugar intermediate produced by GMD, is present in increased amounts in the Lec13 cells. These results suggest that metabolites generated in this pathway may participate in the transcriptional regulation of the FX protein and possibly the GMD protein. The results also suggest that the genomic structure encoding GMD in Lec13 cells likely has a defect different from a point mutation in the coding region.

Fucose is one of the critical carbohydrates in membrane-associated glycoproteins and glycolipids. Carbohydrates containing fucose are often characteristic of different cell types and are determinants for carbohydrate antigens (1). In particular, sialyl Le\(^\alpha\), NeuNAc\(_2\)→3Gal\(_1\)→4(Fuc\(_1\)→3)GlcNAc→R, discovered in granulocytes (2), was found to be a ligand for E- and P-selectin (3–6). The isomer of sialyl Le\(^\alpha\), sialyl Le\(^\beta\), NeuNAc\(_2\)→3Gal\(_1\)→3(Fuc\(_1\)→4)GlcNAc→R, is also a ligand for E- and P-selectin (7, 8). Moreover, sulfated derivatives of sialyl Le\(^\alpha\), were found to be ligands for l-selectin (9, 10), although sialyl Le\(^\beta\) serves as an inefficient l-selectin ligand (10, 11).

Expression of these fucosylated oligosaccharides is dependent on fucosyltransferases and donor substrate GDP-\(\alpha\)-fucose (12). So far at least four fucosyltransferases, FucTIV, -V, -VI, and -VII were found to be capable of forming sialyl Le\(^\alpha\) (13–17). On the other hand, FucTIII was found to be responsible for the expression of sialyl Le\(^\beta\) as well as sialyl Le\(^\alpha\) (12). Among these \(\alpha\)→3/4 fucosyltransferases, FucTVII is present in granulocytes, memory T cells, and high endothelia venules and directs the synthesis of selectin ligands in these cells (14, 15, 17). Recent report on gene knockout of mouse FucTVII clearly demonstrated the role of FucTVII in selectin ligand presentation (18). On the other hand, FucTIII, -V, and -VI are present in a wide variety of cells, and more than one \(\alpha\)→3/4 fucosyltransferase is present in a given tissue or cell.

To address the roles of sialyl Le\(^\beta\) and fucosylated oligosaccharides in general, a mouse lacking each gene encoding a fucosyltransferase must first be established and then such a mutant mouse must breed with another mutant mouse. Such a step has to be repeated three or possibly four times to obtain null mouse which completely lacks sialyl Le\(^\alpha\) in all tissues. In order to overcome this problem, we decided to clone a cDNA encoding an enzyme that is critically involved in fucose metabolism in general. This direction was also prompted by the report on patients with recurrent pneumonia and skin infections (19). These patients lack sialyl Le\(^\beta\) in neutrophils and are defective in the recruitment of neutrophils to sites of inflammation. Because fucose-containing antigens such as ABO blood group antigens are also absent in these patients, it is assumed that a step in the fucose metabolism is defective in these patients (19).

Donor substrate GDP-\(\alpha\)-fucose is synthesized from GDP-\(\beta\)-mannose via three steps; GDP-\(\beta\)-mannose is first converted to

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank/EBI Data Bank with accession number(s) AF040260.†

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1 The abbreviations used are: Le, Lewis; GMD, GDP-\(\beta\)-mannose-4,6-dehydratase; \(\alpha\)1,2-FT, \(\alpha\)1,2-fucosyltransferase; FITC, fluorescein isothiocyanate; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; FX protein, GDP-4-keto-6-D-deoxymannose epimerase/GDP-4-keto-6-L-galactose reductase; CHO, Chinese hamster ovary; FucT, fucosyltransferase; EST, expressed sequence tag.

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GDP-4-keto-D-mannose-4,6-dehydratase, and then to GDP-4-keto-6-L-deoxygalactose, which is further converted to GDP-L-fucose (20, 21). It has been demonstrated that GDP-mannose-4,6-dehydratase, catalyzing the first reaction in the above metabolic pathway, is defective in a mutant CHO cell line Lec13 (22). However, no successful correction of Lec13 phenotype has been reported.

In this report, we first describe the molecular cloning of the human GDP-mannose-4,6-dehydratase (GMD). For this cloning, cDNA was initially enriched by expression cloning strategy using Lec13 as recipient cells. Plasmids rescued from those transfected cells strongly positive for H antigen expression. Plasmid DNA was amplified in the host bacteria Escherichia coli DH10B/P3 in the presence of ampicillin and tetracycline. The plasmid vector contains the supF suppressor tRNA gene, so that DH10B/P3 cells containing pcDNA I are resistant to both ampicillin and tetracycline. In contrast, DH10B/P3 cells having pcDNA3- or pPSVE1-PyE are resistant only to ampicillin. By selection with ampicillin and tetracycline, only bacteria containing pcDNA I were rescued and amplified (26), allowing the isolation of plasmids responsible for fucose expression assessed by the anti-H antibody. From this initial pool of 2×10⁴ plasmids, sibling selection was carried out to isolate a plasmid clone that encodes a GDP-D-mannose-4,6-dehydratase from Arabidopsis thaliana (23), resulting in the isolation of a plasmid DNA containing the human GMD. Introduction of the cloned cDNA into Lec13 cells resulted in the expression of a plasmid DNA containing the human GMD. Introduc-

Experimental Procedures

Vectors and Antibody—α-1,2-Fucosyltransferase (α-1,2-FT) cDNA was excised from pcDNA I-α-1,2-FT (24) by EcoRI and XbaI digestion and cloned into the same sites in pcDNA3, resulting in pcDNA3-α-1,2-FT. α-1,2-FT was shown to add α-1,2-fucose to both type 1 (Galβ1-3GlcNAc) and type 2 (Galβ1-4GlcNAc) oligosaccharides, forming Leb and H structure, respectively (24). pcDNA I-FucTIII was prepared as described previously (25). Anti-H antibody was prepared from a hybridoma cell line obtained from American Type Culture Collections (CLONTECH) as a template and an oligonucleotide 5'-TCAGT-3' complementary to mouse IgM. The cells were then sorted by fluorescence-activated cell sorting using FACStar (Becton Dickinson).

Cloning of GDP-mannose-4,6-dehydratase—Lec13 cells were thus co-transfected with 7 µg of a human fetal brain cDNA library in pcDNA I (26), 7 µg of pcDNA3-α-1,2-FT, and 7 µg of pPSVE1-PyE harboring polyoma large T antigen cDNA (27), using LipofectAMINE™ (Life Technologies, Inc.) as described previously (26). Sixty-two h after the transfection, the transfected cells were dispersed into single cells by the cell dissociation solution (Cell & Molecular Technologies, Levelle, NJ), and then incubated with mouse anti-H antibody followed by FITC-conjugated goat anti-mouse antibody. After washing, the transfected cells strongly positive for H antigen expression were then screened by a human anti-H antibody. From this initial pool of 2×10⁴ plasmids, sibling selection was carried out to isolate a plasmid clone that encodes a full length of the coding region. This cDNA was cloned into pcDNA I, resulting in pcDNA I-hGMD.

| Nucleotide Sequence of Human GDP-D-mannose-4,6-dehydratase | Translated Amino Acid Sequence |
|-----------------------------------------------------------|--------------------------------|
| ATCCTTCACTTTAGTTGCTAGGACAGTGCCTACTGCTGGAGACATCAAAT--- | ALVHGFDK |
| ... | ... |

Fig. 1. Nucleotide and translated amino acid sequences of human GDP-D-mannose-4,6-dehydratase. A potential polyadenylation signal is singly underlined. Translated amino acid residues are shown below the nucleotide sequence. The sequences are numbered relative to the translation initiation site.
Transfection of Lec13 cells with pcDNA I-hGMD—Lec13 cells were transfected with pcDNA3-FucTIII and pcDNA 1-hGMD using LipofectAMINE as described (26). After selection with G418 (Life Technologies, Inc.), the transfected cells were selected by immunofluorescent staining for their expression of Le\(^a\) using anti-Le\(^a\) antibody (Immuno-Dynamics, Inc.), the transfected cells were selected by immunofluorescent staining for their expression of Le\(^a\) using anti-Le\(^a\) antibody (ImmunoTech, Marseille, France) or sialyl Le\(^a\) using CSLEX-1 antibody (Becton Dickinson), as described previously (10, 26).

Northern Blot Analysis—Northern blots of poly(A)\(^+\) RNA from human fetal and adult multiple tissues were purchased from CLONTECH. Northern blots were also made using poly(A)\(^+\) RNA isolated from CHO parent cells, Lec13, HeLa, and HepG2 cells using a FastTrack™ 2.0 kit (Invitrogen). These blots were hybridized with a gel-purified cDNA insert of pcDNA I-hGMD after labeling with \(^{32}\)PdCTP by random oligonucleotide priming (Prime It-II labeling kit, Stratagene). The blots, made in an identical manner, were hybridized with a gel-purified cDNA encoding GDP-4-keto-6-deoxymannose epimerase/NADPH-dependent reductase (FX protein) (29). This cDNA encoding FX protein was obtained as an EST cDNA (AA115440) and purchased from Genome Systems.

In Vitro Assay for Conversion of GDP-D-mannose to GDP-L-fucose—Enzymatic activity of GMD and formation of the GDP-L-fucose from GDP-D-mannose were assayed using a slight modification of procedures published previously (22). The incubation mixture in 100 \( \mu \)l contained 0.1 \( \mu \)mol of GDP-\(^{14}\)C-mannose (0.06 \( \mu \)Ci, NEN Life Science Products), 100 mM Hepes, pH 7.0, 5 mM ATP, 10 mM nicotinamide, 2% glycerol, 0.28 mg/ml phenylmethylsulfonyl fluoride, and 96 \( \mu \)g of cytosolic proteins. In some instances, assays were supplemented with 0.2 mM NADPH. Assays completed in the absence of added NADPH measure the generation of GDP-4-keto-6-deoxymannose intermediate from GDP-D-mannose via the action of GDP-D-mannose-4,6-dehydratase activity (20, 21). This keto intermediate accumulates because its subsequent conversion to GDP-L-fucose by the action of the FX protein is an NADPH-dependent reaction (29). Consequently, assays supplemented with NADPH measure the concerted actions of both steps in this pathway.

The unstable GDP-ketosugar intermediates generated in the assays were converted to their reduced forms and acid-hydrolyzed as described before (22). Free mannose liberated by this hydrolysis procedure was re-phosphorylated with yeast hexokinase and removed by passing through a Dowex 1 (X8-200) column (phosphate counter-ion) as described (22). The eluate was dried by rotary evaporation, resuspended in a small volume of water, and subjected to descending paper chromatography for 6 h on Whatman No. 1 paper in the upper phase of pyridine:ethyl acetate:water (1:0.3:6.1.15, v/v/v). The paper was cut into 3-cm strips, and the radioactivity in each strip was quantitated by scintillation counting. Sugars were identified by their mobilities relative to commercially available standards (mannose, fucose, 6-deoxyglucose, and \( \alpha\)-rhamnose), or 6-deoxytalose standard generously provided by Dr. James Paulson and Katherine Ketchum (Cytel Corp.). The parent CHO (Pro \(^5\)) and Lec13 cells were kindly provided by Dr. Pamela Stanley, Albert Einstein College of Medicine.

RESULTS

Cloning of cDNA Encoding Human GMD—CHO mutant Lec13 cells were co-transfected with a human fetal brain cDNA library in pcDNA I, pcDNA3-\( \alpha\)-1.2-FT, and pPSVE1-PyE. The transfected cells highly positive for H antigen expression were isolated by fluorescence-activated cell sorting. Plasmid DNAs, recovered from the above H-antigen positive Lec13 cells, were initially subjected to sibling selection with sequentially smaller, active pools, attempting to isolate a single clone that directs the synthesis of fucose in Lec13 cells. However, this attempt was not successful (see below).

We then screened the plasmid pool right after the cell sorting with a human cDNA fragment which contains a sequence highly homologous to \( A. \) thaliana GMD (23) and isolated four positive clones. Comparing \( A. \) thaliana GMD to human cDNA sequences cloned, the cloned DNA contained almost all of the coding sequence but lacks the extreme 5′-region. The sequence obtained by 5′-RACE reaction was ligated to the common NeoI site of the cDNA clone to form a cDNA encoding the entire
GMD, resulting in pcDNA I-hGMD. The cDNA sequence of hGMD contains 1466 nucleotides encoding 339 amino acids (Fig. 1). Because poly(A) tail is present in nucleotides 1383–1409, ATTAAA at nucleotides 1355–1360 may have functioned as a polyadenylation signal.

The amino acid and nucleotide sequence of human GMD are similar to those of the A. thaliana GMD (55.0% and 55.4% identity, respectively). The sequence is highly conserved from bacteria to human (see also Ref. 23).

Expression of Various Fucosylated Antigens on Lec13 Cells after Transfection with pcDNA I-hGMD—To confirm that pcDNA I-hGMD directs the expression of fucosylated oligosaccharides on Lec13 cells, Lec13 cells were co-transfected with pcDNA I-hGMD and pcDNA3-FucTIII or pcDNA I-hGMD and pcDNA3-α-1,2-FT. As shown in Fig. 2 (A and B) and Lec13 cells stably transfected with pcDNA I-GMD and FucTIII (C and D) and the parent CHO Pro cells (E and F) were assayed in the absence (A, C, and E) or the presence (B, D, and F) of NADPH. The radioactivity in the origin may be phosphorylated sugars (22). The parent Lec13 cells produced the same results as Lec13-FcTIII cells.

Formation of GDP-4-keto-6-deoxy-D-mannose by hGMD—To confirm that the cloned cDNA encodes GDP-D-mannose-4,6-dehydratase, the enzymatic assay was carried out on the Lec13 cells and Lec13 cells stably transfected with pcDNA I-hGMD. GDP-D-mannose is converted to GDP-4-keto-6-deoxy-D-mannose by GMD. This keto intermediate will be converted to GDP-L-fucose by an epimerase and GDP-4-keto-6-L-deoxygalactose reductase (Fig. 3). As shown previously, the last two reactions are carried out by a single enzyme, FX protein (29). In the absence of NADPH, however, the second and the third reactions do not take place, thus allowing us to measure the activity of the dehydratase by determining the amount of GDP-4-keto-6-deoxy-D-mannose formed (Fig. 3). GDP-4-keto-6-deoxymanose would be converted by NaBH₄ reduction to GDP-6-deoxy-D-talose and GDP-rhamnose, whereas GDP-4-keto-6-deoxy-L-
Lec13 poly(A) detected in testis and small intestine (data not shown). Among various adult tissues, the strongest signal was detected in colon and pancreas and a moderately strong signal was prominent in fetal kidney than in fetal brain, lung, and liver.

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Table I

| Tissue          | No. of kb | Duration (min) |
|-----------------|-----------|---------------|
| CHO(Pro)        | 2.18      | 45            |
| Lec13           | 2.18      | 45            |
| Lec13+FucTIII   | 2.18      | 45            |
| Lec13+FucTIII+GMD | 2.18     | 45            |

Expression of GMD and FX mRNAs in Lec13, Wild-type CHO, Lec13, HeLa, and HepG2 Cells—Northern blots of poly(A)+ RNA derived from various human tissues were examined. A GDm transcript of ~1.7 kilobases was detected in all tissues examined. However, it was more prominent in fetal kidney than in fetal brain, lung, and liver. Among various adult tissues, the strongest signal was detected in colon and pancreas and a moderately strong signal was detected in testis and small intestine (data not shown).

An ~1.4-kilobase transcript of FX protein, on the other hand, was more prominent in fetal liver than in fetal brain, lung, and kidney. Among adult tissues, pancreas, testis, colon, and skeletal muscle expressed more prominently the transcript for the FX protein than other tissues.

Expression of GMD and FX mRNAs in Lec13, Wild-type CHO, HeLa, and HepG2 Cells—Northern blot analysis of poly(A)+ RNA isolated from Lec13 and wild-type CHO cells, inasmuch as fucose was detected from reduced-acid hydrolysed product (Fig. 4D and F; see also Table I). These results establish that pcDNA I-hGMD encodes GDP-D-mannose-4,6-dehydratase, capable of correcting the defect in fucose metabolism in Lec13 cells.

Expression of GMD and FX mRNAs in Human Tissues—Northern blots of poly(A)+ RNA derived from various human tissues were examined. A GDm transcript of ~1.7 kilobases was detected in all tissues examined. However, it was more prominent in fetal kidney than in fetal brain, lung, and liver. Among various adult tissues, the strongest signal was detected in colon and pancreas and a moderately strong signal was detected in testis and small intestine (data not shown).

An ~1.4-kilobase transcript of FX protein, on the other hand, was more prominent in fetal liver than in fetal brain, lung, and kidney. Among adult tissues, pancreas, testis, colon, and skeletal muscle expressed more prominently the transcript for the FX protein than other tissues.

Expression of GMD and FX mRNAs in Lec13, Wild-type CHO, HeLa, and HepG2 Cells—Northern blot analysis of poly(A)+ RNA isolated from Lec13 and CHO cells demonstrated that the transcript for GMD was not detectable in Lec13 cells whereas it was detected in CHO cell (Fig. 5). In contrast, Lec13 cells express more transcripts for FX protein than CHO. The same analysis also showed that the transcript for FX protein is less in HepG2 cells than HeLa cells whereas the transcript for GMD is more in HepG2 cells than HeLa cells (Fig. 5). These results indicate that the transcript for GMD is absent in Lec13 cells. The results also suggest that the transcript for FX protein is increased when GMD is not sufficiently expressed.

DISCUSSION

In the present study, we have isolated a human cDNA encoding GMD using expression cloning strategy and then screening the obtained plasmid pool by EST sequence. The expression of cloned GMD in Lec13 cells corrected the phenotype of Lec13 cells, acquiring fucosylated oligosaccharides. The amino acid sequence of human GMD is highly homologous to those isolated from other organisms including bacteria (23, 30). This situation differs completely from Golgi-associated glycosyltransferases. For example, the amino acid sequences of mammalian polysialyltransferases (26, 31–34) differ entirely from bacterial polysialyltransferase (35). Similarly, human β-1,3-N-acetylglucosaminyltransferase II (36) and β-1,4-N-acetylglucosaminyltransferase II (41). Such a point mutation leads to inactivation of the enzyme, causing defective brain development (41). In contrast, Golgi-associated glycosyltransferases most likely evolved only after organisms reached eukaryotes, acquiring the Golgi-apparatus.

Previously, it has been shown that a point mutation in the coding region of N-acetylglucosaminyltransferase I and V leads into the glycosylation defect in Lecl and Lec4A cells, respectively (39, 40). In carbohydrate-deficient glycoprotein syndrome type II, a point mutation was discovered in the nucleotide sequence encoding the catalytic domain of β-1,2-N-acetylglucosaminyltransferase II (41). Such a point mutation leads to inactivation of the enzyme, causing defective brain development (41). In contrast, Lec13 cells lack the transcript for GMD as shown in the present study. This defect is similar to that discovered in one of HEMPAS (congenital disaccharide deficien ty II) patients, where the transcript for α-mannosidase II is substantially reduced (42). These results strongly suggest that a defect in the genomic structures encoding GMD and α-mannosidase II leads to glycosylation anomaly in Lec13 and HEMPAS patients, respectively. These defects can be due to a defect in the transcriptional regulation, a deletion of part or all of the locus or dramatically decreased mRNA stability caused by nonsense mutation (43). Moreover, the amount of the transcript for the FX protein, which converts the intermediate formed by GMD to GDP-L-fucose, is increased in Lec13 cells as if Lec13 cells try to compensate the low activity of GMD (44, 45). These results suggest that metabolites generated in this pathway may participate in the transcriptional regulation of the FX protein and possibly the GMD protein. It will be significant to determine if patients with the defect in fucose metabolism (19) is due to a genomic defect in GMD or FX protein, and in parallel to determine the genetic defect in Lec13 cells.
In the present study, we have demonstrated that the defect of fucose metabolism in Lec13 cells can be corrected by the expression of GMD. Similarly, the mutant of *A. thaliana* cells regained normal fucose metabolism by expressing GMD in the mutant cell line. A point mutation was identified in the coding sequence of GMD gene, *MUR1* in the latter studies (23). Although an additional gene for GMD is suggested in *A. thaliana* (23), these results strongly suggest that only one gene may be dominant for expressing GMD in *A. thaliana*, and most likely in Lec13 cells. Northern blot analysis indicated that cloned human GMD is expressed in all tissues so far examined. However, we do not know whether the cloned enzyme is solely responsible for fucose metabolism in hematopoietic and endothelial cells. These issues need to be dissolved before attempts for generating knock-out mice defective in the GMD gene will be carried out.

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