Synthesis of GDP-L-fucose by the Human FX Protein*

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FX is a homodimeric NADP(H)-binding protein of 68 kDa, first identified in human erythrocytes, from which it was purified to homogeneity. Its function has been unrecognized despite partial structural and genetic characterization. Recently, on the basis of partial amino acid sequence, it proved to be the human homolog of the murine protein P35B, a tumor rejection antigen. In order to address the biochemical role of FX, its primary structure was completed by cDNA sequencing. This sequence revealed a significant homology with many proteins from different organisms. Specifically, FX showed a remarkable similarity with a putative *Escherichia coli* protein, named Yebf, whose gene maps in a region of *E. coli* chromosome coding for enzymes involved in synthesis and utilization of GDP-α-mannose. Accordingly, a possible role of FX in this metabolism was investigated. The data obtained indicate FX as the enzyme responsible for the last step of the major metabolic pathway resulting in GDP-α-fucose synthesis from GDP-α-mannose in procaryotic and eucaryotic cells. Specifically, purified FX apparently catalyzes a combined epimerase and NADPH-dependent reductase reaction, converting GDP-4-keto-6-α-deoxymannose to GDP-L-fucose. This is the substrate of several fucosyltransferases involved in the correct expression of many glycoconjugates, including blood groups and developmental antigens.

FX has been known for several years as an NADP(H)-binding protein (1–2). It was isolated and purified to homogeneity from human erythrocytes where it is present at concentrations as high as 7 μg/ml of packed red cells (3). Higher FX levels, around 10 μg/ml red cells, were observed in subjects having the Mediterranean type of glucose-6-phosphate dehydrogenase (EC 1.1.1.49) deficiency (4).

The biochemical function of FX has been unrecognized because no apparent enzymatic activity nor regulatory effects on erythrocyte metabolism could be demonstrated. Recently, approximately 90% of its amino acid sequence was determined (5). This led unexpectedly to identifying FX as the human homolog of a putative murine protein, *Tum*- rejection antigen P35B, so designated because a specific point mutation of the normal gene results in an immune rejection response of the corresponding mastocytoma cell line, with complete loss of tumorigenicity (6).

The virtual identity between human erythrocyte FX and murine *Tum*- rejection antigen P35B suggested this protein may be ubiquitous, although its function still remained unknown. In an effort to address the biological role of FX, whose occurrence had been found also in many cells other than erythrocytes, its primary structure was completed by cDNA sequencing. Careful comparative analysis of the FX sequence with those present in data bases identified a remarkably conserved amino acid similarity with a number of proteins that are expressed in a wide range of organisms. Most of these proteins are enzymes catalyzing either NAD(P)H-dependent reduction reactions or nucleoside diphosphate sugar epimerization reactions, this suggesting a related function for FX protein. This finding led to focus on metabolism of nucleoside diphosphate sugars and specifically on a multi-step pathway that converts GDP-α-mannose to GDP-α-fucose (7–14) and is responsible for the largest part of cellular GDP-L-fucose biosynthesis (15). It soon became clear that FX purified to homogeneity from human erythrocytes is an enzyme protein involved in the above pathway, apparently catalyzing a two-step epimerase-reductase reaction that results in GDP-L-fucose synthesis. This result identifies FX as a key enzyme for GDP-L-fucose production in cells. This metabolite is the substrate of a number of fucosyltransferase activities involved in the biosynthesis of blood group ABH antigenic determinants (16–17) and of other fucosylated glycoproteins and glycolipids, which mediate cell adhesion and recognition (17–20).

EXPERIMENTAL PROCEDURES

Materials—All reagents were of purest grade available. GDP-α-mannose, GDP-α-fucose, β-mannose, L-fucose, β-6-deoxyxymannose (ranmanose), β-6-deoxyglucosylase, NADPH, NAD+, ATP, nicotinamide, NaBH₄, and phenylmethylsulfonyl fluoride were obtained from Sigma. GDP[U-¹⁴C]mannose (288 mCi/mmol) was obtained from Amersham Corp. GDP[U-¹⁴C]fucose (272 mCi/mmol) was obtained from DuPont NEN.

cDNA Sequencing—A polymerase chain reaction-based cloning strategy was adopted to obtain cDNA sequence of FX protein, using placental poly(A)⁺ RNA (obtained from Clontech, Palo Alto, CA). Primers (obtained from TIB Mol Biol, Genova, Italy) for polymerase chain reaction and reverse transcription, indicated in Fig. 1, were initially designed on the basis of FX amino acid sequence and of the corresponding sequence of mouse P35B cDNA (6) (primers F1, R1, and R2) and then from the nucleotide sequence derived (primers P2, F3, and B4). B4 and F3 were used as gene-specific primers for 5’ and 3’ rapid amplification of cDNA ends, respectively.

Reverse transcription was achieved using either oligo(dT) or gene-specific primers in reverse transcription buffer containing 2.5 mM MgCl₂, 10 units of RNasin (Promega, Madison, WI), 1 mM dNTP (Phar-macia Biotech, Inc.) and 25 units of reverse transcriptase (Perkin-Elmer), incubated at 37 °C for 1 h. Polymerase chain reaction amplification was performed for 35 cycles in 100-μl reaction volumes containing 1 × PCR Buffer II, 1.5 mM MgCl₂, 200 μM dNTP, 10 pmol of primers and using 1 unit of Ampli-Taq polymerase (Perkin-Elmer). The reaction was carried out on a Perkin-Elmer 2400 thermal cycler.

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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) U58766.

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Synthesis of GDP-L-fucose by Human FX Protein

5' and 3' ends were obtained by the rapid amplification of cDNA ends method (26), using the Marathon cDNA amplification kit (Clonteck) following the manufacturer's instructions.

Amplification products were directly inserted in a pCRII vector, by means of the TA Cloning Kit (Invitrogen, San Diego, CA). Plasmid DNA from positive clones was sequenced with Sequenase 2.0 (U.S. Biochemical Corp.), using supplier recommendations. Due to the possibility of misinsertions by DNA polymerase, sequence was performed on at least three different amplifications of each fragment.

Sequence homologies were searched on SwissProt and GenBank data bases using the FASTA (22) and BLAST (23) programs. Multiple alignments of the sequences were performed by the CLUSTAL program according to the method developed by Higgins and Sharp (24).

Purification of FX Protein and Production of Anti-FX Antibodies—Human erythrocytes were obtained from expired blood derived from the local transfusional centres. Purification was performed as described previously (1), but a further size exclusion chromatography step was introduced using an HPLC system equipped with a Tosoh TSK-gel G2000SW column (Montgomeryville, PA; 5-μm particle size, 7.8 × 300 mm), mobile phase, 50 mM Tris-HCl, pH 7.5, containing 300 mM NaCl, flow rate, 0.7 ml/min. Protein purity was checked by SDS-polyacrylamide gel electrophoresis (25) and by gel filtration. Rabbit polyclonal antibodies against homogenous FX were raised as described previously (3).

Cell Culture and Preparation of Cytosol—The human hepatocarcinoma cell line HepG2, kindly provided by Trendgen (Caserta, Italy) was cultured in RPMI 1640, supplemented with 10% fetal calf serum and 4 mM L-glutamine, and were routinely screened for mycoplasma contamination and found to be negative. Cells (1 × 10^6) growing exponentially were harvested by trypsinisation and washed three times in cold phosphate-buffered saline. The cell pellet was resuspended in 2 ml of cold 50 mM Tris-HCl, pH 8, containing 0.35 mM phenylmethylethylsulfonyl fluoride. Cells were then lysed by sonication, and the suspension was centrifuged at 100,000 g for 30 min. The supernatant was stored at −80°C.

Enzyme Assay—The standard assay for the conversion of GDP-D-mannose to GDP-L-fucose was performed as described previously (12). The incubation mixture contained 50 mM Tris-HCl, pH 8.0, 5 mM MgCl2, 0.2 mM NADP+, 0.2 mM ATP, 10 mM nicotinamide, 10 μM GDP-[U-14C]mannose (specific activity, 6.2 mCi/mmol) and 600 μM GDP-D-mannose, GDP-L-fucose, and the 4-keto-6-deoxysugar standards were co-chromatographed with samples and were detected by the diphenylamine/aniline/phosphoric acid reagent (27). TLC plates were developed three times using acetonitrile:water (95:5).

Identification of Products—GDP-D-mannose, GDP-L-fucose and the 4-keto-6-deoxysugar intermediates were analysed by HPLC, as described previously (26). Samples (250 μl) were injected onto a C18 μBondapack column (Waters, Milford, MA; 3.9 × 300 mm, 10-μm particle size) with 0.5 mM KH2PO4 as mobile phase at 1 ml/min flow rate. The eluate was monitored with a UV detector at 254 nm and for radioactivity using a Packard Radiomatic 500TR flow scintillation analyzer. Retention time of GDP-D-mannose and of GDP-L-fucose formed during the incubation were compared with those obtained for labeled standards (6.0 and 10.8 min, respectively).

To further confirm the identity of the products formed during the reactions, TLC analyses of the monosaccharides derived from acid hydrolysis of the corresponding nucleoside diphosphate sugars were performed. Due to instability of the 4-keto-6-deoxysugars, which prevents their direct identification (11, 12), the intermediates were subjected to chemical reduction with NaBH4 to yield the corresponding 6-deoxysugars (see Fig. 3). 1 mg of NaBH4 was added to 1 ml of the heat-inactivated extracts and incubated for 1 h at room temperature. After acid hydrolysis of the sugar nucleotides (50 μl of 2 N HCl, 20 min at 100°C) followed by neutralization with 2 N NaOH, samples were desalted through a Sephadex G-25 column (1 × 12 cm). After concentration under vacuum, samples were spotted on silica gel 60 TLC plates (Merck) pretreated by spraying with 0.1 M Na2S2O5 and 0.009 M sodium citrate buffer, pH 4.8, followed by drying at 100°C for 1 h (27). TLC plates were developed three times using acetonitrile:water (95:5).

Sugar standards were co-chromatographed with samples and were detected by the diphenylamine/methylphosphonic acid reagent (27). The radioabeled compounds were detected by cutting the TLC plates in 0.5-cm strips, scraping the silica gel, and counting it in a liquid scintillation spectrometer.

RESULTS

The cDNA sequence of FX contains 1330 nucleotides, with a 5'-untranslated region of 74 nucleotides and a 3'-untranslated region of 190 nucleotides, including the highly conserved polyadenylation signal AATAAA (Fig. 1). The coding sequence predicts a polypeptide of 320 amino acids, with a molecular mass of 35.7 kDa, in good agreement with the values previously reported for the human FX protein (1–2). The predicted amino acid sequence confirmed and completed the partial sequence obtained from tryptic peptides (5); the only difference observed was in the first peptide, in which the glutamine at position 5 is replaced by glutamic acid.

Comparison of FX complete sequence with available data bases confirmed a 92.6% identity (with 98% similarity, if conservative amino acid substitutions are considered) with the mouse tumor antigen P35B (6) and a 65% identity (with 79% similarity) with the R01H2.5 gene product of Caenorhabditis elegans. Further, a significant similarity with the nodulation protein NolK of Azorhizobium caulinodans and with the 36.1 kDa hypothetical protein Yebf from Escherichia coli was ob-

2 The abbreviation used is: HPLC, high performance liquid chromatography.
observed (Fig. 2). Unfortunately, no precise function is known for all these proteins. Some evidence indicated a nucleotide sugar epimerase-reductase activity for Nolk protein (28). Furthermore, the CSP ORF 0.9, which codes for Yebf and shares a 51% identity over 670 nucleotides with FX cDNA sequence, is located in a region of E. coli chromosome containing several genes involved in the production and metabolism of GDP-D-mannose, such as phosphomannomutase (EC 5.4.2.8), mannose-1-phosphate guanylyltransferase (EC 2.7.7.22), GDP-D-mannose dehydratase (EC 4.2.1.47) (29), and GDP-D-mannose mannosyl hydrolase (30).

These data prompted us to investigate a possible role of FX protein in GDP-D-mannose metabolism. Fig. 3 summarizes the pathway for the conversion of GDP-D-mannose to GDP-L-fucose, first described by Ginsburg (8), as well as the patterns of reduction with NaBH4 of the two unstable intermediates of the pathway, i.e. GDP-4-keto-6-D-deoxymannose (Fig. 3, compound I) and GDP-4-keto-6-D-deoxygalactose (compound II). Conversion of GDP-mannose to compound I is catalyzed by a dehydratase (13), whereas a single protein, which was purified to homogeneity from porcine thyroid gland, is apparently responsible for the two steps beyond GDP-D-mannose dehydratase, i.e. conversion of compound I to compound II (epimerase) and reduction of compound II to GDP-L-fucose (reductase) (14). The thyroid protein proved to be a 63–68-kDa homodimer (14).

The similarity of M, values between this epimerase/reductase and FX protein and the strict NADPH requirement of the epimerase/reductase (11–12) suggested that investigation of whether FX purified to a homogeneous form from human erythrocytes possesses such enzymatic activities might be beneficial. FX purity was checked by SDS-polyacrylamide gel electrophoresis, which revealed a single band at approximately 40 kDa (Fig. 4A). This value is higher than the 35.7 M, predicted from the amino acid sequence and the 35.9 M, obtained by a matrix-assisted laser desorption ionization-time of flight mass spectrometry analysis. Because the protein is not glycosylated (2), this finding could be explained by an incomplete masking of the intrinsic charge of the protein by SDS or by an atypical conformation induced by the presence of SDS, as previously shown for other proteins (31). HPLC size exclusion analysis against known molecular weight standards indicated for native FX molecular mass of 68 kDa, which is in good agreement with a homodimeric structure (Fig. 4B).

Because the substrate of epimerase/reductase, GDP-4-keto-6-deoxy-D-mannose (Fig. 3, compound I), is not available due to its instability, an assay was developed that allowed us to produce and accumulate this compound. The assay exploited the high activity of the GDP-D-mannose to GDP-L-fucose pathway in the cytosol of HepG2 cells (7.5 nanomoles/h/mg cytosolic protein). The production of GDP-L-fucose was completely blocked upon the addition of polyclonal anti-FX antibodies, which led to the accumulation of a new HPLC peak having a retention time of 8.8 min, intermediate between those of GDP-D-mannose and of GDP-L-fucose (Fig. 5A). Comparable results were observed in experiments in which NADPH was omitted from the incubation mixtures containing HepG2 cytosol and GDP-D-mannose, thus preventing the reductase reaction (11, 12). In both experimental settings, the addition of purified FX together with NADPH to the heat-inactivated incubation mixtures restored the GDP-L-fucose synthesizing activity while resulting in the concomitant disappearance of the 8.8-min HPLC intermediate (Fig. 5B). Neither FX nor NADPH alone exhibited this effect.

To further characterize the 4-keto intermediates formed in this multi-step system, NaBH4 was added to the assay mixtures in order to reduce the various intermediate species to stable products (see Fig. 3), and the nucleotide sugars were acid-hydrolyzed to yield the corresponding monosaccharides, which were analyzed by TLC. Upon incubation of HepG2 cytosol with GDP-D-mannose and NADPH, only fucose and residual mannose were detected by this procedure (not shown). When the incubation was performed in the presence of anti-FX antibodies, most of the total radioactivity co-migrated with authentic rhamnose standard, whereas the remainder corresponded mainly to mannose and to a limited extent to fucose (Fig. 6A). This pattern is consistent with a prevalent accumulation of GDP-4-keto-6-D-deoxymannose upon depletion of FX, because this nucleotide sugar is known to yield 6-deoxy-D-mannose (rhamnose) and 6-deoxy-D-talose (talomethylose) upon NaBH4 reduction and hydrolysis (12). The supplementation of the FX-depleted HepG2 cytosol with homogeneous FX and NADPH, only fucose and residual mannose were detected by this procedure (not shown). The pattern obtained when the NaBH4 reduction step was omitted (not shown), thus implicating fucose as derived from acid hydrolysis of GDP-L-fucose only and excluding its formation from chemical reduction of an intermediate compound (i.e. GDP-4-keto-6-D-deoxygalactose, see Fig. 3). This conclusion is also supported by absence of 6-deoxyglucose (Fig. 6B), which would arise together with fucose from the chemical reduction and hydrolysis of compound II (i.e. GDP-4-keto-6-D-deoxygalactose). Due to the small amount of fucose observed under conditions of FX depletion (Fig. 6A), we cannot exclude that in our experimental system FX activity could be incompletely blocked, particularly in its epimerase component. Consistently with a previous report (14), these data suggest that FX could be directly involved in displaying both activities, i.e. epimerase (converting I to II) and

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3 G. L. Damonte, personal communication.
NADPH-dependent reductase (converting II to GDP-L-fucose) beyond GDP-D-mannose-4,6 dehydratase in the pathway shown in Fig. 3.

**DISCUSSION**

Fucosylated glycoproteins and glycolipids have been identified as important cell surface molecules including blood group and developmental antigens (16–17). In addition, they have been shown to be involved in a number of pathophysiological processes, e.g. selectin-mediated adhesion of neutrophils to endothelial cells and related defects (32–33), and also tumorigenesis. For instance, the levels of fucosylation of cell surface glycoproteins have been reported to correlate with tumor invasiveness (34–35). Moreover, significantly higher levels of urinary fucose were detected in patients having specific tumors than in normal subjects (36). Recently, a new adhesion molecule defect, designated leukocyte adhesion deficiency II syndrome, was described in which a still unidentified defect of fucose metabolism blocks the expression of the fucosylated sialyl-Lewis X (SLeX) determinant on granulocytes (33).

Although considerable attention is being devoted to the enzymatic processes whereby these complex surface structures are modified by a number of fucosyltransferases (37–39), the information is more scanty on the biosynthesis of GDP-L-fucose that represents the substrate of these transferases. Specifically, an evolutionarily conserved multi-step pathway converting GDP-D-mannose to GDP-L-fucose has been described for many years both in procaryotic and eucaryotic cells (7–14, 40). Although the enzymes involved in this pathway in mammalian cells have been isolated and partially characterized (13, 14), they have not been sequenced and cloned. Furthermore, no information is presently available on the mechanisms that control the activity of this pathway, which represents the most important source of GDP-fucose. A fucose “salvage” pathway is also present, which starts from free fucose and leads to the formation of GDP-L-fucose by the action of a L-fucose kinase (EC 2.7.1.52) (41) and of a GDP-L-fucose pyrophosphorylase (EC 2.7.7.31) (42). However, its contribution seems quantitatively lower in supplying the cellular GDP-fucose pool, accounting for approximately 10% of it (15).

In view of the widespread and functionally important biological and pathological roles of fucosylated glycoconjugates, the present identification of a direct role of the long known NADPH-binding protein FX in terminating the major pathway of GDP-fucose biosynthesis seems to open new perspectives in the study of these processes. For instance, in erythrocytes its NADPH requirement points to stringent metabolic correlations with the NADPH-generating enzyme glucose-6-phosphate dehydrogenase in terms of expression of the major ABH group. In this respect, the increased FX levels in erythrocytes from glucose-6-phosphate dehydrogenase-deficient sub-
jects (4) might reflect some compensation for a defective NADPH supply in maintaining a normal rate of biosynthesis of the ABH antigens. Furthermore, besides glycosyl transferase activity and translocation of nucleotide sugars from cytosol, where they are formed, to Golgi lumen (43), another site of regulation of protein glycosylation is represented by the levels of the nucleotide sugars pools inside the cells (44). Some reports in literature indicate that an alteration of GDP-fucose biosynthetic pathway can lead to modifications in membrane fucosylation. For instance, two Chinese hamster ovary cell mutants, showing resistance to pea and lentil lectins, were found to be defective in the conversion of GDP-D-mannose to GDP-L-fucose, and the GDP-D-mannose 4,6-dehydratase-catalyzed reaction was identified as the site of the block (12). Furthermore, during intestinal development in the weaning period in rat, the profound changes observed in fucosylation of glycoconjugates were accompanied by significant alterations in the activity of the enzymes involved in this pathway (45). In particular, in the first days after birth the GDP-4-keto-6-D-deoxymannose epimerase/reductase activity proved to be significantly inhibited, with accumulation of the corresponding keto intermediate inside cells. On the contrary, at a later age this activity was found to be increased with consequent formation of GDP-L-fucose and was paralleled by an increase in fucosyltransferase activity and in the fucose content in glycoconjugates (45).

A better understanding of all these processes and of their alterations requires a detailed biochemical characterization of the GDP-D-mannose to GDP-L-fucose pathway. A related step toward this goal is the definitive assignment of epimerase/reductase activity to the FX protein through experiments ruling out unequivocally alternative yet remote possibilities. These might be, for instance, cooperation of FX with another protein present in the HepG2 cytosol in catalyzing this reaction or in generating a cofactor that might be required by such putative epimerase/reductase protein. The isolation of GDP-D-mannose 4,6-dehydratase, now underway in our laboratory, is required to develop an in vitro assay of this epimerase/reductase activity in which all components are known, thereby allowing the complete reconstruction of the GDP-D-mannose to GDP-L-fucose pathway. Accordingly, both catalytic and regulatory mechanisms involved in GDP-L-fucose biosynthesis should be adequately investigated. In any case, the availability of new experimental tools (e.g., FX cDNA and recombinant FX) should enable to address fucose metabolism and its subsequent fate in several cell types and in physiological and pathological conditions as well.

A similar pathway for the synthesis of 6-deoxy sugars has been described in E. coli for the production of 6-deoxy-1-L-talose (46). This pathway, starting from dTDP-1-glucose, consists of a dehydratase reaction, which leads to a 4-keto intermediate, followed by an epimerization at carbons 3 and 5 and by a reduction by NADPH of the 4-keto group. However, in this case two separate enzymes seem to be involved in the epimerization and in the reduction steps, respectively (47). The evidence

FIG. 5. Identification of 14C-labeled GDP-sugars after HPLC separation on a C18 column: GDP-D-mannose (1), GDP-4 keto intermediate products (2), and GDP-L-fucose (3). GDP-4-keto-6-D-deoxymannose could not be separated from GDP-4-keto-6-L-deoxygalactose by this analysis. Detection was performed using a continuous flow scintillation analyzer. Retention times of GDP-D-mannose and GDP-L-fucose corresponded to those obtained for radiolabeled standards. Incubations and extraction were performed as described under "Experimental Procedures." A, incubation was carried on at 37 °C in the presence of 1 μg of rabbit polyclonal IgG and stopped after 90 min by heating for 2 min at 100 °C. In these conditions, GDP-L-fucose formation was prevented, and the accumulation of an intermediate peak was observed. B, 0.5 μg of purified FX were added to the heat-treated assay mixture, and incubation was performed for further 30 min. This caused the disappearance of the intermediate peak and restored GDP-L-fucose production. Chromatograms were obtained from a representative experiment.
Synthesis of GDP-L-fucose by Human FX Protein

already provided by Chang et al. (14) that two enzymatic activities, i.e. the epimerase and the reductase, are present on a single protein received now additional support by the present data on FX. Thus, FX seems to have unique properties among many enzymes involved in the metabolism of nucleotide diphosphate sugars that share features of epimerases and oxidoreductases (48). These enzymes are characterized by protein-bound NAD+ that mediates the intramolecular transfer of hydrogen in both initiation and termination of enzyme catalysis therefore resulting in epimerization reaction (48). Conversely, although this conclusion awaits the availability of pure GDP-4-keto-6-deoxy-mannose 4,6-dehydratase initiating the complete metabolic pathway of GDP-L-fucose deserve further investigation.

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