The enzyme that catalyzes the formation of GDP-β-l-fucose from GTP and β-l-fucose-1-phosphate (i.e. GDP-β-L-fucose pyrophosphorylase, GFPP) was purified about 560-fold from the cytosolic fraction of pig kidney. At this stage, there were still a number of protein bands on SDS gels, but only the 61-kDa band became specifically labeled with the photoaffinity substrate, azido-GDP-β-[32P]fucose. Several peptides from this 61-kDa band were sequenced and these sequences were used for cloning the gene. The cDNA clone yielded high levels of GFPP activity when expressed in myeloma cells and in a baculovirus system, demonstrating that the 61-kDa band is the authentic GFPP. The porcine tissue with highest specific activity for GFPP was kidney, with lung, liver, and pancreas being somewhat lower. GFPP was also found in Chinese hamster ovary, but not Madin-Darby canine kidney cells. Northern analysis showed the mRNA in human spleen, prostate, testis, ovary, small intestine, and colon. GFPP was stable at 4 °C in buffer containing 50 mM sucrose, with little loss of activity over a 9-day period. GTP was the best nucleotide substrate for the enzyme, purified from kidney or liver, and was utilized by a GDP-β-arabinose-1-P to produce GDP-β-α-arabinose. The product of the reaction with GTP and α-L-fucose-1-P was characterized as GDP-β-L-fucose by a variety of chemical and chromatographic methods.

Fucosylated oligosaccharides with sialic acid on one terminus have recently been demonstrated to be ligands for several members of the selectin family of membrane receptors. In particular, NeuAc2,3Galβ1,4(Fucα1,3)GlcNAc-R, the sialyl Lewisx antigen, is the ligand for the E and P selectins (10–13). In addition, an isomer of the sialyl Lewisx, called sialyl Lewisa, and having the structure NeuAc2,3Galβ1,3(Fucα1,4)GlcNAc-R, is also a ligand for the E selectin (14, 15). I-Selectin also binds to sialyl Lewisx antigen, but whether this, or some other structure, is the natural ligand for this selectin is still not known (16). Specific studies on structure-function of these ligands have suggested that subtle differences in the structures of the sialyated, fucosylated oligosaccharides affect the binding affinities of the selectins for these molecules (17, 18).

The fucosyl donor for these fucosylated oligosaccharides is GDP-β-L-fucose. The major pathway for formation of GDP-fucose involves the oxidation-reduction and epimerization of GDP-α-D-mannose to produce GDP-β-L-fucose (19, 20). This pathway is present in most animal cells and tissues, as well as in plants and microorganisms. An alternate pathway of formation of GDP-β-fucose is present in certain mammalian tissues, such as liver and kidney, and apparently functions as a salvage pathway to reutilize L-fucose arising from the turnover of glycoproteins and glycolipids (21). This pathway involves the phosphorylation of L-fucose by a specific kinase to form β-L-fucose-1-P (22–24), and then condensation of the l-fucose-1-P with GTP by a GDP-fucose pyrophosphorylase to form GDP-β-L-fucose (25, 26).

In the present study, we have purified the GDP-β-L-fucose pyrophosphorylase from porcine kidney, and identified the specific GFPP protein band using the photoprobe N3-GDP-[32P]fucose. Several peptide sequences from this protein were used to clone the encoding cDNA which gave high amounts of the specific GFPP protein band using the photoprobe N3-GDP-[32P]fucose. Several peptide sequences from this protein were used to clone the encoding cDNA which gave high amounts of the specific GFPP protein band using the photoprobe N3-GDP-[32P]fucose. Several peptide sequences from this protein were used to clone the encoding cDNA which gave high amounts of the specific GFPP protein band using the photoprobe N3-GDP-[32P]fucose.

EXPERIMENTAL PROCEDURES

Materials

The abbreviations used are: GFPP, GDP-β-L-fucose pyrophosphorylase; PCR, polymerase chain reaction; HPLC, high performance liquid chromatography; EST, expressed sequence tag.
Purification of Kidney GDP-L-fucose Pyrophosphorylase

Sepharose were purchased from Sigma. Polyethyleneimine-cellulose TLC plates, cellulose TLC plates, and silica gel TLC plates were purchased from EM Science Inc. The following materials were obtained from Bio-Rad: SDS, acrylamide, bisacrylamide, hydroxyapatite, Coomassie Blue, and protein assay reagent. All other chemicals were from reliable chemical sources, and were of the best grade available.

GDP-L-fucose Pyrophosphorylase Assay

The pyrophosphorylase could be assayed in the forward (i.e., formation of GDP-fucose from fucose-1-P and GTP), or in the reverse (i.e., formation of fucose-1-P from GDP-fucose and inorganic pyrophosphate) directions, as indicated below.

For measuring the activity in the forward direction, the incubation mixtures contained the following components in a final volume of 50 μl: 100 mM Tris-HCl buffer, pH 7.6, 8 mM MgCl₂, 100 μM [3H]fucose-1-P (40,000 cpm), 5 mM GTP, 0.5 unit of inorganic pyrophosphatase, and different amounts of the GFPP at various stages of purification. After an incubation at 37 °C for 5 or 10 min, the reaction was terminated by heating the tubes in a boiling water bath for 40 s. The reaction mixture was then applied to a column of DE-52 (about 1 ml of resin in a Pasteur pipette), and the columns were washed with 50 mM (NH₄)HCO₃ to remove sugar phosphates, and then with 200 mM (NH₄)HCO₃ to elute the GDP-fucose. The radioactivity in the various fractions was determined as a measure of the amount of GDP-fucose formed.

The amount of product could also be determined by measuring the amount of radioactivity that bound to DE-52 after treatment of the reactions with 2 units of alkaline phosphatase for 20 min at 37 °C. Alkaline phosphatase removes the phosphate group from any remaining sugar phosphate but does not affect the nucleoside diphosphate sugar that is produced in the reaction. After treatment with phosphatase, the reaction mixtures were placed on DE-52 and the columns were washed with 10 mM (NH₄)HCO₃. The GDP-fucose was eluted from the columns as described above and the radioactivity in the eluate was determined.

In most of the experiments described in this study, pyrophosphorylase activity was determined by measuring the formation of GDP-L-fucose (forward assay), since this assay was considered to be more reliable. The enzyme activity, especially with more purified enzyme fractions, could also be determined by measuring the formation of fucose-1-P from GDP-fucose (reverse assay). For measuring activity in the reverse direction, the incubation mixtures contained the following components in a final volume of 50 μl: 100 mM Tris-HCl buffer, pH 7.4, 5 mM sodium pyrophosphate, 8 mM MgCl₂, 100 μM GDP-[3H]fucose (10,000–15,000 cpm), and various amounts of the GFPP at different stages of purification. Incubations were done at 37 °C for 5 min, and reactions were terminated by the addition of 0.5 ml of ice-cold 5% trichloroacetic acid. The nucleotides were then absorbed on to activated charcoal (150 mg/ml of Darco G-50 in water). The suspension was vortexed vigorously, centrifuged, and the supernatant liquid was removed and saved. The charcoal pellet was washed with 1 ml of water and again centrifuged. The supernatant liquid from this wash was pooled with the first supernatant liquid, and an aliquot of the pooled fraction was counted for the determination of radioactivity converted to [3H]fucose-1-P.

Preparation of Crude Extract

Pig kidneys were obtained from a local slaughterhouse and were kept on ice until used. The kidneys were cut into small pieces and homogenized in Buffer A (10 mM Tris-HCl buffer, pH 7.6, containing 1 mM β-mercaptoethanol, 1 mM EDTA, 50 mM sucrose, and 1 mM phenylmethylsulfonyl fluoride) in a Waring blender for 3 to 4 min, at high speed. The homogenate was centrifuged at 12,000 × g for 20 min at 4 °C, and the supernatant liquid was removed and filtered through several layers of cheesecloth, and centrifuged at 100,000 × g for 45 min. The supernatant liquid was removed and used as the crude extract.

Purification of the GDP-β-L-fucose Pyrophosphorylase

The various steps used in the purification of the enzyme were as follows.

Step 1: Polyethylene Glycol Precipitation—Solid polyethylene glycol (8,000 molecular weight) was added to the crude extract to reach a concentration of 30%. The solution was centrifuged and the supernatant liquid was discarded. The precipitate was dissolved in a minimal volume of Buffer A.

Step 2: Column Chromatography on DE-52—The enzyme from step 1 was applied to a 5 × 18-cm column of DE-52 that had been equilibrated with Buffer A. The column was then washed with Buffer A to remove unbound protein, and the pyrophosphorylase was eluted with 1 liter of a linear gradient of 0 to 250 mM NaCl. Eight-ml fractions were collected and the enzyme emerged between 75 and 150 mM NaCl. Active fractions were pooled and utilized in further purification steps.

Step 3: Chromatography on Phenyl-Sepharose—The active fractions from DE-52 column were pooled and brought to 60% saturation by the addition of solid ammonium sulfate. The precipitate was isolated by centrifugation and dissolved in Buffer A containing 1 M ammonium sulfate. This enzyme preparation was applied to a column of phenyl-Sepharose that had been equilibrated with Buffer A, containing 1 M ammonium sulfate. The column was eluted with a downward linear gradient of ammonium sulfate starting from 1 M to 0 M salt concentration. The pyrophosphorylase activity began to emerge from the column at about 0.3 M ammonium sulfate with the peak of activity eluting at 0.1 M salt. Active fractions were pooled and dialyzed against Buffer B (10 mM sodium phosphate buffer, pH 6.8, containing 1 mM β-mercaptoethanol and 50 mM sucrose).

Step 4: Chromatography on Hydroxylapatite—The dialyzed enzyme from the phenyl-Sepharose column was loaded onto a column of hydroxylapatite (2 × 10 cm), that had been equilibrated with Buffer B. The column was washed with Buffer B to remove unadsorbed protein, and the enzyme was then eluted with a 10–200 mM linear gradient of phosphate buffer. Fractions containing active enzyme were pooled and concentrated to about 2 ml on the Amicon concentrator, using a PM30 membrane.

Step 5: Gel Filtration on Sephacryl S-300—The concentrated enzyme from the hydroxylapatite column was applied to a column of Sephacryl S-300 that had been equilibrated with 20 mM Tris-HCl buffer, pH 7.6, containing 1 mM β-mercaptoethanol and 50 mM sucrose (Buffer C). The enzyme emerged from the column in the area suggesting a molecular mass of about 60 kDa. Active fractions were pooled, concentrated to about 5 ml on an Amicon filtration apparatus, and used in the next step.

Step 6: Chromatography on Reactive Red-Agarose—The concentrated fraction from the Sephacryl column was applied to a column (2 × 10 cm) of Reactive Red-Agarose (Type 3000) that had been equilibrated with Buffer C. The column was washed with the above buffer, and the pyrophosphorylase was eluted with a linear gradient of 0 to 20 mM sodium pyrophosphate in Buffer C. Active fractions were pooled, concentrated to about 5 ml, and dialyzed against Buffer D (25 mM imidazole-HCl buffer, pH 7.4, containing 1 mM β-mercaptoethanol, 1 mM EDTA, and 50 mM sucrose).

Step 7: Chromatofocusing—The enzyme from the previous step was applied to a 0.9 × 14-cm column of Polybuffer exchanger (PBE 94) that had been equilibrated with Buffer D. The enzyme was eluted from the column with Polybuffer 74-HCl, pH 4.4, containing 1 mM EDTA, 1 mM β-mercaptoethanol, and 50 mM sucrose. Fractions of 2 ml were collected, and each fraction was analyzed for protein, for enzymatic activity, and for pH. The enzyme emerged from the column at pH 6.5 to 5.5, with the peak of activity emerging at pH 6.1 to 5.9. Active fractions were pooled, dialyzed overnight against Buffer C, and concentrated.

A summary of the purification procedure is outlined in Table 1, and the discussion of this procedure is presented under “Results.”

dNA Libraries

Human prostate (CLONTECH, HL1140), and testis (Life Technologies, Inc., 10426-013) cDNA libraries, and human prostate Marathon-Ready cDNA (CLONTECH, 7418-1) were purchased. The Epstein-Barr virus transformed B lymphocyte line JY (27) was used to construct a cDNA library in BluescriptII (Stratagene) according to standard protocols (28).

GDP-L-fucose Pyrophosphorylase cDNA Isolation

A PCR procedure was used to isolate GFPP clones from prostate, testis, and B cell cDNA libraries. Purified plasmid DNA prepared from the total cDNA library or Marathon-Ready cDNA was amplified with an amplifier set containing a GFPP primer and a vector primer. The PCR primer set F12805+146: CAGAGCTCGGCTTACAGTCC (nucleotides 1139–1159) with a vector-specific primer was used to amplify the 3’ end of the GFPP from all of the cDNA sources. The 5’ end of GFPP was isolated with the PCR primer set F12830-758: AATGCAGTTTTCG (nucleotides 1314–1295) and a vector primer. PCR products amplified with Taq polymerase were cloned into pCRRII and Pfu polymerase- amplified products were cloned into pCR-blunt (Invitrogen). Both strands of at least two clones from each cDNA source were sequenced. The primers FP-29, TCGGAGCCGATCTTGGCAGG, was used with the human Promoter Finder Walking kit (CLONTECH, K1803-1) to isolate sequences upstream of the GFPP coding region.
DNA Sequencing

Sequencing was performed on an ABI model 373 automated se- quencer in the Core Facilities at San Diego State University.

Northern Analysis

A probe encompassing nucleotides 170–1314 of the GFPP cDNA was radiolabeled with [α-32P]dCTP, using a random priming kit (Boehringer Mannheim). A human multiple tissue Northern blot II (CLONTECH 7759-1) containing poly(A) mRNA from spleen, thymus, prostate, testis, ovary, small intestine, colon, and peripheral blood leukocytes was hybridized and washed at high stringency according to the manufacturer’s protocol.

Generation of NSO Cell Line Stably Expressing GDP-Fucose Pyrophosphorylase

The murine myeloma cell line, NSO (Celltech), does not grow in glutamine-free selection medium. NSO cells were maintained in non-selective media: Iscove’s Dulbecco’s Modified Eagle’s medium supplemented with 10% heat inactivated dialyzed fetal bovine serum and 2 mM L-glutamine (Life Technologies, Inc.). Selective media consisted of Iscove’s modified Eagle’s medium supplemented with 10% heat inactivated fetal bovine serum and 1 mM GS supplement (JRH Bioscience).

The vector pEE12 was obtained from Celltech. This plasmid contains a glutamine synthetase gene under control of SV40 early promoter. Downstream of the SV40-GS transcription unit is the human cytomegalo virus enhancer, promoter and 5'-untranslated region from the major immediate early gene. The multicloning site is located directly downstream of this sequence. The plasmid also contains ampicillin resistance genes for maintenance in bacteria.

A 1.5 kilobase EcoRI fragment containing the entire open reading frame of GFPP was inserted into the Smal restriction site of the plasmid pEE12 generating the plasmid pEE12-GFPP using general molecular biology techniques (28). The orientation of the cloned gene was judged to be correct by restriction mapping.

pEE12-GFPP was linearized with SstI, precipitated, and resuspended in sterile TE at 1 mg/μl. NSO cells were grown to a density of 8 × 10^6 cells/ml and 10^7 cells were pelleted and washed once with cold sterile phosphate-buffered saline, and then pelleted again. The pellet was resuspended in 1 ml of phosphate-buffered saline and stored on ice for up to 1 h prior to electroporation.

Linearized pEE12-GFPP DNA (40 μg) and 0.8 ml of the NSO cell suspension were transferred to an electroporation cuvette and incubated on ice for 5 min. A "gene pulser" electroporation (Bio-Rad) delivered 1500 volts, 5 microfarads to the DNA/cell suspension, and the cuvette was incubated on ice for 5 min. The DNA/cell suspension was diluted in non-selective media to prepare cell suspensions ranging in particle size was 20 mM potassium phosphate (monobasic), 10 mM tetraprol gluamine, 20% FBS and 1% Triton X-100. Ten μl of this extract was added into a 20-μl assay mixture consisting of 50 μl Tris buffer, pH 7.5, 10 mM MgCl_2, 10 μl (from 0.1 M) [α-32P]GTP, 10 μl (from 1 M) P, and 10 milliliters of inorganic pyrophosphatase with an incubation of 5 min at 37°C. Assays were quenched and measured with methyl alcohol, and protein was removed by centrifugation. The supernatant liquid was removed and dried under a vacuum. The residue was dosed in 20 μl for analysis by HPLC. The formation of GDP-fucose was quantitated by measuring its absorbance at 260 nm, for radioactivity by scintillation counting, and for 6-deoxyhexose by assay on 35S-labeled oligosaccharides (29). Pools of cells were pelleted, concentrated on a rotary evaporator, and (NH_4)_2SO_4 was removed by evaporation in the presence of triethylamine. The triethylamine was then removed by evaporation in the presence of methanol.

The reaction products were identified by chromatography either directly or following various treatments such as digestion with phosphodiesterase, or 20% methanol or 20% methanol, and then the mixture was dried under nitrogen in the Core Facilities at San Diego State University.

To quantitatively determine the expression of GFPP cDNA from whole cell lysates, a reverse phase ion pairing HPLC method was developed that resolved GDP-fucose from GDP-mannose as well as GTP, GDP, GMP, and guanosine. An Alltech Adsorbosphere HS C18 150 × 4.6-mm column (Alltech 28775) with a 7-μm particle size was utilized for these assays. The elution buffers providing the best resolution were 20 mM potassium phosphate (monobasic), 10 mM tetraper- gammadominium phosphate, pH 5.0, with H_3PO_4 (Buffer A) and methanol (Buffer B), using a linear gradient of Buffer B from 1 to 20% over 20 min. NSO cells expressing recombinant GFPP were lysed with 50 μl Tris buffer, pH 7.5, containing 1% Triton X-100. Ten μl of this extract was added into a 20-μl assay mixture consisting of 50 μl Tris buffer, pH 7.5, 10 μl (from 0.1 M) MgCl_2, 10 μl (from 1 M) [α-32P]GTP, 10 μl (from 1 M) P, and 10 milliliters of inorganic pyrophosphatase with an incubation of 5 min at 37°C. Assays were quenched and measured with methyl alcohol, and protein was removed by centrifugation. The supernatant liquid was removed and dried under a vacuum. The residue was dosed in 20 μl for analysis by HPLC. The formation of GDP-fucose was quantitated by measuring its absorbance at 260 nm, for radioactivity by scintillation counting, and for 6-deoxyhexose by assay on 35S-labeled oligosaccharides (29). Pools of cells were pelleted, concentrated on a rotary evaporator, and (NH_4)_2SO_4 was removed by evaporation in the presence of triethylamine. The triethylamine was then removed by evaporation in the presence of methanol.

The reaction products were identified by chromatography either directly or following various treatments such as digestion with phosphodiesterase, or 20% methanol or 20% methanol, and then the mixture was dried under nitrogen in the Core Facilities at San Diego State University.

Polyacrylamide Gel Electrophoresis

Native PAGE was performed as described by Davis (31) with an 8% gel and a discontinuous buffer system, under nondenaturing conditions. Two samples were run in parallel, one lane was stained with Coomassie Blue to detect protein bands; the other lane was cut into 0.5-cm pieces and each piece was crushed in 200 μl of Buffer A and incubated overnight in the cold to elute the enzyme. The supernatant liquid was then assayed for enzymatic activity.

SDS-PAGE was done as described previously (32). Prior to electrophoresis, protein samples were mixed with sample buffer (62 mM Tris-HCl, pH 6.8, 5% β-mercaptoethanol, 2% SDS, 10% glycerol, and 0.02% bromphenol blue) and heated in a boiling water bath for 5 min.

Photoaffinity Labeling of the Enzyme

The purified enzyme was photoalbled with 8-azido-GDP-1-[α-32P]fu- cose in the absence or presence of unlabeled GDP-fucose. In these experiments, various amounts of the azido-GDP-1-[32P]fucose were incubated with the enzyme on ice for 2 min, and then the mixtures were exposed to UV light for 90 s to activate the azido group. The protein was then precipitated by the addition of 5% trichloroacetic acid and the precipitate was suspended in SDS-loading buffer, and subjected to SDS-PAGE. Labeled proteins were detected by autoradiography and densitometry for 2–5 days. In some of these incubations, various amounts of unlabeled GDP-fucose were added to determine the specificity of the labeling procedure (33).

The 8-azido-GDP-1-[32P]fucose was prepared as follows: GTP (0.5 μmol containing 1 mCi of [α-32P]GTP) was incubated in 50 mM Tris-HCl buffer, pH 9.0, containing 4 mM MgCl_2, 2 μmol of t-fucose-1-P, 2 units of the GFPP enzyme, and 10 μl of a 10 μg/ml solution of 8-azido-GDP-1-[32P]fucose. After 5 min at 37°C, the reaction was quenched and analyzed by SDS-PAGE and autoradiography. The protein was then precipitated by the addition of 5% trichloroacetic acid and the precipitate was suspended in SDS-loading buffer, and subjected to SDS-PAGE. Labeled proteins were detected by autoradiography and densitometry for 2–5 days. In some of these incubations, various amounts of unlabeled GDP-fucose were added to determine the specificity of the labeling procedure (33).
chemical procedure as follows. To a solution of the \( \alpha-32P \) GDP-fucose in Tris buffer, pH 8.0, water-saturated bromine was added dropwise with stirring to form \( \alpha-32P \)8-bromo-GDP-fucose. The progress of the reaction was followed by monitoring the shift in the UV spectrum from 252 to 262 nm. The reaction mixture was then diluted with 100 ml of 10 mM (NH4)2HCO3 and placed on a column of DE-52 equilibrated with 10 mM (NH4)2HCO3. The column was washed with 10 ml (NH4)2HCO3 and the 8-bromo-GDP-fucose was eluted with a gradient of 10 to 400 mM (NH4)2HCO3. Radiolabeled fractions with absorbance at 262 nm were pooled and concentrated to dryness. The residue was dissolved in 1 ml of water and 4 ml of triethylamine were added. The solution was then evaporated to dryness to remove the triethylamine and (NH4)2HCO3. The product could be stored in methanol at -20 °C until used in further reactions.

The above synthesized \( \alpha-32P \)8-bromo-GDP-fucose was used to prepare the 8-azido derivative as follows. The bromo derivative in anhydrous methanol was placed in a dry flask and the solvent was removed by evaporation. Additional anhydrous methanol was added and the solvent evaporated again. This procedure was repeated several times. Then, 5 ml of a solution of dimethyl formamide, isobutyric acid, and lithium azide were added. This solution was prepared as follows: 5 ml of methanol, saturated with lithium azide, was added to a dry flask and the solvent was removed by evaporation. The residue was then dissolved in 5 ml of a solution of dimethyl formamide and isobutyric acid (4:1), and this mixture was added to the flask containing the 8-bromo-GDP-fucose. The flask was sealed with parafilm and placed in a 50 °C waterbath overnight. At the end of this time, the solution was concentrated to dryness, taken up in water, and placed on a column of DE-52 as described for the 8-bromo derivative. The isolation of this product was based on its migration on the DE-52 column and on thin layer chromatography with Coomassie Blue revealed a number of protein bands with a major protein band at about 61 kDa. On Sephacryl S-300 columns, the active enzyme emerged from the column in the area suggesting a molecular weight of about 61,000. Thus, the GDP-1-fucose pyrophosphorylase appears to be a monomer of about 61 kDa.

That the 61-kDa band is indeed GFPP was demonstrated by the fact that it became radiolabeled when incubated with either the azido-GTP\([32P] \) (data not shown) or azido-GDP-[32P]L-fucose. As seen in Fig. 2, the native enzyme was incubated with NpGDP-[32P]L-fucose, in the presence of Mg2+, for 2 min in an ice bath, and the mixtures were then exposed to UV light. The proteins were then precipitated by the addition of trichloroacetic acid, resuspended in SDS, and separated by SDS-PAGE. Labeled proteins were detected by autoradiography. Fig. 2, lane 2, shows that the 61-kDa band became photolabeled, whereas no labeling was seen in the absence of exposure to UV light (lane 1). Adding increasing amounts of unlabeled GDP-L-fucose to the incubation mixtures (lanes 3-6) caused an increasing inhibition of photoincorporation into the 61-kDa band, demonstrating that photolabeling of GFPP was specific for GDP-fucose. The photolabeling of the 61-kDa band was also inhibited by the addition of unlabeled GTP (lanes 7-10), and here also inhibition was concentration-dependent as expected, since GTP is also a substrate for this enzyme. However, addi-
tion of unlabeled UDP-glucose or UTP did not inhibit the photoaffinity labeling (data not shown).

Stability of the GDP-<i>L</i>-fucose Pyrophosphorylase—The stability of GFPP was examined at various stages of purification and under a variety of conditions. The enzyme from DEAE cellulose lost most of its activity within five days when kept in buffer at 20°C. The addition of <i>L</i>-fucose (50 mM) or <i>D</i>-glucose (50 mM) lost most of its activity within five days when kept in buffer at 20°C. The enzyme from DEAE cellulose was much more effective. Thus, in the presence of 50 mM sucrose, the enzyme remained fairly stable for 9 days on ice. Based on such experiments, it was found that the enzyme could be stored for several months in the freezer (i.e., -20°C) in 50 mM sucrose, with no loss in activity.

cDNA Cloning: Using the Porcine Peptide Sequences to Search TBLASTN Data Base—The 61-kDa band was eluted from the SDS gels at the most purified stage, transferred to polyvinylidene difluoride membranes, and subjected to proteolytic digestion and sequencing. Three peptides obtained by trypsin digestion, and 4 peptides obtained by Endo Lys-C digestion, had the amino acid sequences presented in Table II. The amino acid sequence of the porcine Endo Lys-C peak 123 peptide, SELGLQTGFPPIFPAIPPY, was used to search the six-frame translation of the GenBank nonredundant EST data base with the BLAST algorithm. A 14/18 identity to the ESTs HSC13H111, HSC3EG051, and T75166 was found. The DNA sequence of the human ESTT75166 was used to synthesize primers for screening cDNA libraries as described under “Experimental Procedures” GFPP cDNA clones were obtained from human prostate, testis, and an Epstein-Barr virus trans-translation, and contained ESTs H81173 and N92082. The complete sequence of the human ESTT75166 was used to synthesize primers for screening cDNA libraries as described under “Experimental Procedures.” GFPP cDNA clones were obtained from human prostate, testis, and an Epstein-Barr virus transformed B lymphocyte cell line. The cDNA sequences encompassed 3105 nucleotides and encoded a single open reading frame containing the seven sequenced peptides from the GFP shown in Table II. None of these cDNAs contained the ATG translation start site, so 345 base pairs of upstream DNA were isolated with the Marathon Promoter Finder walking kit. This upstream sequence provided the missing A of the ATG translation codon, the Kozak sequence, the putative GFPP promoter region, and contained ESTs HS41173 and N92082. The complete cDNA sequence (accession numbers AF017445) and the upstream region (accession number AF017446) have been deposited in GenBank.

GFPP Coding Sequence—A 594-amino acid protein having a molecular mass of 66,616 daltons is predicted by the GFP DNA sequence. The transcription start site of the GFPP gene has not been determined and the genomic sequence encodes an in-frame upstream possible translation initiation start site that would add an additional 13 amino acids. We feel that the translation start site shown in Fig. 3 is correct since it best matches the consensus Kozak sequence by having a G at the important positions -3 and +4 (34).

Expression of GFPP Activity in NSO Cells and in Baculovirus System—GFPP activity was expressed in both NSO cells and the baculovirus system with parental constructs. Thus, the control cells had barely detectable GFPP activity whereas transfected NSO cells had a specific activity of about 21 nmol/mg of protein and the baculovirus-infected cells a specific activity of 38 nmol/mg of protein. These activities were determined in the forward direction, although the same activity was determined for the NSO cells in the reverse assay.

That the product formed by the NSO cells was indeed GDP-fucose was demonstrated by HPLC. Fig. 4 shows two HPLC tracings of the GFPP assays done with cellular extracts from NSO cells harboring the GFPP plasmid pEE12-GFPP (Profile B), or a construct encoding β1,4-galactosyltransferase cDNA in the pEE12 plasmid (Profile A). As indicated in the figure, GDP-fucose emerges approximately 1 min after GDP (about 12 min). This peak of GDP-fucose is absent from extracts derived from the cell line harboring the β1,4-galactosyltransferase cDNA. When the GFPP was quantitated, the clonal cell lines examined ranged in activity from 0.03 milliunit/2 × 10⁵ cells to 0.38 milliunits/2 × 10⁵ cells.

The actual elution times vary from run to run, depending on the time allowed for the HPLC column to equilibrate in Buffer A. However, the order of elution and relative retention times do not vary. This analysis was repeated using a nucleotide analysis (Bio-Rad) HPLC column (data not shown). In each case, only those cell lines harboring a GFPP cDNA expression plasmid yielded a unique peak corresponding to the GDP-fucose peak. Thus GFPP cDNA is expressed in active form in the NSO cell line. This evidence along with the co-linearity of the cDNA with peptides derived from purified GFPP strongly indicate that the cDNA isolated in this study encodes GFPP activity.

### Table II

| Peptide sequences from GFPP |
|-----------------------------|
| Pig sequenced peptide | Human deduced sequence |
| A. Trypsin |
| Peak 28 | SIGESEFIR | SIGESEFIR |
| Peak 61 | LPEVYVTXS/SYFY | LDSDFVYVTDLSFY |
| Peak 70 | TQIFLEIALN | EQIFLIEILK |
| B. Endo Lys-C |
| Peak 79 | LDEPEYVYDTS/FYMHDH | LDSDFVYVTDLS/FYMHDH |
| Peak 102 | YESLVLMOQRIFH(L) | YESLVLMOQRIFH(L) |
| Peak 105 | GKPVAAGEFW(D)IVAITA(K/A)/P/D | GKLVARSGFWDIVAITAAD |
| Peak 123 | SELGLQTG(P)/IPPAIP(E)Y | SELGLQISIFPSIFPDIEPC |

**Fig. 2. Photoaffinity Labeling of GFPP.** The purified enzyme was incubated with 8-<i>N</i>-GDP-[<sup>32</sup>P]fucose in the absence or presence of various unlabeled nucleotides to determine the specificity of labeling, and the mixtures were exposed to UV light to activate the photoprobe. The protein was precipitated by the addition of trichloroacetic acid to a final concentration of 5% and the resulting pellet was suspended in SDS buffer and subjected to SDS-PAGE. Radioactive proteins were detected by exposure to film. Lanes are as follows: 1, probe + enzyme, but no exposure to UV; 2, probe + enzyme + UV; 3–6, probe + enzyme + 20, 50, 75, or 100 μM unlabeled GDP-<i>L</i>-fucose + UV; 7–10, probe + enzyme + 20, 50, 75, or 100 μM unlabeled GTP + exposure to UV.
Homology Searches—BLAST and FASTA searches failed to identify any matches to known sequences or motifs in the data bases. A BLAST search of the Caenorhabditis elegans DNA database with the human GFPP sequence yielded one significant match to cosmid K03H1 (p = 5.2e-11, n = 8).

Northern Results and Tissue Distribution—Hybridization of the GFPP cDNA probe to a multiple poly(A) + RNA blot revealed a single 3.5-kilobase hybridizing band of equal intensity in spleen, thymus, prostate, testis, ovary, small intestine, and colon. The GFPP hybridizing band in the peripheral blood leukocyte lane was faint, however, GFPP could be PCR amplified from the human B lymphoblastoid cell line JY. In addition, a BLAST search of the GenBank identified GFPP sequence matches to ESTs derived from human brain.
TABLE III
Demonstration of expression of GFPP activity in NSO cells and the baculovirus system

| Cell lysate                  | Specific activity | nmol/mg protein | Forward direction | Reverse direction |
|------------------------------|-------------------|-----------------|------------------|------------------|
| Control NSO cells (irrelevant construct) | 0                 | 0.09            |                  |                  |
| NSO cells (containing the GFPP construct) | 21.55             | 17.15           |                  |                  |
| Control baculovirus (containing the GFPP construct) | 0.11              | 38.07           |                  |                  |

A

**Fig. 4. Formation of GDP-ß-fucose in NSO cells harboring GFPP cDNA.** Extracts of NSO cells harboring an irrelevant construct (pEE12:GT) for β1,4-galactosyltransferase were incubated as described under “Experimental Procedures” with [³H]GTP and fucose-1-P and the products were examined by HPLC as seen in tracing A. No radioactivity was observed in the GDP-fucose area of the elution. Extracts of NSO cells harboring the GFPP cDNA (pEE12:GFPP) were also tested for their ability to form GDP-ß-fucose as shown in profile B. In this case, a distinct peak was observed in the GDP-fucose area of the column.

(B12805, R38619, T75166, Z43126, Z39211), heart (N89141), B lymphocytes (AA490306, AA490400), and pancreas (AA386117).

Tissue Distribution of GFPP Activity—Various porcine tissues were examined for the presence of GFPP activity as shown in Table IV. The highest specific activity of this enzyme was found in kidney, while spleen showed the next highest activity, and liver, lung, and pancreas were somewhat lower. In order to be sure that these determinations were really measuring pyrophosphorylase activity rather than degradative activity, the assays were run in the forward as well as in the reverse direction. It is clear that the GFPP is widely distributed in various tissues. GFPP activity was also found in CHO cells, but not in Madin-Darby canine kidney cells.

Properties of the GDP-ß-fucose Pyrophosphorylase—The formation of GDP-ß-fucose from GTP and ß-ß-fucose-1-P by the purified GFPP required the presence of a divalent cation for activity, as shown in Fig. 5. Mg²⁺ gave the highest activity and was optimum at about 6 to 8 mM, while Mn²⁺ showed somewhat lower activity, but was optimum at 4 mM and inhibitory at higher concentrations. Co²⁺ showed about the same activating activity as Mn²⁺ at 6 mM, and was also inhibitory at higher concentrations. Of the divalent cations tested for activity with the GFPP, in either the forward or reverse direction, only Mg²⁺, Mn²⁺, and Co²⁺ showed significant activity, whereas Ca²⁺, Cu²⁺, Zn²⁺, Ni²⁺, Hg²⁺, Mo⁷⁺, and Fe²⁺ were without activity. Similar results were obtained with the recombinant enzyme from NSO cells.

The pH optimum for the enzyme was measured using either Tris-HCl or Tris maleate buffers in the assay mixtures, and assays were done in the forward and reverse directions at various pH values. These experiments demonstrated that the pH optimum for this enzyme was about pH 6.8 to 7.8 (data not shown).

The substrate specificity of the GFPP was examined in the forward direction using various nucleoside triphosphates as shown in Table V. With the partially purified enzyme from pig kidney, the best activity was observed with either GTP or ITP as the nucleotide donors, but significant activity was also observed with ATP. The activity with ITP is not surprising but the activity with ATP is unexpected. Attempts were made to isolate the product of the reaction without success. Since it was not clear that these other activities were entirely due to GFPP (since the kidney enzyme is not homogeneous), specificity was also examined with the enzyme expressed in the NSO cells and in the baculovirus system. The table shows that in both of these recombinant systems, GTP was clearly the best nucleotide substrate, and the only other nucleotide showing significant activity was ITP. Control NSO cells or baculovirus transfected with an irrelevant construct had very low activity with fucose-1-P and any nucleoside triphosphate (see Table III).

The specificity of the enzyme for sugar-1-P was also examined with the pig kidney GFPP as well as with the enzymes from NSO cells and from baculovirus, as shown in Table VI. However, in this experiment each of the sugar-1-Ps was not readily available in radioactive form. Therefore, the experiment was done using radioactive ß-fucose-1-P and GTP as substrates, and each of the other sugar-1-Ps was added at 1 mM.

TABLE IV
Tissue distribution of GDP-fucose pyrophosphorylase

| Tissue                          | Specific activity | nmol/mg protein |
|---------------------------------|-------------------|-----------------|
|                                | Forward assay     | Reverse assay   |
|                                | (formation of GDP-Fuc) | (formation of Fuc-1-P) |
| Aorta                           | 1.31              | 1.23            |
| Kidney                          | 2.23              | 4.31            |
| Liver                           | 0.65              | 1.37            |
| Lung                            | 0.48              | 1.05            |
| Heart                           | 1.08              | 1.48            |
| Spleen                          | 1.32              | 2.14            |
| Pancreas                        | 1.07              | 1.47            |
| Chinese hamster ovary cells     | 0.24              |                 |
| Madin-Darby canine kidney cells | 0.00              |                 |

In the forward assay, the reactions contained 400 µM [³H]fucose-1-P (74,000 cpm) and 2 mM GTP with an incubation time of 5 min. In the reverse direction, reactions contained 100 µM GDP-[³H]fucose (27,000 cpm) and 5 mM inorganic pyrophosphate with an incubation time of 5 min.
concentration to determine whether any of them would inhibit the formation of GDP-1-fucose. The idea of this experiment was that if any of the other sugar-1-Ps could act as a substrate, then it should inhibit the reaction with fucose-1-P. The results show that only unlabeled L-fucose-1-P was effective at inhibiting the reaction. The interpretation of this data is that GFPP from any of these macromolecules, such as their role in cell:cell recognition or targeting (36). For example, fucose is a component of the sialyl Lewis x antigen which is the recognition site for selectins that play a key role in inflammation and the interaction of leukocytes with endothelial cells (37).

The major pathway for the biosynthesis of 1-fucose, which is probably present in most eucaryotic and procaryotic cells, involves a pathway that converts GDP-D-mannose to GDP-L-fucose. This fucose nucleotide is the fucosyl donor for polysaccharides in the formation of GDP-D-mannose to GDP-L-fucose. The idea of this experiment was to characterize the product of the reaction in order to elucidate the mechanism of the reaction produced from GDP with the purified enzyme.\footnote{Unlabeled sugar-1-P were added at 1 mM concentrations. Radiolabeled fucose-1-P as the substrate was present at 100 µM}

**Discussion**

1-Fucose is a key sugar in glycoproteins and other complex carbohydrates since it may be involved in many functional roles of these macromolecules, such as their role in cell:cell recognition or targeting (36). For example, fucose is a component of the sialyl Lewis x antigen which is the recognition site for selectins that play a key role in inflammation and the interaction of leukocytes with endothelial cells (37).

The major pathway for the biosynthesis of 1-fucose, which is probably present in most eucaryotic and procaryotic cells, involves a pathway that converts GDP-D-mannose to GDP-L-fucose. This fucose nucleotide is the fucosyl donor for polysaccharides in the formation of GDP-D-mannose to GDP-L-fucose. The idea of this experiment was to characterize the product of the reaction produced from GDP with the purified enzyme.\footnote{Unlabeled sugar-1-P were added at 1 mM concentrations. Radiolabeled fucose-1-P as the substrate was present at 100 µM}
charides, glycoproteins, and glycolipids (38). However, in various animal tissues, especially kidney, lung, and liver, another pathway exists that can lead to the formation of GDP-L-fucose. This pathway involves a fucokinase that catalyzes the transfer of phosphate from ATP to free L-fucose to give L-fucose-1-P, and a GDP-L-fucose pyrophosphorylase that condenses the above L-fucose-1-P with GTP to give GDP-L-fucose. This pathway appears to function as a salvage pathway to recapture L-fucose that arises in these tissues as a result of turnover and degradation of complex carbohydrates.

Although the fucokinase and GDP-fucose pyrophosphorylase had been partially purified from liver in the past (23, 24), neither enzyme was pure enough to obtain amino acid sequence data, or to obtain probes for molecular biological approaches to study these enzymes or their possible regulation. We recently described the purification of the pig kidney L-fucokinase to apparent homogeneity and the sequencing of a number of peptides obtained from the homogeneous protein (25). In the present report, we have purified the pig kidney GDP-fucose pyrophosphorylase to near homogeneity and identified the specific protein band that represents this enzymatic activity using the photoaffinity probe, $8$-$N_3$-GDP-$[32P]$L-fucose. The 61-kDa band that becomes photolabeled in a specific and concentration-dependent manner was eluted from the SDS gels and subjected to amino acid sequencing.

Seven different peptide sequences were obtained and several of these were used to prepare degenerate oligonucleotides for cloning the gene. GFPP cDNA clones were obtained from human prostate, testis, and an Epstein-Barr virus transformed B lymphocyte cell line. The GFPP DNA sequence predicts a 594-amino acid sequence having a molecular mass of 66,616 daltons. When GFPP cDNA is expressed in the myeloma cell line NSO, extracts of this culture catalyze the formation of GDP-L-fucose from fucose-1-P and GTP. BLAST and FASTA searches failed to reveal any matches to known sequences or motifs in the data bases. The GFPP cDNA probe was used to identify the mRNA in various tissues. Hybridization studies revealed a single 3.5-kilobase mRNA of equal intensity in spleen, thymus, prostate, testis, ovaries, small intestine, and colon.

Analysis of endogenous GFPP enzymatic activity from pig tissues revealed the highest activity in kidney, while spleen was next, and liver, lung, and pancreas were somewhat lower.

**Fig. 6. Effect of substrate concentrations on GDP-fucose pyrophosphorylase.** A, various amounts of GTP were added to standard incubations containing 2 mM L-fucose-1-P and other necessary components, and the formation of GDP-fucose was measured as described under “Experimental Procedures.” B, various amounts of the other substrate, L-fucose-1-P were added to standard incubation mixtures containing 5 mM GTP, and the formation of GDP-fucose was determined. The data was plotted according to Lineweaver and Burk, as seen in the insets.
However, it is not clear whether tissues such as testis, ovaries, spleen, etc., actually reutilize free L-fucose, or whether this enzyme and the "salvage" pathway are mostly confined to tissues such as liver and kidney. At any rate, the L-fucokinase and the GDP-L-fucose pyrophosphorylase represent valuable enzymes for biochemists studying fucosylation reactions, since they offer a means to prepare radioactive GDP-fucose in large amounts and for a reasonable cost. In addition, these enzymes may be under some sort of regulation in these tissues, since the amounts and for a reasonable cost. In addition, these enzymes may be under some sort of regulation in these tissues, since the fucokinase was previously shown to be inhibited by GDP-L-fucose. The availability of various probes will allow us to examine the levels of mRNA and protein in various tissues under normal and disease conditions.

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