Bovine UDP-N-acetylglucosamine:Lysosomal-enzyme N-Acetylgalactosamine-1-phosphotransferase

I. PURIFICATION AND SUBUNIT STRUCTURE*

Ming Bao, J. Leland Booth, B. Jean Elmendorf, and William M. Canfield‡

From the W. K. Warren Medical Research Institute and the Department of Medicine, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104

(Received for publication, May 28, 1996, and in revised form, September 4, 1996)

UDP-N-acetylglucosamine:lysosomal-enzyme N-acetylgalactosamine-1-phosphotransferase (GlcNAc-phosphotransferase) catalyzes the initial step in the synthesis of the mannose 6-phosphate determinant required for efficient intracellular targeting of newly synthesized lysosomal hydrolases to the lysosome. The enzyme was partially purified ~30,000-fold by chromatography of solubilized membrane proteins from lactating bovine mammary glands on DEAE-Sepharose, reactive green 19-agarose, and Superose 6. The partially purified enzyme was used to generate a panel of murine monoclonal antibodies. The anti-GlcNAc-phosphotransferase monoclonal antibody PT18 was coupled to a solid support and used to immunopurify the enzyme ~480,000-fold to apparent homogeneity with an overall yield of 29%. The purified enzyme has a specific activity of 10–12 mol of GlcNAc phosphate transferred per h/mg using 100 mM α-methylmannoside as acceptor.

The subunit structure of the enzyme was determined using a combination of analytical gel filtration chromatography, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and amino-terminal sequencing. The data indicate that bovine GlcNAc-phosphotransferase is a 540,000-Da complex composed of disulfide-linked homodimers of 166,000- and 51,000-Da subunits and two identical, noncovalently associated 56,000-Da subunits.

The trafficking of most lysosomal hydrolases in higher eucaryotes is mediated by a mannose 6-phosphate-dependent pathway. Asparagine-linked oligosaccharides on newly synthesized lysosomal hydrolases are sequentially modified by two enzymes to generate a mannose 6-phosphate recognition marker. The initial and determining step in the biosynthesis of the mannose 6-phosphate recognition marker is catalyzed by the enzyme UDP-N-acetylgalactosamine:lysosomal-enzyme N-acetylgalactosamine-1-phosphotransferase (GlcNAc-phosphotransferase). GlcNAc-phosphotransferase catalyzes the transfer of GlcNAc 1-phosphate from UDP-GlcNAc to specific α-1,2-linked mannoses on lysosomal hydrolases (1–3). The second enzyme in the pathway, N-acetylgalactosamine-1-phosphodiester α-N-acetylgalactosaminidase, removes the covering GlcNAc, generating a terminal mannose 6-phosphate (4, 5). Lysosomal hydrolases modified with mannose 6-phosphate then bind to one of two mannose 6-phosphate receptors in the trans-Golgi complex and are transferred to the endosome and subsequently the lysosome (6, 7).

GlcNAc-phosphotransferase activity is deficient in human mucolipidosis II (I-cell disease, McKusick 252500) and mucolipidosis III (pseudo-Hurler polydystrophy, McKusick 252600), and this deficiency is believed to be the primary genetic defect in these disorders (8).

The mammalian GlcNAc-phosphotransferase responsible for lysosomal enzyme targeting has previously been purified 784-fold from rat liver and has been partially characterized (9, 10). The human GlcNAc-phosphotransferase has been purified 167-fold from lymphoblasts (11). Glycoprotein GlcNAc-phosphotransferases have been identified in both the soil amoeba Acanthamoeba castellanii and the vegetative amoeba of the slime mold Dictyostelium discoideum (12). The Acanthamoeba enzyme has been partially purified ~54,000-fold to a specific activity of 154 μmol/h/mg. Although this enzyme exhibits some properties similar to the mammalian GlcNAc-phosphotransferase, its functional role is unknown since it has been determined that Acanthamoeba lacks the second enzyme, N-acetylgalactosamine-1-phosphodiester α-N-acetylgalactosaminidase, required for the complete mannose 6-phosphate-dependent lysosomal targeting system found in higher eucaryotes (13).

In this paper, we describe the identification of bovine GlcNAc-phosphotransferase, partial purification by conventional chromatography, the preparation of specific monoclonal antibodies, and subsequent purification by monoclonal antibody immunoaffinity chromatography to apparent homogeneity. The subunit structure of the enzyme was determined by a combination of gel filtration chromatography, amino-terminal sequencing, and SDS-PAGE. The data indicate that bovine GlcNAc-phosphotransferase is a 540,000-Da complex composed of disulfide-linked homodimers of 166,000- and 51,000-Da subunits and two identical, noncovalently associated 56,000-Da subunits.

EXPERIMENTAL PROCEDURES

Materials

Lactating bovine udders were obtained from Mikkelsen Beef, Inc. (Oklahoma City, OK). Ultrasound ODS columns were obtained from Beckman Instruments. Microsorb MV-NH2 columns were obtained from Rainin Instrument Co., Inc. (Woburn, MA). [γ-32P]ATP (7000 Ci/mmol; end labeling grade), Na125I, and Lubrol (C12H25(CH2CH2O)nH) were obtained from ICN (Costa Mesa, CA). Superose 6 (prep grade), DEAE-Sepharose FF, QAE-Sephadex A-25, molecular mass standards for

* This work was supported in part by Grant HS-024 from the Oklahoma Center for the Advancement of Science and Technology, Grant OK-93-GS-33 from the Oklahoma Affiliate of the American Heart Association. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: University of Oklahoma Health Sciences Center, BSEB 302, 941 Stanton L. Young Blvd., Oklahoma City, OK 73104. Tel.: 405-271-3920; Fax: 405-271-3191; E-mail: Bill-canfield@ouhsc.edu.

1 The abbreviations used are: GlcNAc-phosphotransferase, UDP-N-acetylgalactosamine:lysosomal-enzyme N-acetylgalactosamine-1-phosphotransferase; PAGE, polyacrylamide gel electrophoresis; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; MOPS, 3-[N-morpholino]propanesulfonic acid; PTH, phenylthiodyantoin.

This paper is available online at http://www-jbc.stanford.edu/jbc/
SDS-PAGE. HTrap-protein G columns, and Mono Q columns were obtained from Pharmacia Biotech Inc. 2.5% Emphire Biosupport Medium ABI, IODO-GEN iodination reagent, and the BCA protein assay reagent were obtained from Pierce. Glyceraldehyde, sucrose, α-methylmannoside, α-methylglucoside, reactive green 19-agarose, sodium deoxycholate, benzamidine, UDP-GlcNAc, phenylmethylsulfonyl fluoride, Tris, rabbit anti-mouse IgG, and mouse monoclonal antibody isotyping reagents were obtained from Sigma. POROS 50 HQ was obtained from PerSeptive Biosystems (Cambridge, MA). ProFlotn polyvinylidene difluoride membranes were obtained from Applied Biosystems Inc. (Foster City, CA). A Model Q12 rotary tugger was obtained from LORTONE, Inc. (Seattle, WA). A mouse immunoglobulin standard panel was obtained from Southern Biotechnology Associates, Inc. (Birmingham, AL). Recombinant interleukin-6 was a gift from Dr. Samuel Burstein of this institution. Lactating bovine mammary glands were obtained from a farm in Missouri, Columbia, MO). Other chemicals were reagent grade or better and were from standard suppliers.

Methods

Protein Determination

Protein concentration was estimated by absorbance at 280 nm assuming E_1% = 10.0 or using the Micro-BCA assay standardized with bovine serum albumin (Pierce) (14).

SDS-Polyacrylamide Gel Electrophoresis

SDS-PAGE was carried out in Tris/glycine buffer on 4–12% acrylamide gradient gels (13) or in Tris/Tricine buffer on 6% acrylamide gels (15). Proteins were visualized by silver staining (16).

Buffers

Buffer A contained 0.025 M Tris-HCl, pH 7.4, 250 mM sucrose, 5 mM MgCl2, and 5 mM β-mercaptoethanol. Buffer B contained 0.025 M Tris-HCl, pH 7.4, 5 mM MgCl2, and 5 mM β-mercaptoethanol. Buffer C contained 0.025 M Tris-HCl, pH 7.4, 0.4 M NaCl, 5 mM MgCl2, and 5 mM β-mercaptoethanol. Buffer D contained 0.025 M Tris-HCl, pH 7.4, 5 mM MgCl2, 4% Lubrol, and 0.5% sodium deoxycholate. Buffer E contained 0.025 M Tris-HCl, pH 7.4, 0.3% Lubrol, 20% glycerol, and 5 mM MgCl2. Buffer F contained 0.05 M Tris-HCl, pH 7.4, 5 mM MgCl2, and 0.3% Lubrol. Buffer G contained 0.010 M Tris-HCl, pH 7.4, 5 mM MgCl2, 4% Lubrol, and 0.5% sodium deoxycholate. Buffer H contained 0.100 M Tris-HCl, pH 10.0, 5 mM MgCl2, and 0.3% Lubrol. Buffer J contained 0.05 M Tris-HCl, pH 7.4, 5 mM MgCl2, 150 mM NaCl, 1 mg/ml bovine serum albumin, 0.02% Lubrol, and 0.02% NaN3.

GlcNAc-phosphotransferase Assay

[β-32P]UDP-GlcNAc was synthesized using 250 μmol of glucosamine and 2.5 μCi of [γ-32P]ATP essentially as described (9). [β-32P]UDP-GlcNAc was purified by reverse-phase high pressure liquid chromatography on an Ultrasphere ODS column (0.5 × 30 cm) with a linear gradient from 40 mM KH2PO4, pH 6.0, 5 mM tetrabutylammonium dihydrogen phosphate to 100% acetonitrile at 1 ml/min (17). Alternatively, [β-32P]UDP-GlcNAc was purified by ion-exchange chromatography on a Microsorb MV column (0.25 × 25 cm) equilibrated with 5 mM KH2PO4, pH 4.0, and developed at 1 ml/min with a linear gradient from 5 mM KH2PO4, pH 4.0, to 1.5 mM KH2PO4, pH 3.0, as described (18). Purified [β-32P]UDP-GlcNAc had a specific activity of 10 μCi/μmol and was homogeneous in both chromatography systems.

One unit of GlcNAc-phosphotransferase activity is defined as 1 pmol of GlcNAc-phosphotransferase activity transferred to α-methylmannoside/h in a reaction containing 150 μM UDP-GlcNAc and 100 mM α-methylmannoside at 37°C (9).

Purification of GlcNAc-phosphotransferase: Method 1

Step 1: Homogenization, Subcellular Fractionation, Isolation of Membranes, and Solubilization—Lactating bovine mammary glands (~10 kg) were collected at slaughter, sliced into 10-cm thick slices, and immediately packed in an ice/water slurry. Within 30 min of collection, the tissue was cut into 2-cm cubes and freed of connective tissue and vascular structures. The cubed gland was homogenized for 2 min in 1-kg aliquots with 1 liter of buffer A at 4°C (Waring commercial blender, high setting).

The homogenate was centrifuged at 2,000 × g for 20 min, and the supernatant was collected by decantation, avoiding the floating lipid pad. The post-nuclear supernatant was prepared by centrifugation at 9,500 × g for 15 min. Membranes were collected by centrifugation at 47,900 × g for 45 min. Washed membranes were then solubilized in 1700 ml of buffer B, and subsequently collected by centrifugation at 47,900 × g for 4 h. The membrane pellets were extracted by homogenization in 1100 ml of buffer C and collected by centrifugation at 47,900 × g for 45 min. The supernatant was decanted, and the membrane fragments in the pooled salt extracts were pooled with a second salt extraction and pelleted by centrifugation at 47,900 × g for 4 h. The supernatant was discarded, and GlcNAc-phosphotransferase was solubilized from the membrane pellets by suspension in buffer D, dispersion by brief homogenization (Polytron, Buchler Instruments; standard probe), and stirring for 1 h at 4°C. Insoluble material was removed by centrifugation at 47,900 × g for 4 h, yielding a solubilized membrane fraction.

Step 2: DEAE-Sepharose FF Chromatography—The solubilized membrane fraction was applied to a DEAE-Sepharose FF column (5.0 × 28 cm) equilibrated with buffer E at 5 ml/min. After washing with ~1 liter of buffer E containing 0.075 mM NaCl, the column was developed with a 2.5-liter linear gradient from 0.075 to 0.275 mM NaCl in buffer E. Fractions of 20 ml were collected, and those containing specific activities >20,000 units/ml were pooled.

Step 3: Reactive Green 19-Agarose Chromatography—The pooled fractions from the DEAE-Sepharose FF chromatography were applied to a reactive green 19-agarose column (2.6 × 35 cm) equilibrated with buffer E at 5 ml/min. The column was washed with 200 ml of buffer E and developed with a 200-ml linear gradient from 0 to 0.5 mM NaCl in buffer E. GlcNAc-phosphotransferase was then step-eluted with buffer E containing 1 mM NaCl. Fractions of 10 ml were collected, and those with specific activities >100,000 units/ml were pooled.

Step 4: POROS 50 HQ Chromatography—The pool from the DEAE-Sepharose FF chromatography was applied to a POROS 50 HQ column (1.6 × 20 cm) equilibrated with buffer E at 10 ml/min. The column was washed with 80 ml of buffer E and eluted with buffer E containing 0.5 mM NaCl. Fractions of 10 ml were collected, and the two fractions containing the highest protein concentration were pooled.

Step 5: Mono Q Chromatography—The pool from the POROS 50 HQ column was diluted 5-fold with buffer E and applied to a Mono Q column (0.5 × 5.0 cm) equilibrated with buffer E at 1 ml/min. The column was washed with 1 column volume of buffer E and step-eluted with buffer E containing 0.5 mM NaCl. Fractions of 0.5 ml were collected, and the four fractions containing the highest enzyme activity were pooled.

Step 6: Superose 6 Chromatography—The concentrated Mono Q sample was applied to two Superose 6 columns (1.6 × 50 cm) connected in series and equilibrated with buffer E containing 0.15 mM NaCl at 0.25 ml/min. Fractions of 1.0 ml were collected and assayed for GlcNAc-phosphotransferase activity and protein.

Production of Monoclonal Antibodies

Polyvalent antisera that immunocapture GlcNAc-phosphotransferase activity were raised in BALB/c mice inoculated with 75 μg of partially purified GlcNAc-phosphotransferase from Superose 6 gel filtration chromatography (specific activity ~106 units/mg). Monoclonal antibodies were prepared by fusing spleen cells from an immunized mouse to SP2/0 mouse myeloma cells according to standard procedures (19). Fusions were plated in five 96-well plates and grown in Iscov's medium containing 20% fetal bovine serum and 10 ng/ml recombinant human interleukin-6. Hybridomas were screened for the production of murine IgG by enzyme-linked immunosorbent assay. To identify antibodies of interest, aliquots (100 μl) of supernatants from IgG-producing hybridomas were combined in groups of five and screened for their ability to capture GlcNAc-phosphotransferase activity.

GlcNAc-phosphotransferase (800 units) in a final volume of 200 μl of buffer F was incubated with pooled hybridoma supernatant (500 μl) for 1 h at room temperature and then overnight at 4°C. Protein A-Sepharose (15 μl of a 1:1 slurry of beads in buffer I coated with rabbit anti-mouse IgG) was added, and incubation was continued for 1 h with mixing. The beads were then washed four times with 1 ml of buffer I, and the pellets were assayed for GlcNAc-phosphotransferase activity.

Hybridomas secreting GlcNAc-phosphotransferase-specific monoclonal antibodies were subcloned twice, and antibody was produced in ascites culture. Monoclonal antibodies were purified on protein G-Sepharose and isolated by enzyme-linked immunosorbent assay using a mouse monoclonal antibody isotyping kit (Sigma) standardized with a mouse immunoglobulin standard panel.
Characterization of GlcNAc-phosphotransferase-specific Monoclonal Antibodies

Asic (1 ml) containing monoclonal antibodies PT18, PT19, PT20, and PT21 or the irrelevant isotype control (BP95) were dialyzed against 0.6% citrate, 0.1% MOES, pH 7.4, overnight and coupled to 3M-Emphaze resin overnight at 4 °C. The remaining reactive sites were blocked by incubation with 3 M ethanolamine HCl, pH 9.0, for 4 h. The immobilized monoclonal antibodies were incubated with partially purified GlcNAc-phosphotransferase (200,000 units) and washed at neutral pH to remove unbound GlcNAc-phosphotransferase. Parallel samples were also washed with buffer at pH 10. Bound GlcNAc-phosphotransferase was then quantitated by assay of aliquots of the resin for GlcNAc-phosphotransferase activity.

Purification of GlcNAc-phosphotransferase: Method II

All steps were performed at 4 °C, except chromatography was performed at room temperature.

Step 1: Homogenization, Subcellular Fractionation, Membrane Precipitation, and Solubilization—The post-nuclear supernatant was prepared as described for Method I, except that all solutions were made 1 M in phenylmethylsulfonyl fluoride and benzamidine HCl. The post-nuclear supernatant was made 20 mM in MnCl2 and stirred at 4 °C for 30 min. Membranes were then pelleted by centrifugation at 16,000 g for 20 min. The membrane pellets were combined, and Lubrol and deoxycholate were added to final concentrations of 4 and 0.5%, respectively. After stirring for 30 min, insoluble material was removed by centrifugation at 47,900 × g for 45 min. The supernatant was decanted and combined with 10 ml of monoclonal antibody PT18-3M-Emphaze in a 1.5-liter polyethylene terephthalate bottle. Phenylmethylsulfonyl fluoride and benzamidine were added to 1 mM each, and the PT18-3M-Emphaze suspension was then continuously rotated at 44 rpm on a rotary shaker for 16 h at 4 °C. The PT18-3M-Emphaze was then packed in a 1.6 × 5-cm column with ~50 ml of buffer G containing 1.0 M NaCl at 5 ml/min until the absorbance at 280 nm was <0.05. The column was then equilibrated with buffer G, and GlcNAc-phosphotransferase was eluted with buffer H at a flow rate of 2 ml/min. Fractions of 2 ml were collected and assayed for GlcNAc-phosphotransferase activity and protein.

Radioiodination of GlcNAc-phosphotransferase and Uteroferrin

GlcNAc-phosphotransferase from PT18-3M-Emphaze (10 µg) in buffer H adjusted to pH 8.0 with 1 mM Tris-HCl, pH 6.0, was incubated with 0.5 mM of Na125I for 3 min in a 12 × 75-mm glass tube previously coated with 10 µg of IODO-GEN in dichloromethane (20). 125I-Labeled GlcNAc-phosphotransferase was isolated by gel filtration on Sephadex G-25 blocked with 500 mg of bovine serum albumin and then equilibrated with buffer F containing 0.15 M NaCl. The specific activity of the 125I-labeled GlcNAc-phosphotransferase was 12 µCi/µg. Porcine uteroferrin was radioiodinated with the same procedure to a specific activity of 2 µCi/µg.

Immunoprecipitation of 125I-Labeled GlcNAc-phosphotransferase with Monoclonal Antibodies

125I-Labeled GlcNAc-phosphotransferase (0.5 µCi) and 125I-labeled porcine uteroferrin (0.5 µCi) in 50 µl of buffer F were incubated with 100 µl of hybridoma supernatant for 16 h at 4 °C. Protein A-Sepharose with bound rabbit anti-mouse IgG (20 µl of a 50% slurry in buffer J) was added, and the incubation was continued for 2 h. The Sepharose was then collected by centrifugation at 13,000 × g for 5 min. The supernatant was aspirated, and the pellet was washed three times with 1 ml of buffer J. Bound proteins were released by incubation with 8% SDS, 10% β-mercaptoethanol for 5 min at 100 °C, and the eluted proteins were examined by SDS-PAGE on a 6% polyacrylamide gel in Tris/Tricine buffer. Following electrophoresis, the gel was dried and exposed to Kodak MS film at −70 °C for 12 h with an intensifying screen.

Amino-terminal Protein Sequencing

GlcNAc-phosphotransferase (20 µg) was subjected to electrophoresis on a 6% polyacrylamide gel in Tris/glycine buffer (21) with and without disulfide bond reduction. The gel was electroblotted onto a ProBlott membrane (22), which was washed with 1.0% Coomassie Blue in 10% acetic acid, 50% methanol and briefly destained in 10% acetic acid, 50% methanol. The 340,000-, 166,000-, 102,000-, and 56,000-Da protein bands were excised and subjected to automated Edman degradation in an Applied Biosystems Model 492 protein sequencer. The broad 51,000–56,000-Da band in the reduced sample was excised en block and treated similarly.

RESULTS

Lactating bovine mammary glands were chosen as a tissue source for GlcNAc-phosphotransferase purification because preliminary experiments indicated relatively high GlcNAc-phosphotransferase activity, and the tissue was available in large quantities.

Mammary Gland GlcNAc-phosphotransferase Is a Membrane-associated Protein

To investigate whether mammary gland GlcNAc-phosphotransferase was membrane-associated, the post-nuclear supernatant fraction of mammary gland homogenates was centrifuged at 106,000 × g for 30 min. Greater than 95% of the enzyme activity was pelleted under these conditions. Detergent (4% Lubrol), but not 1 M NaCl, was able to release GlcNAc-phosphotransferase from the membrane into the 106,000 × g supernatant, indicating that the enzyme is membrane-associated, similar to the enzyme activities in rat liver (7, 8) and human lymphoblasts (9).

Partial Purification of GlcNAc-phosphotransferase by Method I

The 30,000-fold partial purification of GlcNAc-phosphotransferase by Method I is summarized in Table I. Crude membranes were precipitated from homogenates of mammary glands by high speed centrifugation. The preparation of a high speed crude membrane pellet from lactating bovine mammary gland was based on a procedure developed by Vijay and Fram (25) for the purification of UDP-N-acetyl-D-glucosamine-dolichyl-phosphate N-acetyl-D-glucosamine-1-phosphotransferase (24). Membrane proteins were solubilized in Lubrol and deoxycholate and chromatographed on DEAE-Sepharose FF. GlcNAc-phosphotransferase bound to the column and was eluted as a symmetric peak near the midpoint of a gradient in sodium chloride (Fig. 1A). Overall, the enzyme eluted from the DEAE column was purified 1,300-fold with a yield of 3.6%. Pooled
fractions from the DEAE column were directly applied to a column of reactive green 19-agarose. The enzyme bound tightly to the column, which was washed with a salt gradient and eluted with 1.0 M NaCl (Fig. 1B). Washing the reactive green 19-agarose with a NaCl gradient resulted in consistently higher specific activity than washing with a fixed concentration of NaCl. The enzyme eluted from the reactive green 19-agarose column was purified 6,500-fold over the starting material in 2.5% yield.

The reactive green 19 pool was concentrated to 2 ml by ion-exchange chromatography on POROS 50 HQ and Mono Q. The concentrated GlcNAc-phosphotransferase from the Mono Q column was then chromatographed on Superose 6. The enzyme was included in the column and eluted as a symmetric peak at a position consistent with a molecular mass of \( \sim 570,000 \) Da (Fig. 1C). Following chromatography on Superose 6, the GlcNAc-phosphotransferase had been purified 30,000-fold to a specific activity of 720,000 units/mg in 0.6% yield. Peak fractions contained GlcNAc-phosphotransferase at specific activities \( >10^6 \) units/mg. SDS-PAGE and silver staining showed that multiple polypeptides were present in the fractions containing GlcNAc-phosphotransferase activity. Protein bands whose intensity correlated with GlcNAc-phosphotransferase activity were not apparent (data not shown). Attempts to utilize affinity chromatography steps for further purification were unsuccessful. As an alternative approach, the partially purified enzyme was used to generate monoclonal antibodies for immunoaffinity purification of the enzyme.
Generation and Identification of GlcNAc-phosphotransferase-specific Monoclonal Antibodies

Mice were immunized with partially purified GlcNAc-phosphotransferase, and spleens were removed and fused with myeloma cells according to standard techniques. Hybridomas secreting antibodies against GlcNAc-phosphotransferase were identified with a two-step screening procedure. First, hybridomas secreting mouse IgG were identified by enzyme-linked immunosorbent assay as described under “Methods.” Monoclonal antibodies specific for GlcNAc-phosphotransferase were then identified by their ability to immunoprecipitate GlcNAc-phosphotransferase activity. Four GlcNAc-phosphotransferase-specific monoclonal antibodies (all of subclass IgG2), designated PT18, PT19, PT20, and PT21, were produced.

Characterization of GlcNAc-phosphotransferase-specific Monoclonal Antibodies

The binding characteristics of these monoclonal antibodies were evaluated by incubation of GlcNAc-phosphotransferase (200,000 units) with 1 ml of various monoclonal antibodies coupled to 3M-Emphaze resin. Following a neutral pH wash to remove unbound GlcNAc-phosphotransferase, aliquots of the loaded resin were subjected to experimental elution conditions. As shown in Fig. 2, the immobilized monoclonal antibodies bound 40–90% of the GlcNAc-phosphotransferase, while the control monoclonal antibody (BP95) (directed against bovine C4b-binding protein) bound <5%. GlcNAc-phosphotransferase was eluted from monoclonal antibodies PT18, PT19, and PT20, but not PT21, with buffer at pH 10. The reduction in GlcNAc-phosphotransferase activity results from elution, not denaturation, since the enzyme is stable to pH 11 (data not shown).

Purification of GlcNAc-phosphotransferase by Method II

The 488,000-fold purification of GlcNAc-phosphotransferase to apparent homogeneity by Method II is summarized in Table II.

Step 1: Homogenization, Subcellular Fractionation, and Solubilization—Membrane proteins were precipitated from the post-nuclear supernatant with MnCl2 (25) and solubilized in 4% Lubrol and 0.5% sodium deoxycholate.

Step 2: Absorption and Elution from PT18-3M-Emphaze—GlcNAc-phosphotransferase was directly absorbed from the detergent-solubilized membrane fraction by incubation for 16 h with PT18 coupled to 3M-Emphaze. The affinity absorbent was then packed in a column and washed with buffer F containing 1 M NaCl at pH 7.4 to remove nonspecifically bound proteins. When buffer at pH 10 was applied to the column, a peak of protein was eluted. Assay of column fractions for GlcNAc-phosphotransferase activity demonstrated an activity peak coincident with the eluted protein (Fig. 3). GlcNAc-phosphotransferase eluted from this column was purified 488,000-fold with an overall yield of 29%. The immunoaffinity matrix was highly selective for GlcNAc-phosphotransferase, with a single step purification of 150,000-fold.

The eluted enzyme was examined by SDS-PAGE under reducing and nonreducing conditions (Fig. 4). Under nonreducing conditions, three protein bands were identified with molecular masses of 340,000, 102,000, and 56,000 Da. Following disulfide bond reduction, a band of 166,000 Da and a broad band from 51,000 to 56,000 Da were observed. Although SDS-PAGE of the purified enzyme demonstrated three protein bands, the evidence suggests this is the result of the subunit structure of the enzyme rather than the presence of impurities or degradation. The PT18-3M-Emphaze chromatogram (Fig. 3) demonstrates a good correlation between the protein concentration and GlcNAc-phosphotransferase activity, suggesting that all of the eluted protein represents GlcNAc-phosphotransferase. On SDS-PAGE, the intensity of staining of each of the three bands is similar, consistent with the presence of a 1:1:1 molar ratio among the proteins. Such a ratio should be common among the components of a multisubunit complex and only rarely ob-

Table II

| Step                | Volume | Protein concentration | Total protein | Activity | Specific activity | Total activity | Yield | Purification |
|---------------------|--------|-----------------------|---------------|----------|------------------|----------------|-------|--------------|
| Cleared homogenate  | 6,400  | 200                   | 1,280,000     | 6,400    | 25               | 52,000,000     | 100   | 1.0          |
| Post-nuclear supernatant | 6,020  | 105                   | 632,000       | 6,800    | 82               | 41,000,000     | 79    | 3.3          |
| Solubilized membrane| 640    | 378                   | 242,000       | 30,843   | 81               | 19,700,000     | 38    | 3.2          |
| PT18-3M-Emphaze eluate | 8      | 0.16                  | 1.24          | 1,900,000| 12,200,000       | 15,200,000     | 29    | 488,000      |

Fig. 2. Characterization of binding and elution properties of GlcNAc-phosphotransferase-specific monoclonal antibodies. Ascites containing GlcNAc-phosphotransferase-specific monoclonal antibodies PT18, PT19, PT20, and PT21 as well as the irrelevant control antibody (BP95) (directed against bovine C4b-binding protein) bound <5%. GlcNAc-phosphotransferase was eluted from monoclonal antibodies PT18, PT19, and PT20, but not PT21, with buffer at pH 10. The reduction in GlcNAc-phosphotransferase activity results from elution, not denaturation, since the enzyme is stable to pH 11 (data not shown).
1. Introduction

Purification of Bovine GlcNAc-phosphotransferase

The GlcNAc-phosphotransferase activity was enriched by precipitation with monoclonal antibodies (PT18, PT19, PT20, PT21) as well as with the irrelevant isotype control monoclonal antibody (BP95). Immunoprecipitates were washed, eluted with SDS at 100 °C, and analyzed by SDS-PAGE under reducing conditions and by autoradiography. As shown in Fig. 5, monoclonal antibodies PT18, PT19, PT20, and PT21 precipitated a complex containing 166- and 56-kDa subunits. In non-reduced samples, precipitation of the 102-kDa polypeptide could not be demonstrated since this subunit is not radioiodinated. The separated proteins were transblotted, and individual bands were subjected to amino-terminal sequencing.

Subunit Identification by Amino-terminal Protein Sequencing

On SDS-PAGE, the purified GlcNAc-phosphotransferase appeared to contain three polypeptides, at least two of which were precipitated by four independent monoclonal antibodies. To define the relationship between the bands visualized on reduced and nonreduced SDS-polyacrylamide gels, GlcNAc-phosphotransferase was subjected to SDS-PAGE and electroblotted, and individual bands were subjected to amino-terminal sequencing.

Purified GlcNAc-phosphotransferase (20 µg) was electrophoresed on an SDS-8% polyacrylamide gel with and without disulfide bond reduction. The separated proteins were transferred to ProBlott, and individual Coomassie Blue-stained bands excised and subjected to automated Edman degradation. The 340- and 166-kDa bands were found to contain the same amino-terminal sequence, Met-Leu-Leu-Lys, suggesting that the 340-kDa band represents a homodimer of 166-kDa subunits. The 102-kDa band on the nonreduced blot had an N-
Purification of Bovine GlcNAc-phosphotransferase

**Stoichiometry between the 56- and 51-kDa Subunits**

Quantitative amino-terminal sequencing was used to determine the molar ratio between the 56- and 51-kDa subunits in the GlcNAc-phosphotransferase complex (26). GlcNAc-phosphotransferase was subjected to SDS-PAGE under reducing conditions, and the proteins were transferred to a ProBlott membrane. The Coomassie Blue-stained broad 51–56-kDa band was excised and subjected to 18 cycles of Edman degradation. The Coomassie Blue-stained broad 51–56-kDa band thus appears to be a homodimer composed of 51-kDa subunits. These results suggest that the broad 51–56-kDa band contains both 56- and 51-kDa subunits. The identification of three amino-terminal sequences is consistent with the proposal that the purified GlcNAc-phosphotransferase contains three distinct subunits.

- Tripeptide sequence of Ala-Lys-Met-Lys. The 56-kDa band on the nonreduced blot had an amino-terminal sequence of Asp-Thr-Phe-Ala. When the broad band from 51 to 56 kDa on the reduced blot was subjected to automated Edman degradation, two PTH-derivatives were identified in each cycle, (Ala/Asp)-(Lys/Thr)-(Met/Phe)-(Lys/Ala). The 102-kDa band on the non-reduced gel thus appears to be a homodimer composed of 51-kDa subunits. These results suggest that the broad 51–56-kDa band contains both 56- and 51-kDa subunits. The identification of three amino-terminal sequences is consistent with the proposal that the purified GlcNAc-phosphotransferase contains three distinct subunits.

**Model of GlcNAc-phosphotransferase Structure**—The data presented here are sufficient to propose a model for the subunit structure of GlcNAc-phosphotransferase. The molecular mass of the GlcNAc-phosphotransferase estimated from gel filtration is ~570,000 Da (Fig. 1C). This estimate may be in error because of bound detergent or if the molecule has an extended structure, but provides an upper limit for the molecular mass. Amino-terminal sequencing demonstrates that each of the three proteins that comprise GlcNAc-phosphotransferase has a unique amino-terminal sequence, suggesting that the proteins

FIG. 6. Determination of the stoichiometry of the 56- and 51-kDa subunits. GlcNAc-phosphotransferase (20 µg) was equilibrated with 5% SDS, 10 mM dithiothreitol and electrophoresed on a 6% polyacrylamide gel in Tris/Tricine. The proteins were electrotransferred to a ProBlott membrane, stained with Coomassie Blue, and subjected to N-terminal sequence analysis. The log of the yield of PTH-derivative in each cycle is plotted. For comparison, data are plotted assuming a 1:2 molar ratio between the 56- and 51-kDa subunits. For each cycle is plotted. For comparison, data are plotted assuming a 1:2 molar ratio between the 56- and 51-kDa subunits (Fig. 6, upper dashed line) and a 2:1 molar ratio (lower dashed line). Comparison of the actual data obtained with the 56-kDa subunit at these hypothetical ratios shows that the data are consistent with a 1:1 molar ratio between the subunits and not a 2:3 or 1:2 ratio.

**DISCUSSION**

We have purified bovine UDP-N-acetylgalactosamine:lysosomal-enzyme N-acetylgalactosamine-1-phosphotransferase ~488,000-fold in 29% yield. Lactating bovine mammary glands were chosen as the starting material because the specific activity of the enzyme was relatively high, large amounts of tissue were available, and protocols for the isolation of a high speed membrane pellet had been described (23). GlcNAc-phosphotransferase was quantitatively membrane-associated in mammary gland extracts. No evidence for a soluble form of GlcNAc-phosphotransferase in mammary gland homogenates was found in contrast to what has been described for mammary gland β-1,4-galactosyltransferase. The use of the lactating bovine mammary gland allowed the scale of the preparation to be increased ~200-fold relative to the rat liver purification method described by Reitman et al. (9). This proved important in allowing the preparation of sufficient partially purified enzyme for monoclonal antibody generation with the inefficient purification Method I.

The purification of GlcNAc-phosphotransferase has been attempted from rat liver (9) and human lymphoblasts (11). These investigators were unsuccessful in isolating this trace enzyme in part because of the inability to successfully utilize affinity columns with substrate acceptor or donor analogs. In this study, immobilized UDP-hexanolamine and lysosomal enzyme columns were also investigated without success (data not shown). In retrospect, although highly substituted affinity columns were used, the GlcNAc-phosphotransferase preparations applied were likely too dilute for successful binding, given the moderate (micromolar) affinity of the enzyme for these ligands. In the absence of an available affinity chromatography step based on a donor or acceptor substrate, dye-ligand chromatography was investigated. Of 10 immobilized dyes investigated, only reactive green 19-agarose demonstrated significant binding of GlcNAc-phosphotransferase. Reactive green 19-agarose has previously been utilized in the purification of UDP-rhamnose:flavanone-7-O-glucoside 2′-O-rhamnosyltransferase (27). Selective elution of the rhamnosyltransferase with UDP and hesperetin 7-O-glucoside was successful, suggesting that the reactive green 19-agarose functioned as a substrate analog. Attempts to elute GlcNAc-phosphotransferase from reactive green 19 with UDP and α-methylmannoside alone and in combination were unsuccessful. GlcNAc-phosphotransferase bound tightly to the immobilized reactive green 19-agarose, requiring 1 M NaCl for elution. Since GlcNAc-phosphotransferase can be eluted from the strong anion exchangers POROS 50 HQ and Mono Q with ~0.3 M NaCl, a pure ionic interaction is unlikely. In preliminary experiments, soluble reactive green 19 was found to be a competitive inhibitor of GlcNAc-phosphotransferase, with a Ki of ~0.5 mg/ml (data not shown), suggesting that the column may function as a substrate analog. The structure of reactive green 19 has not been determined, preventing comparison with the structure of UDP-GlcNAc or α-methylmannoside.
are all distinct. The 166- and 51-kDa proteins are each found as disulfide-linked homodimers. Assuming that each molecule of GlcNAc-phosphotransferase contains at least one of each subunit polypeptide, each molecule must contain both a homodimer of 166-kDa subunits and a homodimer of 51-kDa subunits. Since the 56-kDa protein is not found as a disulfide-linked multimer, other approaches must be used to determine the stoichiometry of this subunit in the GlcNAc-phosphotransferase complex. Quantitative amino-terminal sequencing established that a 1:1 molar ratio exists between the 56- and 51-kDa subunits. Since each molecule contains at least one 51-kDa disulfide-linked homodimer, it must also contain two 56-kDa proteins. Each molecule of GlcNAc-phosphotransferase must then contain a minimum of two each of the 166-, 51-, and 56-kDa proteins. The nominal molecular mass of a complex containing these six polypeptides is 546 kDa, in good agreement with the molecular mass of 570 kDa estimated by gel filtration chromatography. A model for the subunit structure of GlcNAc-phosphotransferase is given in Fig. 7.

The molecular mass of human GlcNAc-phosphotransferase has been estimated by radiation inactivation target size determination as 228 kDa in placenta and 283 kDa in fibroblasts (28). These estimates are very different than those determined in this study for bovine mammary gland GlcNAc-phosphotransferase. Whether these differences reflect species or organ differences, the presence of carbohydrate, or an error in the respective mass determinations must await further studies.

Comparison of the Subunit Structure of GlcNAc-phosphotransferase with Other Glycosyltransferases—With the definition of the subunit structure of bovine GlcNAc-phosphotransferase, it was of interest to compare the subunit structure and molecular mass with other glycosyltransferases that might be predicted to be homologous. UDP-N-acetylglucosamine:glycoprotein N-acetylglucosamine-1-phosphotransferase has been partially purified from A. castellanii (13). The partially purified enzyme contained multiple protein bands, precluding confident identification of bands representing the enzyme, although proteins of 97 and 43 kDa were believed to be components of the enzyme. UDP-N-acetylglucosamine:dolichyl-phosphate N-acetylglucosamine-1-phosphotransferase is an endoplasmic reticulum integral membrane glycoprotein that catalyzes the transfer of N-acetylglucosamine 1-phosphate from UDP-N-acetylglucosamine to the acceptor, dolichol phosphate. This enzyme has been isolated and/or cloned from mouse, hamster, Schizosaccharomyces pombe, and Saccharomyces cerevisiae and consists of a single chain protein of 46–50 kDa.

Five Golgi GlcNAc-transases that differ in acceptor specificity have been described. All of these enzymes utilize UDP-GlcNAc as donor and transfer N-acetylglucosamine to oligosaccharide acceptors. Human GlcNAc-transferase I is a single chain protein of ~50 kDa (29). Rat GlcNAc-transferase II has been purified and characterized as a 42-kDa single chain protein (30). A cDNA clone for human GlcNAc-transferase V that predicts a protein of ~81 kDa has been isolated (32). The cytoplasmic GlcNAc-transferase responsible for the glycosylation of cytoplasmic proteins is a multimeric enzyme composed of subunits of 110 and 78 kDa (33). The GlcN-phosphotransferase isolated in this study is only the second multisubunit GlcNAc-transferase described and is the first multisubunit GlcNAc-transferase in the secretory pathway.

Genetic Defects in GlcNAc-phosphotransferase—I-cell disease (mucolipidosis II) and pseudo-Hurler polydystrophy (mucolipidosis III) are human autosomal recessive disorders caused by complete or incomplete deficiency of GlcNAc-phosphotransferase, respectively. These disorders have been demonstrated to be genetically heterogeneous, and two complementation groups of mucolipidosis II (34) and three of mucolipidosis III (34, 35) have been described. The identification of three subunits in purified GlcNAc-phosphotransferase, together with the genetic data suggesting two or three complementation groups, is consistent with the proposal that each subunit of the enzyme could be the product of an individual gene.

Future Directions—The results of this study suggest a number of areas for further investigation. Comparison of the GlcNAc-phosphotransferase protein sequences with those of other glycosyltransferases will provide a more definitive answer about the relationship of the various enzymes. Determination of the number of cDNAs may allow a better understanding of the genetic complementation groups observed in human mucolipidosis II and III. It remains possible that the polypeptides of 56 and 51 kDa found in the purified GlcNAc-phosphotransferase either are impurities remaining in the preparation or result from proteolysis of the intact GlcNAc-phosphotransferase protein. Confirmation of the model for the subunit structure of this complex enzyme will likely need to await the isolation and characterization of the GlcNAc-phosphotransferase cDNA(s). These studies are currently in progress.

Acknowledgments—We thank Chuck Mikkelson, Jr. and Mikkelson Beef, Inc. for invaluable assistance in the collection of lactating bovine udders, and the excellent technical assistance of Rachel Yudovich is appreciated. We also thank Dr. Ken Jackson and the Molecular Biology Resource Facility of the W. K. Warren Medical Research Institute for assistance with protein sequencing and Michele Arcade for secretarial expertise.

REFERENCES
1. Reitman, M. L., and Kornfeld, S. (1981) J. Biol. Chem. 256, 11977–11980
2. Reitman, M. L., and Kornfeld, S. (1981) J. Biol. Chem. 256, 4275–4281
3. Waheed, A., Hasilik, A., and von Figura, K. (1982) J. Biol. Chem. 257, 12322–12327
4. Varki, A., and Kornfeld, S. (1981) J. Biol. Chem. 256, 9937–9943
5. Waheed, A., Hasilik, A., and von Figura, K. (1981) J. Biol. Chem. 256, 5717–5721
6. Kornfeld, S., and Mellman, I. (1989) Annu. Rev. Cell Biol. 5, 483–525
7. Dahlm, N. M., Lobel, P., and Kornfeld, S. (1989) J. Biol. Chem. 264, 12115–12118
8. Reitman, M. L., Varki, A., and Kornfeld, S. (1981) Clin. Invest. 67, 1274–1279
9. Reitman, M. L., Lang, L., and Kornfeld, S. (1984) Methods Enzymol. 107, 163–172
10. Lang, L., Reitman, M., Tang, J., Roberts, R. M., and Kornfeld, S. (1984) J. Biol. Chem. 259, 12302–12307
11. Zhao, K. W., Yeh, R., and Miller, A. L. (1992) Glycobiology 2, 119–125
12. Lang, L., Couso, R., and Kornfeld, S. (1986) J. Biol. Chem. 261, 6320–6325
13. Ketcham, C. M., and Kornfeld, S. (1992) J. Biol. Chem. 267, 11645–11653
14. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goece, N. M., Olson, B. J., and Klenk, D. C. (1985) Anal. Biochem. 160, 76–85
15. Schagger, H., and van Jagow, G. (1987) Anal. Biochem. 166, 368–379
16. Blum, H., Beier, H., and Gross, H. (1987) Electrophoresis 93–99
17. Rupp, P. A., and Cheng, F.-W. (1990) Anal. Biochem. 187, 104–108
18. Brown, E. G., Newton, R., and Shaw, N. M. (1985) Anal. Biochem. 142, 378–388
19. Harlow, E., and Lane, D. (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
20. Fraker, P. J., and Speck, J. C., Jr. (1976) Biochem. Biophys. Res. Commun. 80, 849–857
21. Laemmli, U. K. (1970) Nature 227, 680–685
Purification of Bovine GlcNAc-phosphotransferase

22. Matsudaira, P. (1987) J. Biol. Chem. 262, 10035–10038
23. Vijay, I. K., and Fram, S. R. (1977) J. Supramol. Struct. 7, 251–265
24. Shailubhai, K., Dong-Yu, B., Saxena, E. S., and Vijay, I. K. (1988) J. Biol. Chem. 263, 15964–15972
25. Bendiak, B., and Schachter, H. (1987) J. Biol. Chem. 262, 5775–5783
26. Smithies, O., Gibson, D., Fanning, E. M., Goodflesh, R. M., Gilman, J. G., and Ballantyne, D. L. (1971) Biochemistry 10, 4912–4921
27. Bar-Peled, M., Lewinsohn, E., Fluhr, R., and Gressel, J. (1991) J. Biol. Chem. 266, 20953–20959
28. Ben-Yoseph, Y., Potier, M., Pack, B. A., Mitchell, D. A., Melancon, S. B., and Nadler, H. L. (1986) Biochim. J. 235, 883–886
29. Kumar, R., Yang, J., Larsen, R. D., and Stanley, P. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 9948–9952
30. D’Agostaro, G. A. F., Zingoni, A., Moritz, R. L., Simpson, R. J., Schachter, H., and Bendiak, B. (1995) J. Biol. Chem. 270, 15211–15221
31. Ibara, Y., Nishikawa, A., Tohma, T., Soejima, H., Niikawa, N., and Taniguchi, N. (1993) J. Biochem. (Tokyo) 113, 692–698
32. Saito, H., Nishikawa, A., Gu, J., Ibara, Y., Soejima, H., Wada, Y., Sekiya, C., Kikawa, N., and Taniguchi, N. (1994) Biochem. Biophys. Res. Commun. 198, 318–327
33. Haltiwanger, R. S., Blomberg, M. A., and Hart, G. W. (1992) J. Biol. Chem. 267, 9005–9013
34. Shows, T. B., Mueller, O. T., Honey, N. K., Wright, C. E., and Miller, A. L. (1982) Am. J. Med. Genet. 12, 343–353
35. Honey, N. K., Mueller, O. T., Little, I. E., Miller, A. L., and Shows, T. B. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 7420–7424