Biochem. J. (1987) 248, 151–159 (Printed in Great Britain)

Properties of *N*-acetylglucosamine 1-phosphotransferase from human lymphoblasts

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Human lymphoblast and fibroblast cell lines from a patient with I-cell disease and normal individuals were characterized with respect to certain properties of UDP-*N*-acetylglucosamine:lysosomal enzyme precursor *N*-acetylglucosamine phosphotransferase. The enzyme isolated from normal lymphoblast and fibroblast cell lines expressed similar kinetic properties, substrate specificities and subcellular localizations. Coincident with the severe reduction of *N*-acetylglucosamine phosphotransferase activity in both I-cell fibroblast and lymphoblast cell lines, there was an increased secretion of several lysosomal enzymes compared to normal controls. Subsequent examination of *N*-acetyl-β-D-hexosaminidase secreted by the I-cell lymphoblasts demonstrated a significant increase in adsorption of the I-cell enzyme to *Ricinus communis* agglutinin, a galactose-specific lectin. However, the I-cell lymphoblasts did not exhibit the significant decrease in intracellular lysosomal activities seen in I-cell fibroblasts. Our results suggest that lymphoblasts not only represent an excellent source for the purification of *N*-acetylglucosamine phosphotransferase, but in addition, represent a unique system for studying alternate mechanisms involved in the targeting of lysosomal enzymes.

INTRODUCTION

I-cell disease is an inherited childhood neurometabolic disorder which has been associated with alterations in the intracellular segregation of lysosomal enzymes (Hickman *et al.*, 1974). Cultured fibroblasts derived from I-cell patients are deficient in the UDP-*N*-acetylglucosamine:lysosomal enzyme precursor *N*-acetylglucosamine phosphate transferase (Reitman *et al.*, 1981; Hasilik *et al.*, 1981). This enzyme specifically recognizes lysosomal enzymes and transfers α-*N*-acetylglucosamine 1-phosphate residue(s) to their high-mannose oligosaccharide chains resulting in the formation of a diester bond (Tabas & Kornfeld, 1980). The terminal *N*-acetylglucosamine residue(s) is subsequently removed by α-*N*-acetylglucosamine 1-phosphodiester-*N*-acetyl-β-D-glucosaminidase exposing a mannose 6-phosphate monoester (Varki & Kornfeld, 1980; Varki & Kornfeld, 1981). This latter structure is preferentially recognized by a specific transport receptor protein within the cell and results in delivery of the enzymes to lysosomes (Sly & Fischer, 1982). Cultured I-cell fibroblasts are incapable of synthesizing the mannose 6-phosphate marker on enzymes destined for the lysosome resulting in the conversion of the enzymes' high-mannose-type chains to complex-type prior to secretion from the cell (Miller *et al.*, 1981).

We have previously demonstrated genetic and biochemical complementation of *N*-acetylglucosamine phosphotransferase within I-cell disease and pseudo-Hurler polydystrophy (Mueller *et al.*, 1983; Little *et al.*, 1986). In order to evaluate the molecular basis for each complementation group, the enzyme must first be purified in sufficient quantities for biochemical characterizations. We chose human lymphoblast cell lines to study the structure and function of this regulatory enzyme. Lymphoblasts are cell lines established by the transformation of human lymphocytes with Epstein–Barr virus. Large quantities of these transformed cells can be obtained in a minimum of space and time. These cell lines retain their unique genotype in continuous culture for long periods of time allowing cellular material of stable genotype to be grown in large quantities (Conover *et al.*, 1970). The present study establishes that lymphoblasts represent a good system from which to isolate, purify, and study, the properties of *N*-acetylglucosamine phosphotransferase. A preliminary report of these findings has appeared elsewhere (Miller *et al.*, 1985).

EXPERIMENTAL

Materials and methods

Materials were obtained from the following sources: Scint A (United Packard, Downer Grove, IL, U.S.A.); concanavalin A–Sepharose, 10 mg/ml (Pharmacia); *Ricinus communis* agglutinin I (RCA_I) (EY Laboratories, San Mateo, CA, U.S.A.); Bio-Gel P-6 (BioRad, Richmond, CA, U.S.A.); [γ-^{32}P]ATP, 5000 Ci/mol (Amersham); UDP [^{3}H]*N*-acetylglucosamine (New England Nuclear, Boston, MA, U.S.A.); QAE-Sephadex, α-methyl D-mannoside, bovine thyroglobulin, D-galactose, neuraminidase (*Candida utilis*) and other reagents (Sigma); Pronase (Calbiochem-Behring, La Jolla, CA, U.S.A.); yeast hexokinase (EC 2.7.1.1; Boehringer-Mannheim, Indianapolis, IN, U.S.A.); and Nutriclone H serum-free liquid medium (Techniclone International, Santa Ana, CA, U.S.A.). All procedures were carried out at 0–4 °C unless otherwise stated.

Culture conditions

The original normal lymphocytes were derived from healthy individuals whereas the I-cell lymphocytes were

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obtained from a 6-year-old (patient MD). The normal and I-cell-disease lymphocytes were transformed according to previous published procedures (Harms *et al.*, 1981) in the laboratory of Dr. J. E. Seegmiller, Department of Medicine, University of California, San Diego, U.S.A. Lymphoblasts were maintained in 75 cm^2 flasks using RPMI 1640 medium with 10% (v/v) fetal bovine serum, 0.3% (v/v) glutamine, and 0.3% (w/v) penicillin/streptomycin until a density of approx. 2.5×10^6 cells/ml was obtained. Cells were harvested by centrifugation at 1500 *g* for 20 min in a Beckman table-top TJ-6 centrifuge using a TH-4 rotor. The fibroblasts were cultured under conditions previously reported (Miller *et al.*, 1981, 1986). I-cell (MD) and normal control cultures were used for enzyme assays within 1 day of reaching confluency. The cells were harvested by scraping with a rubber policeman in phosphate (10 mM)-buffered saline (pH 7.4) and pelleted at 1500 *g* as described above.

Assay for lysosomal hydrolase activities

Collected cells were resuspended (1:10, w/v) in glass-distilled water and sonicated with a Kontes micro-ultrasonic cell disruptor for three 10 s bursts (setting 8). Using these homogenates, lysosomal hydrolase activities were assayed fluorometrically by using the corresponding 4-methylumbelliferyl substrates (Koch-Light Laboratories, Colinbrook, Bucks., U.K.) as described previously (Miller *et al.*, 1981, 1986). In addition, α-D-galactosidase was assayed using a final concentration of 10 mM of the 4-methylumbelliferyl substrate that had been prepared in 100 mM-phosphate/citrate, pH 4.6.

Assay for neuraminidase activity

Fibroblasts and lymphoblasts were grown, harvested, and pelleted as described above. The fibroblasts were resuspended in 250 mM-sodium acetate, pH 4.6 (1:4 w/v), while the lymphoblasts were resuspended in a 1:7 (w/v) dilution in the same buffer. Both cell suspensions were sonicated for two 1 s bursts. Up to 20 μl of cell homogenate containing 1–3.5 mg/ml of protein was added to 20 μl of 250 mM-sodium acetate and reaction was initiated with 20 μl of 4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid (Sigma) at an assay temperature of 37 °C. Prior to assay, the substrate was dissolved in glass-distilled water and extracted twice each time with an equal volume of ethyl acetate to remove impurities. The upper phase was removed and a stream of nitrogen was used to remove any residual ethyl acetate. The substrate (0.96 mM final concentration) was then used to assay enzyme activity in fibroblasts. The enzyme from lymphoblasts was also assayed with purified substrate to which taurocholate (Koch-Light Laboratories) and Triton X-100 (Sigma) were added to a final concentration of 10 mg/ml and 8 mg/ml, respectively.

Protein determination

Protein assays were performed by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Secretion of lysosomal enzymes from cultured I-cell and normal lymphoblasts

Secretion from cultured I-cell and normal fibroblasts was measured according to procedure previously published (Miller *et al.*, 1981). Briefly, the complete growth medium was removed from near confluent cells grown in 75 cm^2 flasks and replaced with chemically defined Waymouth's medium. The cells were incubated for 4, 24 and 48 h. At each time-point the medium was removed and concentrated 10–20-fold with an Amicon concentrator fitted with a PM-10 filter. Lysosomal enzyme activities were assayed as previously reported (Miller *et al.*, 1981, 1986). Lymphoblast cells were grown in suspension using 75 cm^2 flasks to a density of $(1–2) \times 10^6$ cells/ml before initiating secretion. The cells were first conditioned for 24 h by the addition of Nutriclone defined medium to the growth medium. The medium was then removed by centrifugation of the cells for 15 min at 1500 *g* in a Beckman TJ-6 centrifuge followed by resuspending the cells in the defined medium, Nutriclone. At 4, 24 and 48 h, the medium was removed from the cells by centrifugation and concentrated in a similar manner as described above for the fibroblast medium. The concentrated samples were used for assays of selected lysosomal enzymes.

Column chromatography of *N*-acetyl-β-D-hexosaminidase on *Ricinus communis* agglutinin before and after neuraminidase treatment

Preparations of normal and I-cell, and secreted and concentrated, *N*-acetyl-β-D-hexosaminidase were applied separately to a column (1 cm × 3 cm) of RCA_I–agarose (3–5 mg of lectin bound per ml of gel), and equilibrated in 15 mM-sodium phosphate buffer, pH 6.0, containing human serum albumin (1 mg/ml) and 0.02% (w/v) NaN_3. After standing for 15 min, the column was washed with several column volumes of the aforementioned buffer. The lectin-bound material was eluted with 15 ml of 100 mM-galactose prepared in the equilibration buffer.

Samples of normal and I-cell, and secreted and concentrated, *N*-acetyl-β-D-hexosaminidase were treated for 5 h with 0.5 units of *Clostridium perfringens* neuraminidase type IX (1 unit will liberate 1.0 nmol of *N*-acetylneuraminic acid/min) in 100 mM-acetate buffer, pH 5.0, containing human serum albumin (1 mg/ml) and 0.02% (w/v) NaN_3 (final volume 110 μl) at 37 °C. The neuraminidase-treated and control samples were subjected to column chromatography on RCA_I as described above.

Subcellular fractionation of normal lymphoblast and fibroblast cell lines

Roller bottles of normal lymphoblasts were grown for 1 week (approx. 2.5×10^6 cells/ml) according to procedures described above. The cells were harvested by centrifugation at 1500 *g* for 20 min in a Beckman TJ-6 table-top centrifuge using a TH-4 rotor. After discarding the supernatant fluids, the cell pellets were resuspended in 2.5 ml of 250 mM-sucrose followed by centrifugation as above. The resulting cell pellets were resuspended in 2.5 ml of 250 mM-sucrose prior to the breakage procedures described below. Two roller bottles of normal fibroblasts were grown to confluency according to previous methodology (Miller *et al.*, 1981). The growth medium was poured off and the cells rinsed twice with 20 ml of 0.9% (w/v) NaCl; 5 ml of 2.5% (w/v) trypsin and 20 ml of 0.9% (w/v) NaCl were added to each roller

bottle. After rotating the bottles for 10 min at 37 °C, the cells were collected by centrifugation at 1500 *g* for 20 min. The resulting supernatant fluids were discarded and the cell pellets were suspended for 15 min in 20 ml of growth medium containing 15% (v/v) bovine serum albumin at room temperature. The cells were then centrifuged as before, washed with 0.9% (w/v) NaCl and recentrifuged. The resulting fibroblast cell pellets were resuspended in 2.5 ml of 250 mM-sucrose prior to breakage. The breakage procedure involved the separate passage of the lymphoblast and fibroblast suspensions through a syringe attached to an 18-gauge syringe needle. This procedure was carried out using only hand pressure. Unbroken cells and cell debris were pelleted in each case by centrifugation in a SS34 rotor at 1500 *g* for 10 min. The resulting supernatant fluids were saved and the pellets suspended in 2.0 ml of 250 mM-sucrose. The suspensions were then passed through a syringe attached to a 20-gauge syringe needle. The samples were centrifuged as above and the resulting supernatant fluids saved. The pellets were again suspended in 1.5 ml of 250 mM-sucrose. The suspensions were subsequently passed through a syringe attached to a 22-gauge syringe needle and centrifuged for 10 min in a SS34 rotor at 1500 *g*. The resulting pellets were discarded and the combined supernatant fluids from passages through the 18, 20 and 22-gauge syringe needles were fractionated on a colloidal silica gradient. The preparation of the gradient and the centrifugation procedure employed were identical to that previously described (Rome *et al.*, 1979). The resulting fractions were assayed for marker enzymes according to previous methods (Miller *et al.*, 1981, 1986). The *N*-acetylglucosamine phosphotransferase activity was determined using the exogenous substrate, α-methyl D-mannoside, as an acceptor according to the procedure described below.

Substrate preparations for assay of *N*-acetylglucosamine phosphotransferase

[β-^{32}P]UDP-*N*-acetylglucosamine synthesis. [^{32}P]UDP-*N*-acetylglucosamine was prepared and identified by the modified method of Owada & Neufeld (1982) as reported previously (Little *et al.*, 1986). The yield of ^{32}P-labelled substrate was 15–20%. [^{32}P]UDP-*N*-acetylglucosamine was used in endogenous acceptor assay described below, while UDP-[^{3}H]*N*-acetylglucosamine was used in both high-mannose glycopeptide acceptor and α-methyl D-mannoside acceptor assays.

High mannose glycopeptides. Bovine thyroglobulin (500 mg) was incubated for 48 h at 56 °C in 10 ml of 100 mM-Tris/HCl buffer, pH 8.0, containing 2 mM-$CaCl_2$ and 5 mg of Pronase. The resulting mixture was boiled and centrifuged for 10 min at 2000 *g*. The supernatant fluid was run in 5 ml portions (20% volume) on a 1.5 cm × 50 cm G-25 column in 100 mM-NH_4HCO_3. Fractions (1 ml) were collected and screened for carbohydrate content with the phenol/sulphuric acid assay (Dubois *et al.*, 1956). Fractions containing carbohydrate were pooled, concentrated with a rotary evaporator, and applied to a 1.5 cm × 95 cm P-6 column. The column was run in 100 mM-NH_4HCO_3 and 1 ml fractions were collected and screened for carbohydrates as described above. The second broad peak was collected and pooled. The amount of mannose in the sample was determined by gas chromatography (Kress *et al.*, 1982).

Assays of *N*-acetylglucosamine phosphotransferase

Endogenous acceptor assay. Collected cells were sonicated with a Kontes micro-ultrasonic cell disruptor at 0–4 °C for five 10 s bursts in a buffer containing 25 mM-Tris/HCl, pH 7.5, 0.3% (w/v) Lubrol and 0.2 mM-dithiothreitol. The resulting homogenates were dialysed against this buffer at 0–4 °C overnight and then centrifuged for 15 min at 10000 *g*. The resulting supernatant fluids were used for the following assay. UDP-*N*-acetylglucosamine (100 μM), 500000 c.p.m. of [β-^{32}P]-UDP-*N*-acetylglucosamine, 13 mM-$MgCl_2$, 13 mM-$MnCl_2$, 1.6 mM-UDP, 1.6 mM-ATP, 500–750 μg of cell supernatant fluid, 25 mM-Tris/HCl, pH 7.5, and 0.35% (w/v) Lubrol in a final volume of 100 μl were incubated for 1 h at 37 °C. This preparation was boiled and subjected to two washes with chloroform/methanol (2:1 v/v) followed by a wash with glass-distilled water. The resulting pellet was incubated with 0.3 ml of 100 mM-Tris/HCl, pH 8.0, 100 mM-glucose 6-phosphate, 20 mM-$CaCl_2$, and 1 mg/ml of Pronase for at least 1 h at 37 °C. This preparation was boiled for 10 min and centrifuged. The resulting supernatant fluid was diluted to 1.5 ml with water and applied to a 1 ml concanavalin A–Sepharose 4B column. The column was washed with 100 ml of phosphate-buffered saline, pH 7.4, and the product eluted with 4 ml of 500 mM-α-methyl-D-mannoside at 60 °C.

High-mannose glycopeptide acceptor assay. All homogenates containing 400–500 μg of protein were incubated for 1 h at 37 °C with 100 μM-UDP-*N*-acetylglucosamine, approx. 300000 c.p.m. of UDP-*N*-[^{3}H]acetylglucosamine, 13 mM-$MgCl_2$, 13 mM-$MnCl_2$, 1.6 mM-UDP, 1.6 mM-ATP, and 5–10 μg of glycopeptide (based on μg of mannoside present) in a final volume of 100 μl. The reactions were stopped at 0–4 °C and washed twice with chloroform/methanol (2:1, v/v). The remaining pellet was subsequently resuspended twice in 20 mM-Tris/HCl, pH 8.0, with 500 mM-NaCl, 0.1 mM-$CaCl_2$, 0.1 mM-$MgCl_2$, 0.1 mM-$MnCl_2$, and 0.02% (w/v) NaN_3 (Buffer A) and centrifuged. The resulting supernatant fluids were applied to 2.5 cm × 0.5 cm columns containing concanavalin A–Sepharose. These are rinsed with 100 ml of phosphate buffered saline, pH 7.5, and eluted with 4 ml of Buffer A containing 500 mM-α-methyl-D-mannoside at 60 °C.

α-Methyl D-mannoside acceptor assay. Prepared cell homogenates of 250–300 μg of protein were incubated in a 25 mM-Tris/HCl, pH 7.5, buffer with 100 mM-α-methyl D-mannoside, 100 μM-UDP-*N*-acetylglucosamine, 300000 c.p.m. of UDP-*N*-[^{3}H]acetylglucosamine, 13 mM-$MgCl_2$, 13 mM-$MnCl_2$, 1.6 mM-UDP, 1.6 mM-ATP, and 0.3% (w/v) Lubrol PX at a final volume of 100 μl. This mixture was incubated for 30 min at 37 °C. The reaction was stopped by boiling for 3 min, and then the mixture was diluted to 1.1 ml with glass-distilled water, and centrifuged for 2.5 min in a Fisher Model 235B microcentrifuge. The resulting supernatant fluid was applied to a 0.5 × 2.5 cm column containing QAE-Sephadex which had been well equilibrated in 2 mM-Tris. The columns were rinsed with 4 ml of 2 mM-Tris and the product eluted with 4 ml of 2 mM-Tris containing 30 mM-NaCl.

RESULTS

Intracellular levels of lysosomal hydrolases in normal and I-cell-disease lymphoblasts

Three normal lymphoblast cell lines and those from an I-cell patient (MD) were grown and intracellular levels of several lysosomal hydrolases were measured. The enzyme levels in the I-cell lymphoblast cell line were within the range of the values found in normal lymphoblast cell lines. These results were in contrast to the level of enzyme activities from cultured I-cell fibroblasts where the expected decrease in lysosomal enzyme activities was seen (Table 1).

Secretion of lysosomal enzymes from normal and I-cell-disease lymphoblasts

Secretion of *N*-acetyl-β-D-hexosaminidase was followed in two normal cell lines and the I-cell lymphoblast line for 48 h (Fig. 1). *N*-Acetyl-β-D-hexosaminidase from the I-cell line was secreted at a much higher rate than the normal lines (Fig. 1*a*), analogous to I-cell fibroblasts which also secrete the enzyme at increased rates (Fig. 1*b*). In addition, α-D-mannosidase and α-L-fucosidase were assayed in the lymphoblast medium at 24 and 48 h. The extracellular levels of these enzymes were 2–8-fold higher in the I-cell lymphoblast medium when compared to the same enzymes obtained from the medium of normal controls (results not shown).

RCA_I binding of secreted *N*-acetyl-β-D-hexosaminidase

Secretion of lysosomal enzymes into the medium by I-cell cultured fibroblasts has been shown to be accompanied by a conversion of their high-mannose-type oligosaccharide units to complex-type (Miller *et al.*, 1981). Therefore secreted *N*-acetyl-β-D-hexosaminidase collected from a normal and the I-cell lymphoblasts were tested for binding to the galactose-specific lectin, agarose-

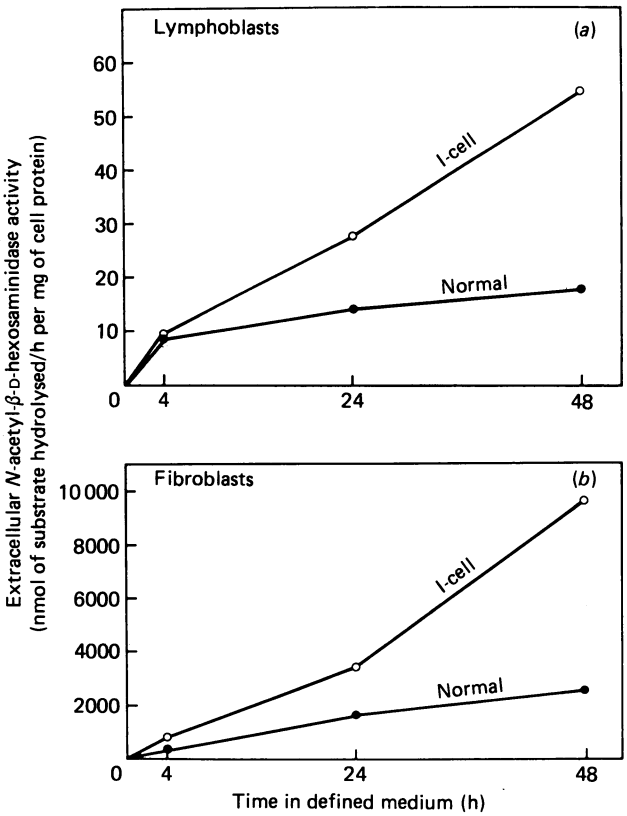

Fig. 1. Examination of the rate of secretion of *N*-acetyl-D-hexosaminidase from normal and I-cell cultured lymphoblasts and fibroblasts

Normal (●) and I cells (○) were incubated with chemically defined culture medium for 4, 24 and 48 h (as described in the Experimental section). The medium was then removed and assayed fluorometrically for *N*-acetyl-β-D-hexosaminidase.

Table 1. Intracellular hydrolase activities of lymphoblasts and fibroblasts

Up to 20 μl of cell homogenate containing 2–3 mg/ml of protein were added to 50 μl of substrate–buffer solution of the following constitutions: 5 mM-4-methylumbelliferyl-α-D-mannopyranoside in 100 mM-phosphate/citrate, pH 4.2; 5 mM-4-methylumbelliferyl-β-D-glucuronide in 200 mM-sodium acetate, pH 4.0; 5 mM-4-methylumbelliferyl-β-D-glucopyranoside in 200 mM-phosphate/citrate, pH 5.4, 8 mg/ml of Triton X-100 and 10 mg/ml of sodium taurocholate; 6 mM-4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside in 40 mM-phosphate/citrate, pH 4.4; 1 mM-4-methylumbelliferyl-α-L-fucopyranoside in 1 mM-sodium citrate, pH 5.0 and 10 mg/ml bovine serum albumin; 0.5 mM-4-methylumbelliferyl-β-D-galactopyranoside in 22 mM-phosphate/citrate, pH 4.35; 20 mM-4-methylumbelliferyl-α-D-galactopyranoside in 100 mM-phosphate/citrate, pH 4.6. The assays ran for 15 and 30 min at 37 °C. Normal lymphoblast data are the results of multiple determinations using three different normal cell lines ranging from 6 to 7 days in age except α-D-neuraminidase where two normal cell lines were used. Normal fibroblast data are the results of multiple determinations using eight different cell lines ranging from 14 to 26 days in age except α-D-neuraminidase where two normal cell lines were used. Abbreviation: N.D., not detected.

	Activities (nmol/h per mg of cell protein) in:			
	Lymphoblasts		Fibroblasts	
Enzyme	Normal	I-cell	Normal	I-cell
N-Acetyl-D-hexosaminidase	540–1326	799	4428–15355	506
β-D-Glucosidase	5–7	13	42–162	145
α-L-Fucosidase	22–56	36	60–307	4
α-D-Glucosidase	6–15	6	51–117	74
β-D-Galactosidase	16–67	25	472–1189	22
α-D-Galactosidase	20–30	25	90–274	7
α-D-Mannosidase	49–146	45	52–207	14
β-D-Glucuronidase	58–80	86	285–394	30
α-D-Neuraminidase	12–30	12	60–86	N.D.

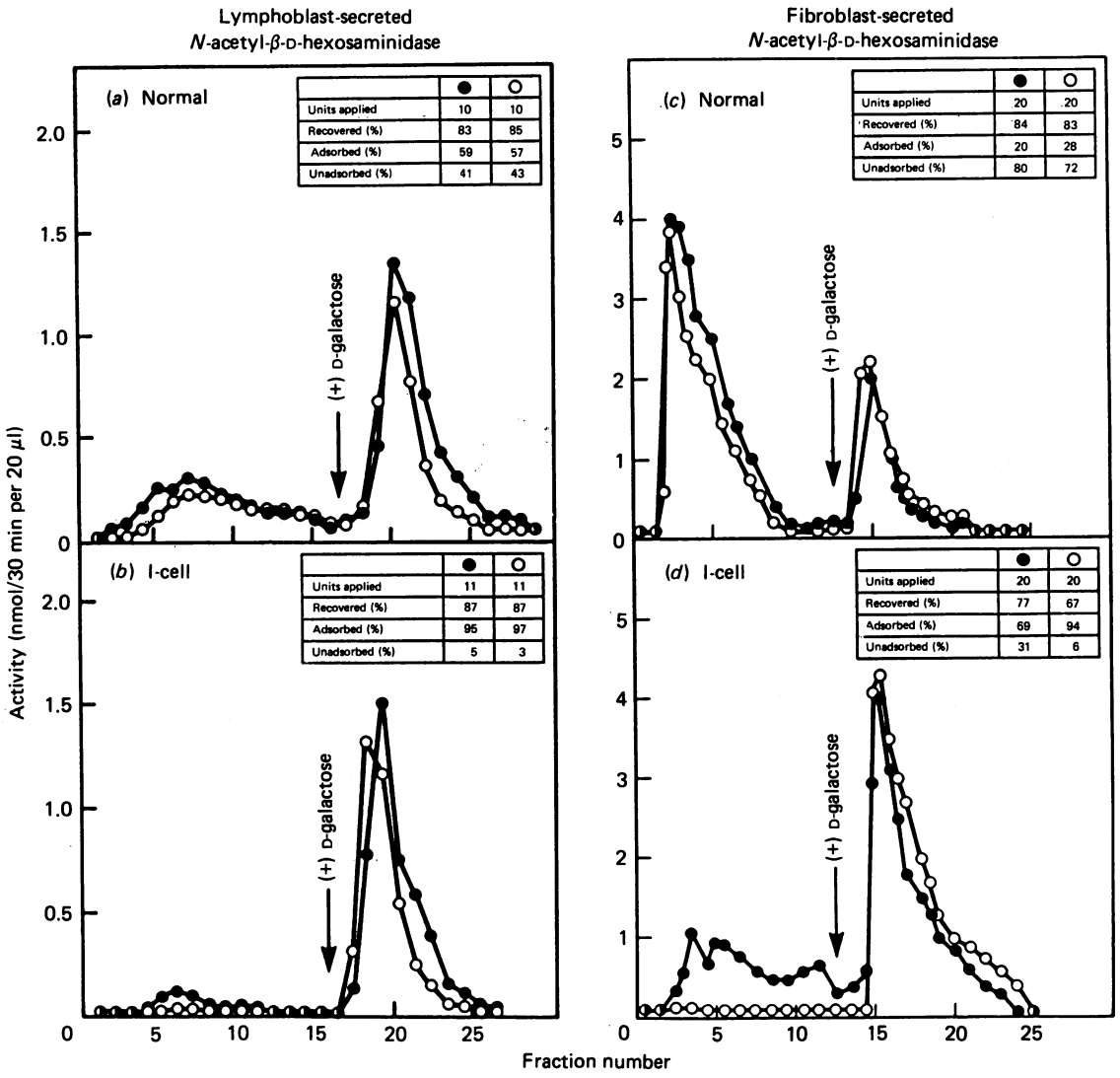

Fig. 2. Binding of secreted *N*-acetyl-β-D-hexosaminidase activity from normal and I-cell cultured lymphoblasts and fibroblasts to RCA

Concentrated portions of extracellular *N*-acetyl-β-D-hexosaminidase from all cell types were separately incubated in the presence (●) and absence (○) of 0.5 units of *Clostridium perfringens* neuraminidase for 5 h at pH 5.0 as described in the Experimental section. These preparations were subsequently chromatographed on the agarose-bound lectin. The percentage adsorbed and percentage unadsorbed are ratios of recovered enzymes.

bound RCA_I, before and after treatment with neuraminidase (Fig. 2). The enzyme secreted from the I-cell lymphoblasts exhibited a significant increase in adsorption to the galactose-specific lectin when compared to the enzyme secreted by normal lymphoblasts (Figs. 2*a* and *b*). This pattern of increased adsorption is similar to that exhibited by secreted *N*-acetyl-β-D-hexosaminidase collected from I-cell and normal fibroblasts (Figs. 2*c* and *d*).

Activity level of *N*-acetylglucosamine phosphotransferase

N-acetylglucosamine phosphotransferase has been shown to be the primary defect responsible for this I-cell disease (Reitman *et al.*, 1981; Hasilik *et al.*, 1981). *N*-Acetylglucosamine phosphotransferase activity levels in normal and I-cell lymphoblasts were measured using α-methyl-D-mannoside as well as high-mannose glycopeptides, and endogenous acceptors. Normal lymphoblast lines demonstrated specific activities of *N*-acetylglucosamine phosphotransferase well within the range of normal fibroblasts (Table 2). The I-cell lymphoblast line showed significant decreases in enzyme activity with all acceptors tested indicating that the transformation of the lymphoblast cells does not affect the level of expression of the *N*-acetylglucosamine phosphotransferase in either the normal or the diseased lymphoblast lines.

Kinetic characterization of normal lymphoblast *N*-acetylglucosamine phosphotransferase

Kinetic properties of *N*-acetylglucosamine phosphotransferase were evaluated in both normal lymphoblasts and fibroblasts. Apparent K_m values for *N*-acetylglucosamine phosphotransferase with respect to α-methyl D-mannoside and high-mannose glycopeptides

Table 2. *N*-acetylglucosamine 1-phosphotransferase activity with three acceptors

Preparation of the high-mannose glycopeptides and the assay procedures employed for *N*-acetylglucosamine 1-phosphotransferase activity with respect to the three substrates are described in the Experimental section. Abbreviation: N.D., not determined.

Cell lines	Acceptor ...	Activity (pmol/h per mg of cell protein)		
		Endogenous	High-mannose glycopeptides	α-Methyl D-mannoside
Lymphoblasts				
(*a*) Normal ($n = 3$)		0.8–4	118–122	113–170
(*b*) I-cell		<0.02	37	14
Fibroblasts				
(*c*) Normal ($N = 3$)		1–5	166–194	98–238
(*d*) I-cell		<0.02	N.D.	4.5

Table 3. Kinetic characteristics of *N*-acetylglucosamine 1-phosphotransferase

The apparent K_m of UDP-GlcNAc was determined using 0.1 M-exogenous α-methyl D-mannoside and varying concentrations of UDP-GlcNAc (10 to 100 μM). All subsequent assays for other kinetic constants contained 100 μM-UDP-GlcNAc and varying concentrations of either α-methyl D-mannoside (0.3–2.5 M), or high-mannose glycopeptides (1–10 mM). Kinetic values were estimated from Lineweaver–Burk plots using linear regression. The results were an average of three or four assays run in triplicate.

Kinetic measurement	Normal lymphoblast	Normal fibroblast
Apparent K_m of UDP-GlcNAc (μM)	167±50	67±33
Apparent K_m of α-methyl D-mannoside (mM)	29±7	30±12
Apparent K_m of high-mannose glycopeptides (mM)	1.5±0.5	1.1±0.2
Apparent K_i (nM) of α-methyl D-mannoside phosphorylation with purified placental hexosaminidase A	45	25
Apparent K_i (nM) of α-methyl D-mannoside phosphorylation with purified placental hexosaminidase B	35	80

revealed no significant variations between cell types (Table 3) suggesting that transformation did not alter the enzyme's active site.

Subcellular fractionation of normal lymphoblast and fibroblast cell lines

The subcellular localization of *N*-acetylglucosamine phosphotransferase in normal fibroblasts and lymphoblasts was determined by fractionation of cell homogenates on a colloidal silica gradient. Golgi, mitochondrial and lysosomal marker enzymes were then assayed (Figs. 3*a*–3*h*). Both lymphoblast cell lines had light and heavy lysosomes in agreement with previous studies demonstrating that primary and secondary lysosomes were present in lymphoblasts (Harms *et al.*, 1981). The *N*-acetylglucosamine phosphotransferase was located at similar densities indicating similar subcellular locations for the enzyme in both cellular systems. These results strongly suggest a similar biological role for the enzyme in both cell types.

Inhibition of α-methyl D-mannoside phosphorylation with highly-purified human lysosomal hydrolases

Previous studies have shown that lysosomal enzymes are endogenous substrates for *N*-acetylglucosamine phosphotransferases in human cultured fibroblasts. We wanted to determine if the *N*-acetylglucosamine phosphotransferase within lymphoblasts also recognizes lysosomal enzymes as substrates. Our results indicate that phosphorylation of the α-methyl D-mannoside acceptor was inhibited by three apparently homogenous lysosomal hydrolases, *N*-acetyl-β-D-hexosaminidase A and B, and α-fucosidase (Fig. 4). The absence of any inhibitor activity using bovine serum albumin and thyroglobulin indicate the specificity of the transferase reaction for lysosomal enzymes. In contrast, glycopeptides obtained from thyroglobulin do interact with the enzyme (Tables 2 and 3) confirming earlier results (Reitman & Kornfeld, 1981; Little *et al.*, 1986) that *N*-acetylglucosamine phosphotransferase possesses a site for recognizing carbohydrate residues on lysosomal and non-lysosomal

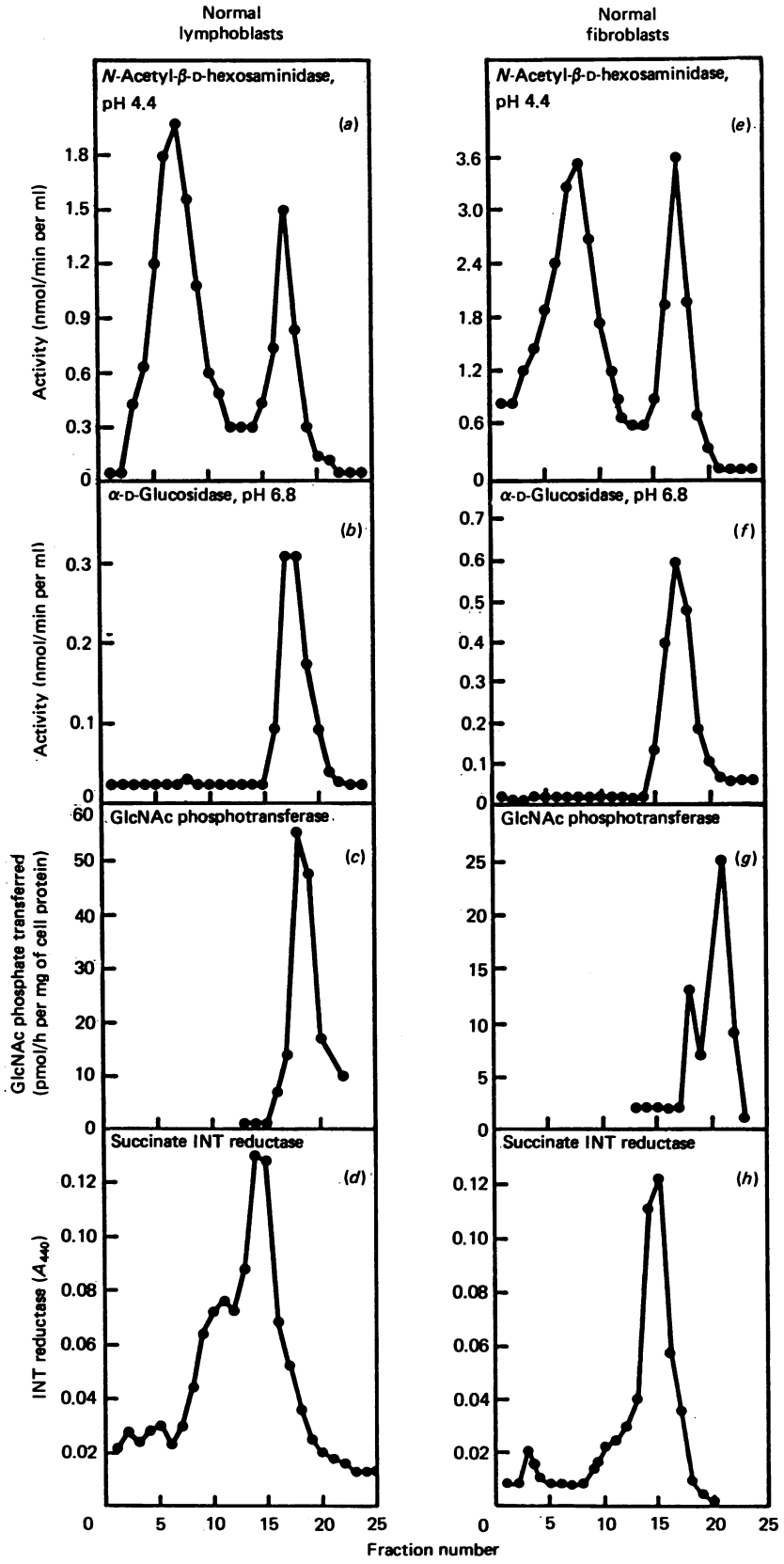

Fig 3. Distribution of marker enzyme activities for lysosomal and non-lysosomal cellular organelles from normal lymphoblasts and normal fibroblasts in a colloidal silica gradient

Cell homogenates were prepared in 250 mM-sucrose by passing cell suspensions through 18-, 20- and 22-gauge syringe needles successively as described in the text. The supernatant fluids (5 ml) were layered on a freshly prepared gradient (25 ml) of polyvinylpyrrolidone and Ludox H5-40 on a 3 ml saturated sucrose cushion. Samples of 1.0 ml were collected after centrifugation for 2 h.

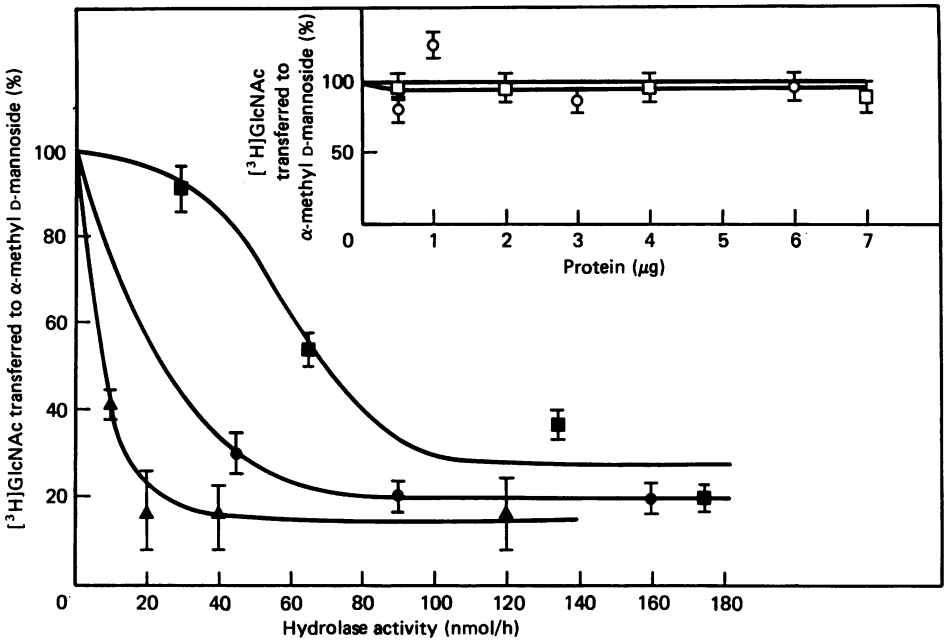

Fig. 4. Inhibition of the *N*-acetylglucosamine 1-phosphotransferase phosphorylation of α-methyl D-mannoside by lysosomal and non-lysosomal proteins

The phosphorylation of the exogenous acceptor α-methyl D-mannoside was measured in the presence of various amounts of human serum albumin (○), bovine thyroglobulin (□), purified human placental *N*-acetyl-β-D-hexosaminidase A (●), purified human placental *N*-acetyl-β-D-hexosaminidase B (■) and purified human liver α-1-fucosidase (▲).

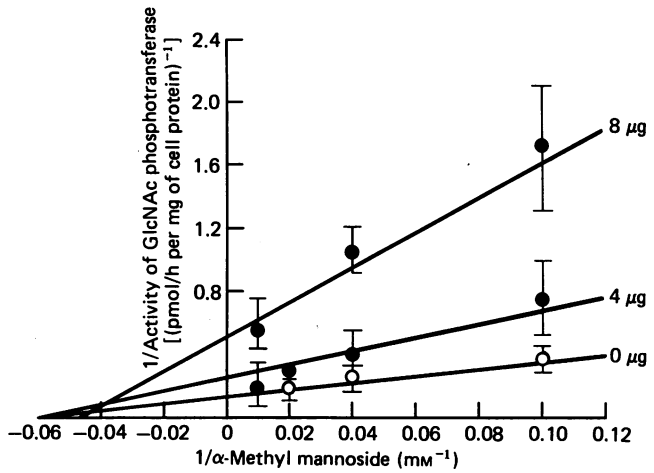

Fig. 5. Effect of *N*-acetyl-β-D-hexosaminidase B on the apparent K_m of phosphorylation of α-methyl D-mannoside by *N*-acetylglucosamine 1-phosphotransferase

Prepared cell homogenates were incubated with varying amounts of α-methyl D-mannoside in the absence (○) and the presence (●) of 4 and 8 μg of purified human placental *N*-acetyl-β-D-hexosaminidase B.

molecules and a second site which recognizes specific protein portion(s) of only lysosomal enzymes. In addition the apparent K_i values were 45 nM and 35 nM for *N*-acetyl-β-hexosaminidases A and B, respectively, using the *N*-acetylglucosamine phosphotransferase from lymphoblast homogenates. This is compared to apparent K_i values 25 nM and 80 nM, obtained for similar experiments using cultured normal fibroblast homogenates as a source of the enzyme (Table 3). These results indicate that *N*-acetylglucosamine phosphotransferase in lymphoblasts, like fibroblasts, is involved in the synthesis of mannose 6-phosphate residues on lysosomal enzymes.

Effect of the addition of lysosomal hydrolases on the phosphorylation of α-methyl D-mannoside

The apparent K_m of α-methyl D-mannoside phosphorylation within normal lymphoblasts was measured in the presence of two different concentrations of *N*-acetyl-β-D-hexosaminidase B (Fig. 5). The apparent K_m was unaltered by the addition of this lysosomal enzyme while the apparent $V_{max.}$ was decreased. This result suggests that *N*-acetyl-β-D-hexosaminidase B is a non-competitive inhibitor of the *N*-acetylglucosamine phosphotransferase from lymphoblasts, analogous to results obtained by us with cultured fibroblasts (Little *et al.*, 1986). These observations suggest that the lysosomal enzyme recognition site on *N*-acetylglucosamine phosphotransferase isolated from either fibroblasts or lymphoblasts is apparently located at a site other than the active site.

DISCUSSION

This study indicates that cultured normal lymphoblasts provide a convenient and economical system for purification and characterization of *N*-acetylglucosamine phosphotransferase. The enzymes obtained from normal lymphoblasts and fibroblasts are similar with respect to their subcellular localization, kinetic parameters, substrate specificities and specific activities. These results indicate that the transformation process does not significantly alter the lymphoblast *N*-acetylglucosamine phosphotransferase. Furthermore, our data indicate that

I-cell lymphoblasts, like their I-cell fibroblast counterparts, are deficient in *N*-acetylglucosamine phosphotransferase activity. This deficiency results in an increased secretion of lysosomal enzymes from both cell types (Fig. 1). Altered binding patterns of these secreted enzymes to RCA_I suggest that the lysosomal enzymes secreted from I-cell (MD) lymphoblasts, like those of I-cell (MD) fibroblasts, now contain additional interacting galactose residues, indicative of an increase in the processing of high-mannose-type oligosaccharide chains to complex-type carbohydrate units. This latter result also suggests that the severe reduction of the *N*-acetylglucosamine phosphotransferase activity leads to an altered segregation pattern of lysosomal enzymes as they are transported through the endoplasmic reticulum–Golgi system. Similar results (as those reported in Tables 1 and 2 and Fig. 1) have recently been found for pseudo-Hurler lymphoblasts (results not shown).

I-cell disease cultured fibroblasts are characterized by a reduction in the intracellular activities of multiple lysosomal enzymes accompanied by an increase of these enzymes in the cell medium (Leroy *et al.*, 1972; Miller *et al.*, 1980; Shows *et al.*, 1982; Mueller *et al.*, 1983). A significant finding in the current study was the observation that although I-cell lymphoblasts secrete increased levels of lysosomal enzymes, they maintain normal or near normal intracellular levels of these enzymes. Analogous results have been reported for leucocytes derived from I-cell patients (Varki *et al.*, 1982) and for lymphoblasts derived from a pseudo-Hurler polydystrophy patient (results not shown). In all cases the intracellular lysosomal enzyme activities were normal in spite of the fact that the *N*-acetylglucosamine phosphotransferase activity was severely depressed or absent. Recent results employing subcellular fractionation on I-cell and normal lymphoblasts revealed normal levels of many lysosmal enzyme activities in I-cell lysosomes (results not shown). The maintenance of normal or near normal levels of lysosomal enzyme activities could have resulted from an overall increased synthesis of lysosomal enzymes leading to increased amounts of these enzymes which are in various stages of secretion. Alternatively these enzymes may be transported to the lysosome by another mechanism used for lysosomal enzyme targeting which has not yet been identified. Support for this latter proposal comes from the existence of the mannose/*N*-acetylglucosamine recognition system for lysosomal enzymes in macrophages (Stahl *et al.*, 1978). In addition, it is evident from previous studies that isolated I-cell lysosomes do contain varying levels of lysosomal enzymes with high-mannose-type chains which do not possess the mannose 6-phosphate marker (Miller *et al.*, 1981). It is also possible that the mannose 6-phosphate receptor is capable of recognizing the high-mannose-type chains on lysosomal enzymes and binds them with a poorer affinity than it does with mannose 6-phosphate (Fischer *et al.* 1982). Our current results do not allow us to differentiate between the aforementioned alternatives.

This study has established a well-characterized system for further purification and characterization of *N*-acetylglucosamine phosphotransferase. Additionally, it provides a practical system to study the question of an alternative recognition system for lysosomal enzyme targeting.

We thank Laura Christian and Dorie Kehew for their expert assistance in preparing the manuscript, Inga Jansen for transforming the lymphocytes and Mary Sundsmo for maintaining all cell cultures. In addition, we acknowledge the advice and comments made by Dr. Barry Kress during the writing of the manuscript. A.L.M. was supported by NIH Grant NS12138.

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Received 25 February 1987/26 May 1987; accepted 30 July 1987