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Heterogeneity of *N*-Acetylglucosamine 1-Phosphotransferase within Mucolipidosis III*

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The primary defect responsible for mucolipidosis III is a deficiency of UDP-*N*-acetylglucosamine:lysosomal enzyme *N*-acetylglucosamine 1-phosphotransferase activity (GlcNAc phosphotransferase). Genetic complementation analysis of cultured fibroblasts derived from 12 patients with mucolipidosis III identified complementation groups A, B, and C (Honey, N. K., Mueller, O. T., Little, L. E., Miller, A. L., and Shows, T. B. (1982) *Proc. Natl. Acad. Sci. U. S. A.* 79, 7420–7424). The GlcNAc phosphotransferase activity present in the cell lines comprising the complementation groups was characterized with respect to endogenous substrates and two exogenous acceptors, α-methyl-D-mannoside and high mannose glycopeptides. All group C cell lines and one group A cell line were found to have normal GlcNAc phosphotransferase activity levels at 37 °C when screened with these exogenous acceptors. The enzyme activity in group A cell lines was within normal range when assayed at 23 °C. Inhibition of the phosphorylation of α-methyl-D-mannoside in the presence of increasing amounts of endogenous substrate *N*-acetyl-β-D-hexosaminidase B was demonstrated in normal cell lines at 23 and 37 °C and in group A cells at 23 °C. However, group C cell lines did not show any inhibition at either temperature. This suggests that the alteration of the GlcNAc phosphotransferase from individuals in group C affects the recognition site for the protein portion of lysosomal enzymes, whereas group A individuals have mutations which result in a temperature-sensitive enzyme.

Mucolipidosis III (ML III,[1] pseudo-Hurler polydystrophy) is an autosomally inherited disorder characterized by an inability to assemble the correct recognition marker on lysosomal enzymes, resulting in an increase of extracellular enzyme activities of these enzymes (1–6). The primary defect responsible for this disorder is the severe reduction or absence of the UDP-GlcNAc:lysosomal enzyme precursor GlcNAc 1-phosphotransferase activity (7, 8). This enzyme transfers the *N*-acetylglucosamine 1-phosphate residues to the high mannose oligosaccharide units of lysosomal enzymes (9, 10). The terminal α-*N*-acetylglucosamine residues are subsequently removed by *N*-acetylglucosamine 1-phosphodiester α-*N*-acetylglucosaminidase (phosphodiester glycosidase) (11, 12) exposing mannose 6-phosphate residues required for recognition of the enzymes by a specific transport receptor for targeting to the lysosome (13).

Although a single enzyme deficiency has been proposed for ML III, clinical and biochemical heterogeneity within the disorder is well established (2, 14–17). This heterogeneity could be due to either allelic variation in the expression of defects in a single gene or mutations in distinct genes. Genetic complementation analysis of 12 ML III cell lines has identified three complementation groups (A, B, and C) (14). Additional results indicate that at least three genes contribute to the activity of the GlcNAc phosphotransferase and that a mutation in any of these genes may result in the ML III phenotype (15, 16).

Our studies demonstrate that the cell lines comprising all three complementation groups have low endogenous GlcNAc phosphotransferase activity when compared to normal cell lines. When the ML III cell lines were screened for enzyme activity using α-methyl-D-mannoside as an exogenous acceptor, one cell line in group A and the six cell lines comprising group C had GlcNAc phosphotransferase activity comparable to normal values. Enzyme derived from these cell lines was characterized with the use of this and other exogenous substrates. Our studies suggest that the mutation(s) responsible for group A results in an altered conformational state of GlcNAc phosphotransferase which is unable to recognize the endogenous acceptor protein at 37 °C but is active and capable of endogenous substrate recognition at 23 °C. The mutation in group C results in an enzyme unable to recognize a unique protein portion of endogenous substrates (lysosomal enzymes) at any of the assay temperatures screened. A preliminary report of this work has been presented (18).

MATERIALS AND METHODS

Materials were obtained from the following sources: Scint A (United Packard); concanavalin A Sepharose, 10 mg/ml (Pharmacia); Bio-Gel P-6 (Bio-Rad); [γ-^{32}P]ATP, 5000 Ci/mmol (Amersham Corp.); UDP-[^{3}H]GlcNAc, 5–25 Ci/mmol (New England Nuclear); and QAE-Sephadex 25-120, α-methyl-D-mannoside, bovine thyroglobulin, Pronase, and other reagents (Sigma). Purified human placental *N*-acetyl-β-D-hexosaminidase B was purified by the method of Hirani *et al.* (19).

[β-^{32}P]UDP-GlcNAc was prepared and identified by the modified method of Owada and Neufeld (20). Briefly, yeast hexokinase (EC 2.7.1.1) was dialyzed against 500 μM $MgCl_2$. Two mCi of [γ-^{32}P]ATP were then incubated with 100 units of dialyzed hexokinase, 5 μM β-mercaptoethanol, 5 μM $MgCl_2$, 2 mg/ml bovine serum albumin at a final volume of 1 ml for 30 min at 37 °C. This mixture was then incubated with 0.5 ml of crude bakers' yeast extract, 0.5 ml of *Candida utilis* yeast extract in 200 μM Tris-HCl, pH 7.5, 10 μM $MgCl_2$, 10 μM β-mercaptoethanol, 12 μM acetyl-CoA, 32 μM UTP, 0.8 μM glucose

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[1] The abbreviation used is: ML III, mucolipidosis III.

1,6-diphosphate, and 12 μM inorganic sodium pyrophosphate. This mixture was incubated at 37 °C for 90 min and was purified by paper chromatography in Leloir Buffers at pH 3.8 and pH 7.0.

High mannose glycopeptides were obtained by incubation of 500 mg of bovine thyroglobulin in 10 ml of 10 mM Tris-HCl, pH 8.0, 2 mM $CaCl_2$, and 5 mg of Pronase at 56 °C for 24–48 h. The reaction mixture was boiled and centrifuged, and the supernatant fluid was desalted on Sephadex G-25. The glycopeptide fractions were pooled and fractionated on a 1.5 × 95-cm Bio-Gel P-6 column in 100 mM ammonium acetate.

Protein was determined by the method of Lowry *et al.* (21) using bovine serum albumin as a standard. Glycopeptide fractions were monitored by the phenol/sulfuric acid method for detection of hexoses (22).

Cell Lines—Fibroblasts were derived from clinically diagnosed ML III subjects. Normal fibroblasts GM3348 and GM3349 and ML III fibroblasts GM2559, GM2425, GM1759, and GM3391 were obtained from the Human Genetic Mutant Cell Respository (Camden, NJ). Cultures were maintained with Coon's F-12 medium containing 10% fetal bovine serum, 2 mM glutamine, and antibiotics (100 μg/ml penicillin, 100 μg/ml streptomycin, and 0.25 units/ml Fungizone).

Preparation of Cell Homogenates—Cultures were used for enzyme assays within 1 day of reaching confluency and harvested by scraping with a rubber policeman in 20 mM Tris-HCl, pH 7.4, 155 mM NaCl. The cells were centrifuged and resuspended in buffer A containing 25 mM Tris-HCl, pH 7.5, 0.3% (v/v) lubrol, 25 mM dithiothreitol and sonicated. The resulting homogenate was then dialyzed against buffer A at 0–4 °C.

Assay of GlcNAc Phosphotransferase—The transfer of GlcNAc 1-phosphate to the 6-hydroxyl of mannose residues was measured in three ways. The first, involving the transfer of [β-^{32}P]UDP-GlcNAc to endogenous glycoprotein acceptors, was performed as previously described (10). The second method, measuring the transfer of UDP-[^{3}H]GlcNAc to exogenous α-methyl-D-mannoside acceptor, was performed by a modified method to be published elsewhere (23). Briefly, 100 mM α-methyl-D-mannoside, 100 μM UDP-GlcNAc, 200,000–300,000 cpm of UDP-[^{3}H]GlcNAc, 13 mM $MgCl_2$, 13 mM $MnCl_2$, 1.6 mM UDP, 1.6 mM ATP, and 250–300 μg of cell homogenate in a final volume of 100 μl were incubated at 37 °C (unless otherwise specified) for 30 min. The reaction was stopped at 0–4 °C, boiled, and then diluted with 1.0 ml of glass-distilled water and centrifuged for 5 min. The supernatant fluid was applied to 0.5 × 2.5-cm columns of QAE-Sephadex 25–120 equilibrated with 2 mM Tris. Columns were washed with 4 ml of 2 mM Tris and eluted with 4 ml of 30 mM NaCl in 2 mM Tris base. And finally, the third method for measuring the transfer of GlcNAc 1-phosphate, involving the transfer of UDP-[^{3}H]GlcNAc to high mannose glycopeptides, was performed by a modified method of Reitman and Kornfeld (24). Briefly, cell homogenates (400–500 μg) were incubated at 37 °C for 1 h with 100 μM UDP-GlcNAc, approximately 300,000 cpm of UDP-[^{3}H]GlcNAc, 13 mM $MgCl_2$, 13 mM $MnCl_2$, 1.6 mM UDP, 1.6 mM ATP, 5–10 μg of glycopeptide in a final volume of 100 μl. The reactions were stopped at 0–4 °C and twice washed with a 2:1 (v/v) chloroform/methanol mixture. The remaining pellet was then twice resuspended in 10 mM sodium phosphate, pH 6.8, 0.5 M NaCl, 0.1 mM $MgCl_2$, 0.1 mM $CaCl_2$ (buffer B) and centrifuged, and the resulting supernatant fluids were applied to 0.5 × 2.5-cm concanavalin A-Sepharose columns. The columns were then rinsed with 100 ml of phosphate-buffered saline and eluted with 4 ml of 60 °C buffer B containing 0.5 M α-methyl-D-mannoside.

Inhibition of α-Methyl-D-mannoside Phosphorylation with N-Acetyl-β-D-hexosaminidase B—Assays were carried out as described above with the exception that 2.5 mM α-methyl-D-mannoside was incubated with varying amounts of purified placental *N*-acetyl-β-D-hexosaminidase B (0–8 μg). This enzyme was purified in our laboratory according to previously published procedures (19, 25).

Determination of Kinetic Constants—Kinetic constants were determined with 100 mM exogenous α-methyl-D-mannoside with varying concentrations of UDP-GlcNAc (10–100 mM). Other assays were performed varying the α-methyl-D-mannoside concentrations (2.5–300 mM) and varying high mannose glycopeptide concentrations (0–200 μg) per 100-μl assay volume at 100 μM UDP-GlcNAc. All kinetic values were estimated from Lineweaver-Burk plots using linear regression. The results were an average of three or four assays.

RESULTS

GlcNAc Phosphotransferase Activity with Three Acceptors—Representative cell lines from all ML III complementation groups and several normal cell lines were assayed for GlcNAc phosphotransferase activity with three different acceptors including high mannose glycopeptides, endogenous protein, and α-methyl-D-mannoside (Table I). All ML III cell lines demonstrated significantly lower endogenous acceptor activity (0.02–0.06 pmol/mg/h) when compared with normal cell lines (0.76–5.37 pmol/mg/h). This is as expected for ML III lines with the primary defect in the GlcNAc phosphotransferase. In contrast is the finding that group C cell lines and a single cell line in group A exhibit normal enzyme activities when assayed using glycopeptides and α-methyl-D-mannoside. These findings have led to the suggestion by ourselves and others (15, 16, 26) that the enzyme present in these cell lines has a functioning active site which is incapable of recognizing the protein portion of endogenous substrates.

Kinetic Properties of UDP-GlcNAc Phosphotransferase—Kinetic properties of the enzyme, with respect to UDP-GlcNAc and two exogenous acceptors α-methyl-D-mannoside and high mannose glycopeptides, were measured in four normal cell lines, MB of group A because of its high activity with α-methyl-D-mannoside, and TA and TR of group C (Table II). The kinetic values, with respect to the acceptors and substrates, were within normal ranges for the GlcNAc phos-

TABLE I

GlcNAc phosphotransferase activity of three acceptors at 37 °C

The assay conditions were as described under "Materials and Methods." Incubation was performed for 30 min.

Sample	α-Methyl-D-mannoside	High mannose glycopeptides	Endogenous
	pmol/mg cell protein/h		
Normal $n = 4$	98–238	166–194	0.76–5.37
Group A			
CW	14	19	<0.02
MB	97	95	<0.02
GM2425	2	18	<0.02
GM1759	0	20	<0.02
GM2559	18	ND[a]	<0.02
Group B			
RW	9	0	<0.02
Group C			
TR	190	130	<0.02
TA	157	180	<0.02
GM3391	200	73	<0.06

[a] ND, not determined.

TABLE II

Kinetic characteristics of GlcNAc phosphotransferase

Apparent K_m and V_{max} values were determined from Lineweaver-Burk plots.

Sample	Apparent K_m UDP-GlcNAc	Apparent K_m α-methyl-D-mannoside	Apparent V_{max}[a]	Apparent K_m high mannose glycopeptides	Apparent V_{max}[a]
	μM	*mM*		*mM*	
Normal $n = 4$	52 ± 20	30 ± 12	108 ± 50	13 ± 6	57 ± 20
Group A MB	21 ± 1	37 ± 12	125 ± 40	17 ± 8	17 ± 8
Group C TR, TA	45 ± 20	35 ± 10	112 ± 22	43 ± 2	25 ± 10

[a] pmol/mg of cell protein/h.

photransferase from all tested ML III cell lines, indicating that there was no apparent change in the catalytic activity of the enzyme.

Enzyme Activity as a Function of Temperature—Cultured normal cells, cell line MB from group A, and cell lines TA and TR from group C were assayed for GlcNAc phosphotransferase at various temperatures for 30 min using α-methyl-D-mannoside as the acceptor (Fig. 1). The enzyme from normal and group C cell lines had a temperature optimum at 37 °C, whereas the cell line MB from group A expressed the highest GlcNAc phosphotransferase activity at 23 °C. This suggested that the mutation responsible for group A results in a temperature-dependent activity change.

Temperature Progressions—Normal, group A, and group C cell lines were assayed at 23, 30 and 37 °C for various times using α-methyl-D-mannoside (Fig. 2). Group C and normal cells expressed higher GlcNAc phosphotransferase activity at 37 than 30 or 23 °C (*Panels I* and *III*). In contrast, the group A cell line expressed highest activity at 23 °C at time points of 30 min or longer, then followed by 30 and 37 °C (*Panel II*). Preincubation of the homogenate at 37 °C before assaying at 23 °C had no effect on the increased activity of group A, suggesting that the temperature-dependent change in activity of the GlcNAc phosphotransferase is reversible (data not shown).

Comparative α-Methyl-D-mannoside Acceptor Activities at 23 and 37 °C—The near normal activities of GlcNAc phosphotransferase at 37 °C from the cell line MB raised the question as to the existence of heterogeneity within group A. Additional cell lines comprising groups A, B, and C and normal controls were subsequently assayed for GlcNAc phosphotransferase activity at 23 and 37 °C (Fig. 3). All group A cell lines, with one exeption (GM1759), had higher enzyme activity at 23 °C. This suggests that the cell lines within group A express different mutations within the same gene, resulting in slightly different biochemical phenotypes.

Comparative Endogenous Acceptor Activity at 23 and 37 °C—Various ML III cell lines in groups A, B, and C and normal cell lines were assayed for GlcNAc phosphotransferase activity at 23 and 37 °C with the endogenous substrate (Fig. 4). All normal cell lines expressed a decreased enzyme activity at 23 °C. Group B and C activities remained unchanged, whereas group A activities were slightly higher at 23 °C.

Effects of Sucrose—Previous experiments have shown that

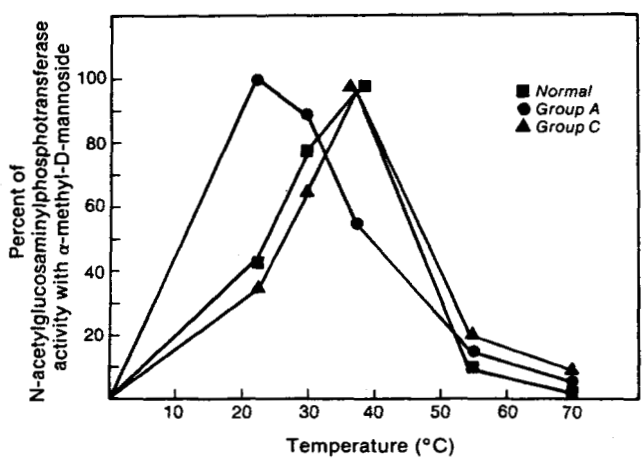

FIG. 1. **Enzyme activity as a function of temperature.** Cells were prepared as described under "Materials and Methods." Cell lines from normal (■——■), complementation group A (●——●), and complementation group C (▲——▲) were incubated at various temperatures with α-methyl-D-mannoside as an acceptor.

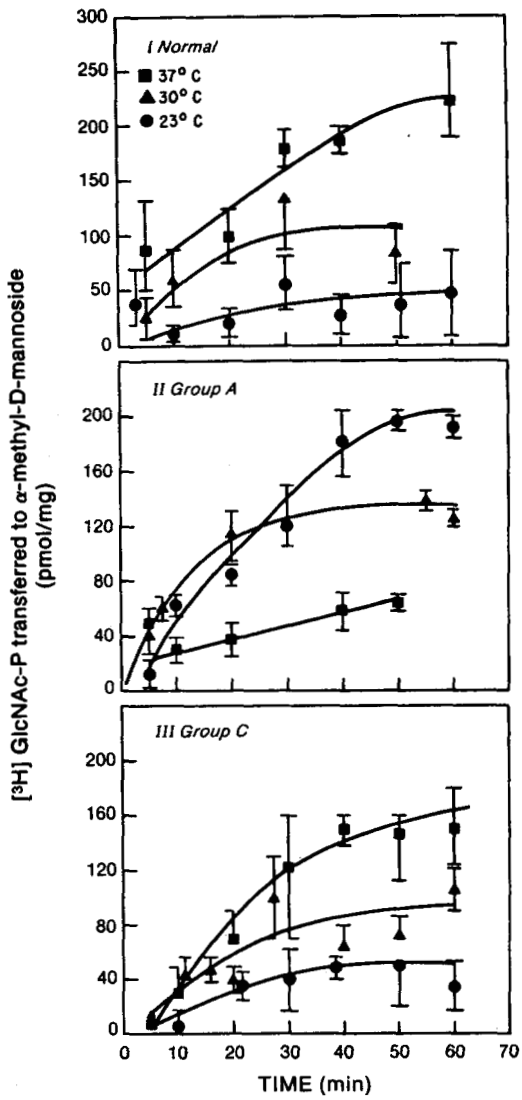

FIG. 2. **Temperature progression curves.** Cell homogenates from normal, group A (MB), and group C (TR) cell lines were incubated at 37 °C (■), 30 °C (▲), and 23 °C (●) for the indicated times with α-methyl-D-mannoside as an acceptor.

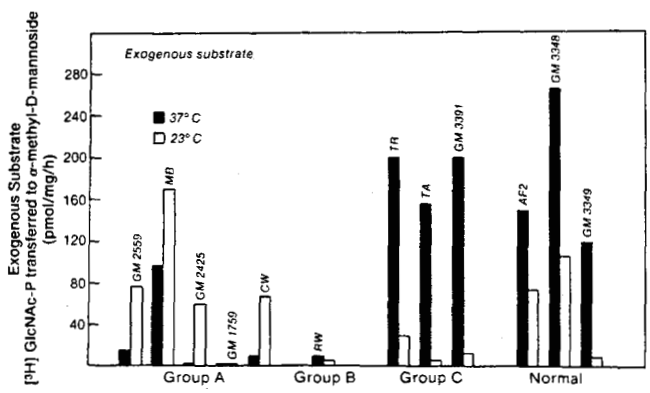

FIG. 3. **Comparative GlcNAc phosphotransferase activity from normal and group A, B, and C cell lines using α-methyl-D-mannoside as the exogenous acceptor.** Prepared homogenates were incubated at 37 °C (■) and 23 °C (□) in the presence of α-methyl-D-mannoside for 30 min.

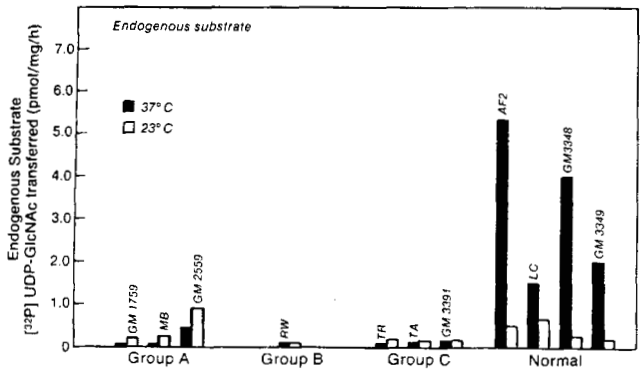

FIG. 4. **Comparative GlcNAc phosphotransferase activity from normal and group A, B, and C cell lines using endogenous acceptors.** Prepared homogenates were incubated for endogenous activity as described under "Materials and Methods" at 37 °C (■) and 23 °C (□) for 60 min.

TABLE III

Effect of sucrose on GlcNAc phosphotransferase activity assayed with the α-methyl-D-mannoside acceptor

Sample	Activity, −sucrose	Activity, +88 mM sucrose
	pmol/mg cell protein/h	
Normal $n = 4$	187 (100)[a]	127 (68)[a]
Group A		
CW	56 (100)	140 (250)
MB	61 (100)	202 (331)
Group C		
TA	158 (100)	168 (106)
TR	225 (100)	43 (19)

[a] Numbers in parentheses indicate per cent activity with or without sucrose.

the addition of sucrose to I-cell disease fibroblasts increased lysosomal enzyme activities, suggesting that this compound may correct the biochemical defect in the disorder (27). Prepared homogenates of normal, group A, and group C cell lines were tested for GlcNAc phosphotransferase activity in the presence of 0.088 M sucrose. Normal- and group C-derived enzyme demonstrated lower enzyme activity when assayed with sucrose present (Table III). The group A cell lines, however, exhibited 200–400% increases in GlcNAc phosphotransferase activity when assayed in the presence of sucrose. This is analogous to the increase in GlcNAc phosphotransferase activity when these cell lines were assayed at 23 °C (Fig. 3). This identical pattern of increased activity suggests that either the addition of sucrose to the assay mixture or lowering the assay temperature may result in a more stable conformation of the altered enzyme.

Inhibition of α-Methyl-D-mannoside Acceptor Activity by Purified Placental N-Acetyl-β-D-hexosaminidase B at 37 and 23 °C—The GlcNAc phosphotransferase activities of those cell lines, comprising group C and MB from group A, have been shown to be normal with respect to exogenous acceptors but inactive toward endogenous substrates. These results are consistent with the proposal that the GlcNAc phosphotransferase in these cell lines is incapable of recognizing the protein portion of the endogenous substrates.

Support for this proposal was obtained by examining the inhibition of the transfer of *N*-acetylglucosamine 1-phosphate to α-methyl-D-mannoside in the presence of increasing amounts of a purified *N*-acetyl-β-D-hexosaminidase B. This transfer, by the GlcNAc phosphotransferase from normal cells, was inhibited up to 80% at 37 °C (Fig. 5, *Panel A*). The enzyme-catalyzed transfer to α-methyl-D-mannoside in the ML III cell lines from groups A and C was unaffected. However, at 23 °C the transfer catalyzed by the GlcNAc phosphotransferase in group A cell lines was now inhibited by hexosaminidase B. This gives further support to our suggestion that at 23 °C the GlcNAc phosphotransferase from group A cell lines exhibits catalytic properties more characteristic of the normal enzyme. Transfer of *N*-acetylglucosamine 1-phosphate to α-methyl-D-mannoside by the enzyme in normal cell lines was 50% inhibitable at 23 °C, whereas the GlcNAc phosphotransferase from group C cell lines remained unaffected (Fig. 5, *Panel B*). These latter results confirm our suggestion that the GlcNAc phosphotransferase from group C cell lines is incapable of recognizing endogenous substrates.

Inhibition of α-Methyl-D-mannoside Phosphorylation by Purified Placental N-Acetyl-β-D-hexosaminidase B—The type of inhibition of phosphate transfer to methyl-D-mannoside by *N*-acetyl-β-D-hexosaminidase was determined in normal cell lines by studying the apparent K_m of α-methyl-D-mannoside in the presence and absence of *N*-acetyl-β-D-hexosaminidase B (Fig. 6). The addition of *N*-acetyl-β-D-hexosaminidase B exhibited no effect on the apparent K_m of the enzyme for α-methyl-D-mannoside but preferentially decreased the V_{max}, indicating a noncompetitive mode of inhibition. This would be consistent with the inhibition of phosphorylation of α-methyl-D-mannoside by *N*-acetyl-β-D-hexosaminidase B occurring at a site other than the active site and correlates with the previous results indicating that the GlcNAc phospho-

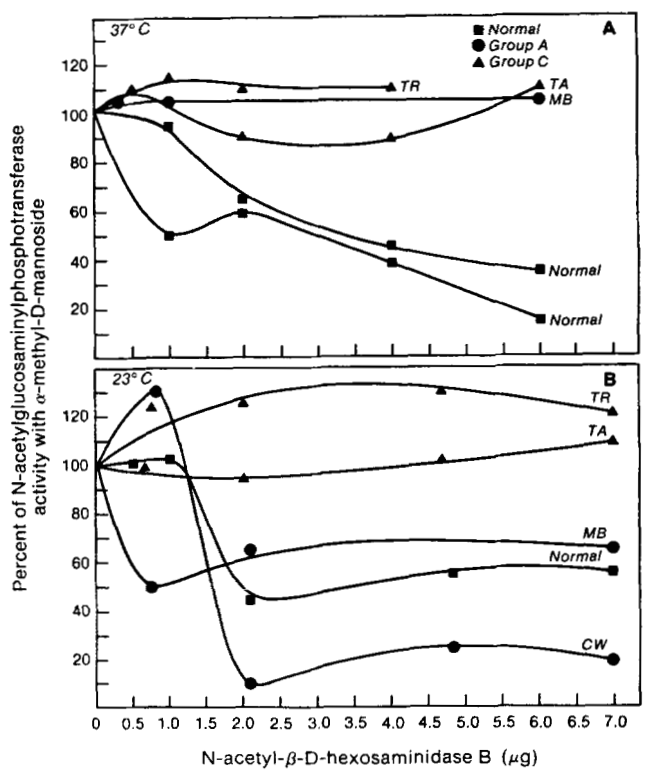

FIG. 5. **Inhibition of the GlcNAc phosphotransferase phosphorylation of α-methyl-D-mannoside by purified human placental *N*-acetyl-β-D-hexosaminidase B at 37 and 23 °C.** Cell homogenates from normal (■) and complementation group A (●), and C (▲) cell lines were prepared as described under "Materials and Methods." *Panel A*, homogenates were incubated at 37 °C in the presence of 2.5 mM α-methyl-D-mannoside and increasing quantities of the lysosomal enzyme (0–7 μg). *Panel B*, homogenates were incubated at 23 °C with other conditions as stated above.

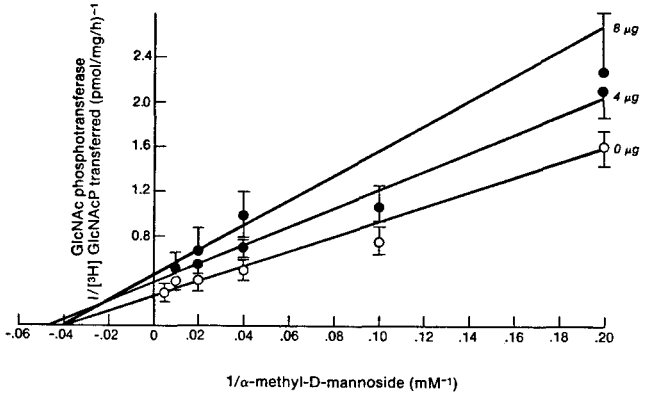

FIG. 6. **Effect of *N*-acetyl-β-D-hexosaminidase B on the apparent K_m for phosphorylation of α-methyl-D-mannoside by GlcNAc phosphotransferase.** Prepared normal cell homogenates were incubated with varying amounts of α-methyl-D-mannoside in the presence and absence of purified human placental *N*-acetyl-β-D-hexosaminidase B (0, 4, and 8 μg) for 30 min.

transferase from group C has a normal active site but is incapable of recognizing endogenous substrates. A mutation altering the lysosomal enzyme recognition site of the GlcNAc phosphotransferase would also explain the inability of *N*-acetyl-β-D-hexosaminidase B to inhibit the phosphorylation of α-methyl-D-mannoside (Fig. 5).

DISCUSSION

Mucolipidosis III is a lysosomal disorder resulting from a decrease in GlcNAc phosphotransferase activity (7, 8). This enzyme catalyzes the transfer of α-*N*-acetylglucosamine 1-phosphate residues to high mannose oligosaccharide unit(s) of lysosomal enzymes. We previously reported three complementation groups within ML III cell lines demonstrating the existence of genetic heterogeneity and the involvement of at least three genes for the production of an enzymatically active GlcNAc phosphotransferase (14, 15). All three groups, A, B, and C, have low endogenous GlcNAc phosphotransferase activity.

Distinguishing characteristics between the cell lines from groups A and C were first seen in temperature studies. Group A cell lines have higher GlcNAc phosphotransferase activities at 23 than 37 °C (Figs. 1 and 3). Furthermore, group A cell lines were found to have increased enzyme activity in the presence of sucrose (Table III). These results are in sharp contrast with normal and group C cell lines which exhibit lower GlcNAc phosphotransferase activity when assayed at 23 °C or in the presence of sucrose (Fig. 2 and Table III). These results are consistent with our proposal that the group A mutation causes a conformational weakness which is less deleterious in the presence of sucrose or at 23 °C (Fig. 7). This is supported by the *N*-acetyl-β-D-hexosaminidase B inhibition of *N*-acetylglucosamine 1-phosphate transfer to α-methyl-D-mannoside at 37 (Fig. 5, *Panel A*) and 23 °C (Fig. 5, *Panel B*). The GlcNAc phosphotransferase in cell lines from group A is insensitive to *N*-acetyl-β-D-hexosaminidase B inhibition at 37 °C; however, at 23 °C the enzyme is now inhibitable by *N*-acetyl-β-D-hexosaminidase B. Furthermore, the return of GlcNAc phosphotransferase activity to normal levels at 23 °C within group A is not uniform (Fig. 3). This is probably a result of different mutations occurring within the same gene, some of which generate more severe conformational changes than others. As a result, the enzyme present in the various group A cell lines may not undergo the proper three-dimensional folding, the proper processing of the proenzyme, or exhibit an increased sensitivity to proteolysis. The present results do not allow us to discern the biochemical basis for the observed heterogeneity in group A.

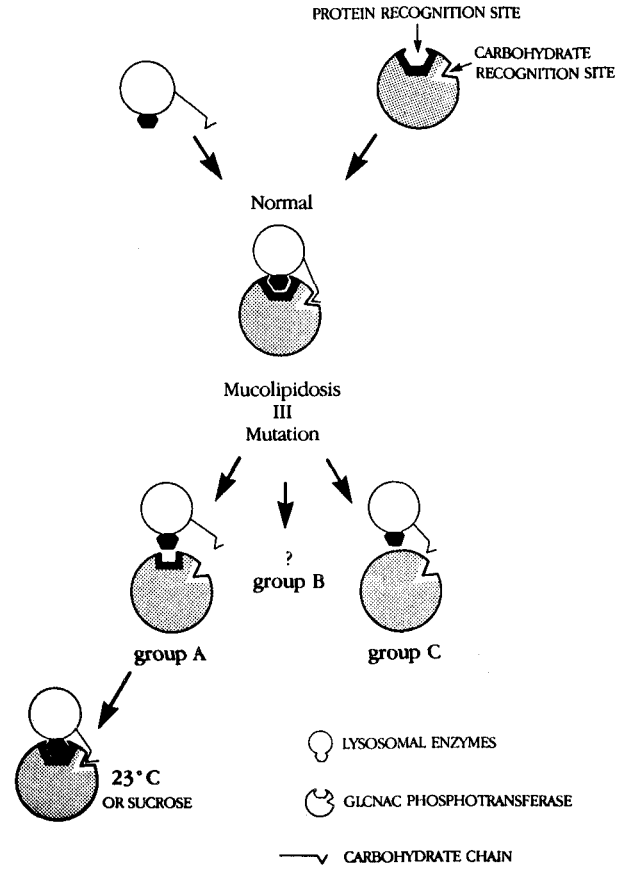

FIG. 7. **Schematic representation of the mutations resulting in ML III complementation groups A and C.**

The gene mutation responsible for complementation group C results in an enzyme which resembles normal GlcNAc phosphotransferase with respect to temperature optima and exogenous substrate activity. However, it lacks the ability to recognize endogenous substrates (lysosomal enzymes) under any tested conditions. In normal cell lines, the transfer of *N*-acetylglucosamine 1-phosphate to α-methyl-D-mannoside is inhibited by the addition of the lysosomal enzyme *N*-acetyl-β-D-hexosaminidase B, whereas in all three ML III cell lines tested, the transfer was almost completely unaffected (Fig. 5, *Panel A*). Furthermore, the inhibition of GlcNAc phosphotransferase from normal cell lines was studied and found to be noncompetitive (Fig. 6). This indicates that inhibition occurs by interaction of the *N*-acetyl-β-D-hexosaminidase B with a site other than the active site of the GlcNAc phosphotransferase. Since our data demonstrate that the active site, which recognizes the carbohydrate moiety of the endogenous substrate, is not the site of inhibition, they strongly suggest that the GlcNAc phosphotransferase contains a site which specifically recognizes a protein portion of its endogenous substrates. This is consistent with the results of Varki *et al.* (26). In addition, we have observed altered cation requirements for the GlcNAc phosphotransferase from group C cell lines when compared to normal cell lines (17). Since divalent cations often function in substrate binding for transferases (28, 29), this provides additional evidence that the affected gene in group C codes for the portion of the enzyme which binds or alters the binding to its endogenous substrate.

This study has resulted in the assignment of functional

roles for two of the gene products required for the synthesis of an enzymatically active GlcNAc phosphotransferase. Fig. 7 represents a schematic model of our current thinking concerning the structure of the GlcNAc phosphotransferase. Based on our study of the complementation mutants, there appears to be at least two binding sites required for an active GlcNAc phosphotransferase. One site recognizes a protein structure within the lysosomal enzymes, while a second site interacts with the carbohydrate portion of these endogenous substrates. The gene product whose mutation results in group C individuals appears to alter only the endogenous substrate recognition by the enzyme. This suggests that these alterations may occur within the actual protein recognition site. The gene product which is altered in group A individuals appears to allow the GlcNAc phosphotransferase to retain its active conformation. The expression of the mutations in most group A cell lines (except MB) interferes with this normal interaction of the lysosomal enzyme at both the protein and carbohydrate recognition sites. This suggests that these mutations occur at a position other than these two sites but which can cause a conformational change at both sites. It seems probable that another gene product would be responsible for active site function. As yet, however, this has not been determined.

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