

# Fibroblasts from Patients with I-Cell Disease and Pseudo-Hurler Polydystrophy Are Deficient in Uridine 5'-Diphosphate-N-Acetylglucosamine: Glycoprotein N-Acetylglucosaminylphosphotransferase Activity

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**ABSTRACT** Newly synthesized acid hydrolases, destined for transport to lysosomes, acquire a phosphomannosyl targeting signal by the transfer of *N*-acetylglucosamine 1-phosphate from uridine 5'-diphosphate (UDP)-*N*-acetylglucosamine to a mannose residue of the acid hydrolase followed by removal of the outer, phosphodiester-linked *N*-acetylglucosamine to expose 6-phosphomannose. This study demonstrates that fibroblasts from patients with the lysosomal enzyme storage diseases, I-cell disease (mucopolipidosis II) and pseudo-Hurler polydystrophy (mucopolipidosis III), are severely deficient in UDP-*N*-acetylglucosamine:glycoprotein *N*-acetylglucosaminylphosphotransferase, the first enzyme of the sequence. The *N*-acetylglucosaminylphosphotransferase activity (assayed using endogenous acceptors) in cultures from six normal subjects ranged from 0.67 to 1.46 pmol *N*-acetylglucosamine-1-phosphate transferred/mg protein per h, whereas five pseudo-Hurler polydystrophy and five I-cell disease cultures transferred <0.02 pmol/mg protein per h. The activity in five other pseudo-Hurler cultures ranged from 0.02 to 0.27 pmol transferred/mg protein per h. The activity of  $\alpha$ -*N*-acetylglucosaminyl phosphodiesterase, the enzyme responsible for phosphomonoester exposure, is normal or elevated in cultured fibroblasts from both I-cell disease and pseudo-Hurler polydystrophy patients. The deficiency of UDP-*N*-acetylglucosamine:glycoprotein *N*-acetylglucosaminylphosphotransferase explains the biochemical abnormalities previously observed in I-cell disease and pseudo-Hurler polydystrophy.

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## INTRODUCTION

I-cell disease and pseudo-Hurler polydystrophy are autosomal recessive lysosomal storage diseases with a Hurler syndromelike presentation. I-cell disease is clinically more severe, often noted shortly after birth, whereas pseudo-Hurler polydystrophy has a later onset and a slower course. Both diseases are characterized by decreased activities of many lysosomal enzymes in connective tissue cells and by markedly elevated enzyme levels in body fluids (1). Cultured skin fibroblasts from these patients secrete acid hydrolases into the medium and cannot take up these enzymes; yet they maintain the ability to endocytose lysosomal enzymes from normal cells. Normal fibroblasts cannot take up I-cell-derived acid hydrolases. These observations led Hickman and Neufeld (2) to propose that the defect in I-cell disease and pseudo-Hurler polydystrophy is the lack of the recognition marker necessary for lysosomal enzyme targeting. It is now established that 6-phosphomannosyl residues on high mannose type oligosaccharide units serve as an essential component of this recognition marker (3-5). Hasilik and Neufeld (6) have demonstrated that I-cell fibroblasts, in contrast to normal fibroblasts, fail to incorporate [<sup>32</sup>P]phosphate into newly synthesized  $\beta$ -hexosaminidase, cathepsin D, and  $\alpha$ -glucosidase. These observations and those by Bach et al. (7) support the hypothesis that the defect in I-cell disease and pseudo-Hurler polydystrophy is in the biosynthesis of the phosphomannosyl signal responsible for the targeting of newly synthesized acid hydrolases to lysosomes.

We have reported that biosynthetic intermediates of the acid hydrolase  $\beta$ -glucuronidase contain phos-

phate groups in diester linkage between mannose residues of the underlying oligosaccharide and outer  $\alpha$ -linked *N*-acetylglucosamine residues (8). A similar observation has been made by Hasilik et al. (9). Because the phosphate is present in mature enzymes as a phosphomonoester moiety (4, 5), we proposed that the phosphorylation of acid hydrolases occurs by transfer of  $\alpha$ -*N*-acetylglucosamine 1-phosphate to mannose residues of high mannose type oligosaccharides followed by removal of the *N*-acetylglucosamine residues to expose 6-phosphomannosyl groups. This, in turn, would allow binding of the acid hydrolases to the specific receptors that mediate the transport of the enzymes to lysosomes (Fig. 1) (8). We have recently developed assays for UDP-*N*-acetylglucosamine:glycoprotein *N*-acetylglucosaminylphosphotransferase (11) and  $\alpha$ -*N*-acetylglucosaminyl phosphodiesterase (12), the enzymes catalyzing the first two reactions in this sequence. We demonstrate that fibroblasts from patients with I-cell disease and pseudo-Hurler polydystrophy are severely deficient in *N*-acetylglucosaminylphosphotransferase activity, thus identifying the biochemical basis for these diseases.

## METHODS

**Materials.** The sources of the materials are described in reference 11. Bovine thyroglobulin was digested with Pronase as described (11) and the high mannose glycopeptide was isolated by affinity chromatography on concanavalin A-Sepharose ( $1 \times 25$  cm). The column was eluted with 170 ml of 20 mM Tris-Cl, pH 8.0/155 mM NaCl, 90 ml of the same buffer containing 10 mM  $\alpha$ -methylglucoside, and 75 ml of the buffer containing 100 mM  $\alpha$ -methyl mannoside at 60°C. The  $\alpha$ -methyl mannoside fraction was subjected to gel filtration on Sephadex G-25 to separate the haptene from the glycopeptides which were then used as described below.

**Cells.** Fibroblast cultures from patients with I-cell disease, pseudo-Hurler polydystrophy, and Sanfilippo B were obtained from the Human Genetic Mutant Cell Repository, Camden, N. J. Dr. William Sly, Washington University, generously provided fibroblasts from T.M. Dr. Eugene Bauer and Dr. Nancy Baenziger, Washington University, supplied the normal human diploid fibroblasts. Cells were grown

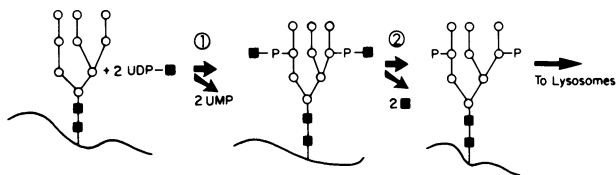


FIGURE 1 Pathway for the phosphorylation and targeting of newly synthesized acid hydrolases to lysosomes. Although two *N*-acetylglucosamine phosphate moieties are shown, one, two, or possibly three of these groups may be attached to a single high mannose unit (9, 10). Enzymes in the pathway are one UDP-*N*-acetylglucosamine: glycoprotein *N*-acetylglucosamine-1-phosphotransferase and two  $\alpha$ -*N*-acetylglucosaminyl phosphodiesterase. ■, *N*-acetylglucosamine; ○, mannose.

at 37°C in a CO<sub>2</sub> incubator in tissue culture dishes (176 cm<sup>2</sup>) using  $\alpha$ -modified Eagle's minimal essential medium (Flow Laboratories, Inc., Rockville, Md.) supplemented with 15% heat-inactivated fetal calf serum (K. C. Biological, Inc. Lenexa, Kan.), penicillin (50 U/ml), and streptomycin (50  $\mu$ g/ml). Cultures were split 1:2 or 1:3 and fed weekly.

**Preparation of cell extracts.** Fibroblasts were mechanically harvested from confluent monolayer cultures 24–72 h after feeding (at least 1 wk after last passage). The cells were washed in 20 mM Tris-Cl, pH 7.45/155 mM NaCl. 2 vol of 50 mM Tris-Cl, pH 7.45, containing 0.75% (wt/vol) Triton X-100 were added to the cell pellet, and the cells were disrupted with 10 passes on a motor-driven Potter-Elvehjem homogenizer. Protein concentrations were determined by a modification of the method of Lowry et al. (13), including 0.5% sodium dodecyl sulfate.

**Enzyme assays.** UDP-*N*-acetylglucosamine:glycoprotein *N*-acetylglucosamine-1-phosphotransferase activity was determined exactly as described (11) except that the incubation time was 30 min, and the concanavalin A-Sepharose was extruded into a scintillation vial and counted after the addition of 1 ml H<sub>2</sub>O and 10 ml scintillation fluid. The assay measures the transfer of *N*-acetylglucosamine 1-<sup>32</sup>P-phosphate from [ $\beta$ -<sup>32</sup>P]UDP-*N*-acetylglucosamine (0.8–1.6  $\times 10^3$  cpm/pmol) to endogenous glycoprotein acceptors that contain high mannose-type oligosaccharides. Each set of assays included a boiled homogenate as control, which gave an assay background of 0–10 cpm over the machine background. One unit of enzyme activity is defined as the transfer of 1 pmol *N*-acetylglucosamine-<sup>32</sup>P]phosphate/h. The limit of sensitivity of the standard assay is 0.02 unit of activity. When structure analysis of the product was performed, [ $\beta$ -<sup>32</sup>P]UDP-*N*-acetyl<sup>3</sup>H]glucosamine was used as the substrate.

$\alpha$ -*N*-Acetylglucosaminyl phosphodiesterase was measured as described (12) except that the reaction mixtures contained 50 mM Tris-Cl, pH 7.45, 0.5% Triton X-100, 5 mM EDTA, and 10 mM *N*-acetylmannosamine. The EDTA and *N*-acetylmannosamine inhibit lysosomal  $\alpha$ -*N*-acetylglucosaminidase (12). One unit of activity is defined as the amount of enzyme catalyzing the cleavage of 1% of the covering *N*-acetylglucosamine from the substrate in 1 h.

$\beta$ -Galactosidase reaction mixtures contained 2.5 mM *p*-nitrophenol- $\beta$ -D-galactoside, 0.1 M Na acetate, pH 4.6, 1% (wt/vol) Triton X-100, and cell homogenate protein (10–250  $\mu$ g) in a final volume of 0.05 ml. Reactions were stopped after 3 h by the addition of 0.05 ml of ice-cold 20% (wt/vol) trichloroacetic acid, incubated five min on ice, and centrifuged at 12,000 *g* for five min. A 0.075-ml aliquot of the supernate was added to 0.3 ml of 0.5 M Na<sub>2</sub>CO<sub>3</sub>, and the A<sub>410</sub> was measured. One unit is defined as 1 nmol *p*-nitrophenol liberated per h, using a molar extinction coefficient of 18,600 liters/mol per cm for *p*-nitrophenol.

## RESULTS

*Normal human diploid fibroblasts have N-acetylglucosaminylphosphotransferase.* Fig. 2 shows that extracts of normal human fibroblasts transfer *N*-acetylglucosamine-<sup>32</sup>P]phosphate from [ $\beta$ -<sup>32</sup>P]UDP-*N*-acetylglucosamine to endogenous acceptors. Under the conditions used, transfer is a linear function of protein concentration (Fig. 2) and time of incubation (data not shown).

In our previous study with Chinese hamster ovary cells, we established that the <sup>32</sup>P-labeled products of incubations using endogenous acceptors were glyco-

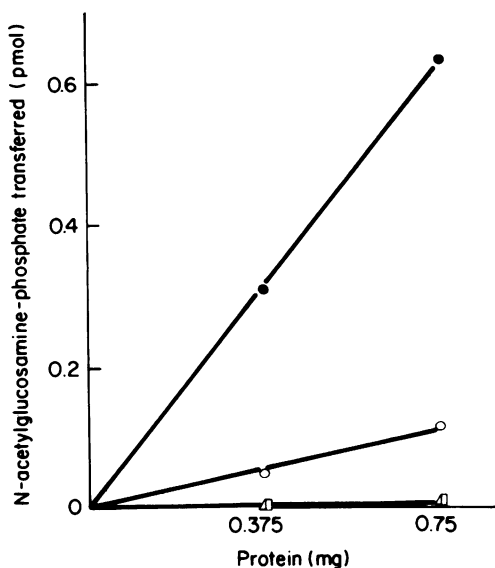


FIGURE 2 Protein concentration dependence of *N*-acetylglucosamine- $^{32}\text{P}$ phosphate transfer to endogenous acceptors. Homogenates from normal (A.G., ●), pseudo-Hurler polydystrophy (GM1494, ○; GM 2065, □), and I-cell disease (GM3066, △) fibroblasts were assayed as described under Methods.

proteins that contained high mannose-type oligosaccharide units with phosphate in diester linkage between a mannose residue of the underlying oligosaccharide and an outer  $\alpha$ -linked *N*-acetylglucosamine residue (11). To prove that a similar product was being formed by normal human fibroblasts, a large scale incubation was performed using  $[\beta\text{-}^{32}\text{P}]\text{UDP-}N\text{-acetyl-}[\text{H}]\text{glucosamine}$  as donor. The double-labeled product had the following properties: (a) it was not extracted with chloroform:methanol (2:1), water or chloroform:methanol:water (10:10:3), but was solubilized by Pronase digestion. These properties are characteristic of a glycoprotein and argue against the product being a lipid-linked oligosaccharide or a free oligosaccharide; (b) the Pronase-solubilized material bound tightly to concanavalin A-Sepharose and required 0.1 M  $\alpha$ -methyl mannoside for elution, indicating that it was a glycopeptide with a high mannose-type oligosaccharide unit. This was confirmed by showing that the glycopeptide was cleaved by endo- $\beta$ -*N*-acetylglucosaminidase H, an enzyme that acts specifically on high mannose-type glycopeptides (14); (c) the endo- $\beta$ -*N*-acetylglucosaminidase H-released oligosaccharides bound to QAE-Sephadex and required NaCl for elution, demonstrating that they had a net negative charge.<sup>1</sup>

<sup>1</sup> 60% of the oligosaccharide material was eluted with 20 mM NaCl which indicates the presence of a single phosphodiester unit, whereas 40% was eluted with 100 mM NaCl which indicates the presence of two phosphate units per oligosaccharide (10).

Mild acid treatment (pH 2, 100°C, 30 min) of the endo- $\beta$ -*N*-acetylglucosaminidase H released material released 92% of the  $^3\text{H}$  as free *N*-acetylglucosamine while the  $^{32}\text{P}$  remained with the oligosaccharide which now bound more tightly to the QAE-Sephadex. This behavior is expected when a phosphodiester moiety is converted to a phosphomonoester; (d) strong acid hydrolysis (1 N HCl, 100°C, 4 h) of the product quantitatively yielded a  $^{32}\text{P}$ -labeled compound which comigrated with mannose 6-phosphate on paper chromatography and which was totally converted to 6-phosphogluconate by a mixture of phosphomannose isomerase, phosphoglucose isomerase, and glucose 6-phosphate dehydrogenase.

These data, taken together, prove that the assay is measuring the transfer of *N*-acetylglucosamine 1-phosphate from UDP-*N*-acetylglucosamine to the 6-hydroxyl of mannose residues of high mannose-type oligosaccharides of endogenous glycoproteins.

*N*-acetylglucosaminylphosphotransferase activity in I-cell and pseudo-Hurler fibroblasts. Because fibroblasts from patients with I-cell disease synthesize lysosomal enzymes that are deficient in phosphate content (6, 7), we assayed fibroblasts from these patients and from patients with pseudo-Hurler polydystrophy for *N*-acetylglucosaminylphosphotransferase activity. The results of these assays are shown in Fig. 2 and Table I. All 5 I-cell and 5 of 10 pseudo-Hurler fibroblast homogenates had no detectable *N*-acetylglucosaminylphosphotransferase activity. The other five pseudo-Hurler patients exhibited 2–28% of the mean normal activity. One patient with mucopolysaccharidosis III B (Sanfilippo B, GM2931) had a normal level of activity (1.13 U/mg protein).

Mixing experiments between normal homogenates and nine of the I-cell or pseudo-Hurler homogenates that were devoid of activity demonstrated the activity expected of appropriately diluted normal homogenate. This suggests that the presence of an inhibitor or the lack of an activator is not the basis for the enzyme deficiency in the abnormal fibroblasts. Similarly, mixing of the GM1494 homogenate with homogenates from nine of the other abnormal fibroblast lines yielded only the activity expected of the GM1494 line. No activity was seen in 12 other mixing experiments between affected fibroblasts.

In our previous study we establish that the high mannose-type glycopeptide of thyroglobulin can act as an exogenous acceptor for the *N*-acetylglucosaminylphosphotransferase (11). Therefore this acceptor was tested with the fibroblast extracts to exclude the possibility that the defect in the abnormal cell lines was in the endogenous acceptor rather than in the *N*-acetylglucosaminylphosphotransferase. As shown in Table II, normal fibroblast extracts are able to utilize the thyroglobulin glycopeptide as an acceptor. The

TABLE I  
*N*-Acetylglucosaminylphosphotransferase, *N*-Acetylglucosaminyl  
 Phosphodiesterase, and  $\beta$ -Galactosidase Activity in Normal,  
 pseudo-Hurler Polydystrophy, and I-Cell Disease  
 Fibroblast Homogenates

Fibroblasts	<i>N</i> -Acetylglucosaminyl phosphotransferase*	<i>N</i> -Acetylglucosaminyl phosphodiesterase†	$\beta$ -Galactosidase
	<i>U/mg protein</i>		
Normal			
A.G.	1.46	2.7	231
F.N.	1.30	5.5	—§
J.R.	0.72	2.5	—
R.B.	0.70	—	403
G.M.	0.96	—	291
R.M.	0.67	—	225
Mean	0.97		
pseudo-Hurler			
GM113	0.16	6.1	157
GM1494	0.27	6.2	126
GM1759	<0.02	9.2	7.4
GM2065	<0.02	6.6	5.7
GM2425	<0.02	8.6	10.1
GM2558	<0.02	5.7	17.8
GM2559	<0.02	6.2	21.2
GM3391	0.05	—	36.8
GM3392	0.04	—	40.7
GM3685	0.02	—	18.6
I-cell			
T.M.	<0.02	13.4	2.5
GM2013	<0.02	8.2	2.7
GM2273	<0.02	8.6	3.8
GM2933	<0.02	8.7	4.5
GM3066	<0.02	8.0	2.4

\* Results of duplicate determinations performed on at least two different sub-cultures, except for R.B., G.M., R.M., GM3391, GM3392, and GM3685, which are duplicate assays on a single culture. Each assay contained 0.75 mg cell homogenate protein.

† Results of duplicate determinations performed on at least two different sub-cultures except for GM113 and GM2933, which are duplicate assays on a single culture.

§ Indicates not assayed.

results obtained using I-cell and pseudo-Hurler extracts were similar to those obtained in the assay using endogenous acceptor; i.e., the pseudo-Hurler lines that had partial activity toward the endogenous acceptors also displayed some activity toward exogenous acceptor, and the abnormal lines that were inactive toward endogenous acceptor were inactive toward exogenous acceptors.

*$\alpha$ -N-acetylglucosaminyl phosphodiesterase assays.* We reported that fibroblasts from a single patient with I-cell disease have normal levels of  $\alpha$ -N-acetylglucosaminyl phosphodiesterase activity (12). To extend this

observation we measured this enzyme activity in the I-cell disease and pseudo-Hurler polydystrophy fibroblast cultures. As shown in Table I, the enzyme activity was normal or elevated in all of the affected fibroblasts.

*$\beta$ -Galactosidase assays.* All of the abnormal fibroblasts were deficient in the acid hydrolase  $\beta$ -galactosidase (Table I). The levels were more severely depressed in the I-cell fibroblasts than in pseudo-Hurler polydystrophy fibroblasts. This result is consistent with a previous report (15) and supports the clinical assignment of the patients to each disease category.

TABLE II  
*N*-Acetylglucosaminylphosphotransferase Activity Using  
 Exogenous Thyroglobulin Glycopeptide Acceptor

Fibroblasts	Type	Exogenous acceptor	Mannose 6-[ <sup>32</sup> P]Phosphate  cpm
G.M.	Normal	-	123
G.M.	Normal	+	623
R.B.	Normal	+	224
GM113	pseudo-Hurler	+	42
GM1494	pseudo-Hurler	+	26
GM2558	pseudo-Hurler	+	0
GM2559	pseudo-Hurler	+	18
T.M.	I-cell	+	0
GM2933	I-cell	+	0

Duplicate reaction mixtures including thyroglobulin glycopeptide (92 nmol hexose) and  $3.1 \times 10^6$  cpm of [ $\beta$ -<sup>32</sup>P]UDP-*N*-acetylglucosamine, otherwise as described (11), were incubated for 60 min and then extracted. The water extracts were chromatographed on Con-A-Sepharose and the bound material eluted with 4 ml of 100 mM  $\alpha$ -methyl mannoside at 60°C. The eluates from the duplicates were pooled, desalted on Sephadex G-25 (0.9  $\times$  24 cm), hydrolyzed (1 N HCl, 100°C, 4 h), and chromatographed on Whatman 3 MM paper in ethyl acetate:pyridine:acetic acid:water (5:5:1:3) for 10 h. An internal [<sup>3</sup>H]mannose 6-phosphate standard was added before acid hydrolysis, providing for correction of mannose 6-phosphate hydrolysis (14–19%) and allowing precise localization of the mannose 6-[<sup>32</sup>P]phosphate in the paper chromatograms. A boiled enzyme blank of 46 cpm has been subtracted from each assay.

## DISCUSSION

These data demonstrate that fibroblasts from patients with I-cell disease and pseudo-Hurler polydystrophy have drastically reduced levels of UDP-*N*-acetylglucosamine:glycoprotein *N*-acetylglucosaminylphosphotransferase activity. This decrease in the phosphotransferase activity explains the lack of phosphorylation of acid hydrolases synthesized by the abnormal fibroblasts (6, 7). We believe that this failure to generate the phosphomannosyl recognition signal prevents the receptor-mediated targeting of the newly synthesized acid hydrolases to lysosomes, and consequently the enzymes can be secreted into the extracellular milieu (2). Other abnormalities of the fibroblasts may be secondary to the generalized acid hydrolase deficiency. For example, the increased sensitivity of these fibroblasts to freezing appears to be due to the deficiency of the lysosomal enzyme neuraminidase (16).

Recently, Ben-Yoseph et al. (17) have reported that I-cell and pseudo-Hurler fibroblasts do not transfer <sup>32</sup>P from [ $\gamma$ -<sup>32</sup>P]ATP to yeast mannan whereas normal fibroblasts do. The relationship of this observation to our results is not clear at this time.

Clinically, pseudo-Hurler polydystrophy is less severe than I-cell disease. The most probable biochemical explanation for this observation is that pseudo-Hurler patients have slightly higher levels of *N*-acetylglucosaminylphosphotransferase activity than do I-cell patients. This finding is suggested by the data in Table I, but a larger series and/or a more sensitive assay is needed to confirm this hypothesis.

*Note added in proof.* After this paper was accepted for publication, Hasilik et al. reported that two patients with I-cell disease are deficient in UDP-*N*-acetylglucosamine: lysosomal enzyme *N*-acetylglucosamine 1-phosphate transferase (1981. *Biochem. Biophys. Res. Commun.* **98**: 761–767).

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