Altered molecular size of *N*-acetylglucosamine 1-phosphotransferase in I-cell disease and pseudo-Hurler polydystrophy

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The size of the mutant *N*-acetylglucosamine 1-phosphotransferase in Golgi membranes from fibroblasts of patients with I-cell disease and classical pseudo-Hurler polydystrophy, which comprised one complementation group characterized by deficiency towards both artificial and natural acceptor substrates, was significantly smaller than the normal enzyme, 151–174 kDa compared with 225–278 kDa. The size of the mutant enzyme from cell lines of patients with variant forms of pseudo-Hurler polydystrophy, which comprised another complementation group characterized by normal activity towards mono- and oligo-saccharide substrates, was significantly larger than the normal enzyme, ranging from 321 to 356 kDa in two families and from 528 to 547 kDa in a third family. These findings suggest that the mutations in I-cell disease and classical pseudo-Hurler polydystrophy result in a missing enzyme component, which renders the enzyme catalytically inefficient toward any type of acceptor substrate. In contrast, the mutations in the variant forms of pseudo-Hurler polydystrophy produce a larger enzyme molecule which is active toward small substrates but is incapable of binding natural lysosomal glycoprotein substrates.

INTRODUCTION

I-cell disease and pseudo-Hurler polydystrophy (mucopolysidoses II and III) comprise a heterogeneous group of autosomal recessive disorders of lysosomal protein processing and targeting, caused by deficiency of the enzyme UDP-*N*-acetylglucosamine:lysosomal protein precursor *N*-acetylglucosamine 1-phosphotransferase (GlcNAc-PO₄ transferase; EC 2.7.8.17) (Hasilik *et al.*, 1981; Reitman *et al.*, 1981). When an absence or a deficiency of this enzyme activity occurs, high-mannose-type oligosaccharides of lysosomal protein precursors are not phosphorylated. This results in their conversion into complex or hybrid-type oligosaccharides, and the lysosomal protein precursors are secreted from the cells rather than targeted into lysosomes (Neufeld & McKusick, 1983).

The heterogeneity within I-cell disease and pseudo-Hurler polydystrophy has previously been demonstrated biochemically by different patterns of intracellular and extracellular lysosomal enzyme activities of cultured skin fibroblasts (Honey *et al.*, 1981), as well as by qualitative and quantitative differences in the electrophoretic mobilities (Honey *et al.*, 1981) and lectin-binding properties (Miller *et al.*, 1981) of these altered lysosomal enzymes, and in their responsiveness to sucrose loading (Okada *et al.*, 1983). Varki *et al.* (1981), using α -methyl mannoside as an acceptor in the assay of *N*-acetylglucosamine 1-phosphotransferase, have shown that fibroblasts from patients with the classical form of pseudo-Hurler

polydystrophy had higher residual activities than those from patients with I-cell disease. They have also identified a variant form of pseudo-Hurler polydystrophy in which the mutant enzyme demonstrated deficiency only toward lysosomal enzyme acceptors while retaining normal activity toward α -methyl mannoside. The apparent K_m values of this mutant enzyme toward α -methyl mannoside, high-mannose-type oligosaccharides and UDP-GlcNAc were normal. Complementation studies by Shows *et al.* (1982), Honey *et al.* (1982) and Mueller *et al.* (1983) have indicated the presence of two subgroups in I-cell disease and three distinct complementation groups among patients with pseudo-Hurler polydystrophy. The first complementation group (A) was identical with I-cell disease; the second group (B) consisted of a single cell line; and the third (C) included the variant form described by Varki *et al.* (1981). The mutant enzymes of the two major complementation groups (A and C) differed from each other by the optimum temperature of the enzymic activity (Little *et al.*, 1986) and by the susceptibility of the phosphorylation of mono- and oligo-saccharide acceptors to inhibition by lysosomal enzyme preparations (Little *et al.*, 1986; Ben-Yoseph *et al.*, 1986a).

In the present study we have determined the molecular size of the mutant GlcNAcPO₄ transferase *in vivo*. The results indicate that the mutant enzyme in the first complementation group is smaller than the normal enzyme and those of cell lines from the second complementation group are larger than the normal enzyme.

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MATERIALS AND METHODS

Materials

[γ - 32 P]ATP, 4800 Ci/mmol, and UDP-[1- 14 C]GlcNAc, 35 mCi/mmol, were from ICN Radiochemicals. Concanavalin A-Sepharose, QAE-Sephadex, Q-25-120, Pronase, yeast UDP-glucose pyrophosphorylase, yeast inorganic pyrophosphatase, ATP, UTP, ADP, CDP-choline, glucosamine, glucose 1,6-bisphosphate, α -methyl mannoside, iodoacetamide, leupeptin and acetic anhydride were from Sigma. Yeast hexokinase and rabbit muscle phosphoglucomutase were from Boehringer-Mannheim. [β - 32 P]UDP-GlcNAc was synthesized according to the method of Lang & Kornfeld (1984). Hexosaminidases A and B were purified from human placenta by affinity chromatography as previously described (Geiger *et al.*, 1974), and monitored with 4-methylumbelliferyl derivatives of β -*N*-acetylglucosamine 6-sulphate (Ben-Yoseph *et al.*, 1985) and β -*N*-acetylglucosamine (Sigma), respectively.

Cell lines

Cultured skin fibroblasts from patients with I-cell disease and pseudo-Hurler polydystrophy, and from apparently normal subjects, were obtained, maintained and harvested as previously described (Ben-Yoseph *et al.*, 1983). Cell lines with the prefix GM were obtained from the NIGMS Human Genetic Mutant Cell Repository (Camden, NJ, U.S.A.).

Preparation of Golgi-rich membranes

Cell pellets were homogenized in 4 vol. of 0.25 M-sucrose containing 10 mM-Tris/HCl buffer, pH 7.4, 1 mM-iodoacetamide and 1 mM-leupeptin, by eight strokes in a Con-Torque homogenizer. Golgi-rich membranes were isolated by the method of Sandberg *et al.* (1980) using a sucrose density gradient. The Golgi membrane fraction obtained from the gradient was diluted to 0.25 M-sucrose, and the membranes were pelleted by centrifugation at 105000 *g* for 90 min and washed twice in 0.25 M-sucrose/0.25 M-NaCl. Protein was determined by the dye-binding method of Bradford (1976) using bovine γ -globulin as standard.

Radiation inactivation

Golgi membrane preparations were suspended in water by homogenization, and portions containing 1 mg of protein/ml were freeze-dried in 1.5 ml Eppendorf micro test tubes. The test tubes containing the freeze-dried material were flushed with N₂, capped, and irradiated at 38 °C in a 60 Co irradiator at a dose rate of about 2 Mrad/h (Gammacell model 220, Atomic Energy of Canada) as described previously (Beauregard & Potier, 1982; Beauregard *et al.*, 1983). The freeze-dried samples were kept on solid CO₂ or at -20 °C except for the time of the radiation inactivation experiments. After exposure to radiation for various periods of time, the samples were resuspended in 1% (v/v) Triton X-100 and assayed for enzyme activities. Non-irradiated samples retained more than 90% of their initial enzyme activities. The relative molecular size was calculated according to the equation:

$$\log(\text{Molecular size}) = 5.89 - \log D_{37,t} - 0.0028t(^{\circ}\text{C})$$

which is valid at any temperature *t* between -200 and +200 °C (Beauregard & Potier, 1984). $D_{37,t}$ is the radiation dose (in Mrad) that inactivates the enzyme to

37% of its initial activity. Control enzyme preparations of measured $D_{37,t}$ values and known molecular sizes were used for calibration of the 60 Co source (Beauregard & Potier, 1982).

GlcNAc-PO₄ transferase assays

Whole-cell extracts and Golgi-rich membrane preparations were adjusted to 1% (v/v) Triton X-100. The samples were sonicated by three pulses, 5 s each, with an Ultrasonics cell disruptor with a microtip at setting 7. The assay of GlcNAc-PO₄ transferase activity toward lysosomal enzyme acceptors was based on the methods of Reitman & Kornfeld (1981) and Waheed *et al.* (1982). The reaction mixture contained 5 nmol of [β - 32 P]UDP-GlcNAc (500–2000 c.p.m./pmol), 20–100 μ g of lysosomal enzyme preparation and 50–150 μ g of Golgi membrane protein, or 200–600 μ g of whole cell protein, in a final volume of 50 μ l of 35 mM-sodium phosphate buffer, pH 6.8, containing 1% (v/v) Triton X-100, 10 mM-MgCl₂, 5 mM-dimercaptopropanol, 10 mM-CDP-choline, 2 mM-ADP and 0.125 mM-leupeptin. Following incubation for 60 min at 37 °C, the reaction was terminated, the glycoprotein fraction was digested by Pronase and the phosphorylated high-mannose glycopeptides were isolated by affinity chromatography on immobilized concanavalin A and quantified as described (Reitman & Kornfeld, 1981). Activity toward α -methyl mannoside acceptor was assayed by a modification of previously described methods (Reitman & Kornfeld, 1981; Waheed *et al.*, 1982; Ben-Yoseph *et al.*, 1984, 1986b). The composition of the reaction mixture was as described above, except for UDP-[1- 14 C]GlcNAc (35 mCi/mmol) replacing [β - 32 P]UDP-GlcNAc, and 5 μ mol of α -methyl mannoside replacing lysosomal enzyme preparation. Following incubation for 30 or 60 min at 37 °C, the reaction was terminated, the phosphodiester product of the transfer reaction ([1- 14 C]GlcNAc- α -1-phospho-6- α -1-methylmannose) was separated from the breakdown product ([1- 14 C]GlcNAc) on a QAE-Sephadex column (0.5 cm $\times$ 5.0 cm) and quantified as described (Ben-Yoseph *et al.*, 1984). Specific activities of GlcNAc-PO₄ transferase were expressed as pmol of GlcNAc-1-phosphate transferred/h per mg of cell protein.

RESULTS

Residual GlcNAc-PO₄ transferase activity in mutant cell lines

Cultured skin fibroblasts from the various patients with I-cell disease and pseudo-Hurler polydystrophy, and from normal controls, were assayed for GlcNAc-PO₄ transferase activity toward natural and artificial acceptor substrates (Table 1). Endogenous glycoproteins and exogenously-added hexosaminidase preparations served as natural acceptors, and the monosaccharide α -methyl mannoside served as an artificial acceptor. All the mutant cell lines examined demonstrated severe deficiency of GlcNAc-PO₄ transferase activity toward natural acceptor substrates. The low levels of these residual activities were found to be insufficient for assessment of the molecular size of the mutant enzyme by the radiation-inactivation method. Higher residual activities were determined in cell lines from patients with I-cell disease and classical pseudo-Hurler polydystrophy

Table 1. GlcNAc-PO₄ transferase activities in normal and mutant cell lines

The activities toward natural (endogenous glycoprotein and exogenously-added hexosaminidase A preparation) and artificial (α -methyl mannoside) acceptor substrates were assayed in whole sonicates of cultured skin fibroblasts or in Golgi-rich membrane fractions as described in the Materials and methods section. Abbreviation: ND, not determined.

Cell type	Cell line	Activities (pmol/h per mg)			
		Endogenous (whole cell)	Exogenous (Golgi)	α -Methyl mannoside	
				Whole cell	Golgi
Normal	<i>n</i> = 5	1.05–4.88	12.3–32.6	128–332	497–1520
Complementation group I					
I-cell disease	1060	< 0.02	0.36	3.14	13.80
	1225	< 0.02	0.19	0.65	1.83
	1407	< 0.02	0.80	4.32	19.00
	1519	< 0.02	0.11	1.07	5.02
	2067	< 0.02	0.42	2.99	14.10
	GM3066	< 0.02	0.27	0.88	2.20
Pseudo-Hurler	1919	ND	0.54	ND	44.30
	GM1494	0.04	3.81	32.70	112.00
	GM2559	< 0.02	0.78	10.90	58.50
Complementation group II					
Variant	1902	0.05	1.09	254	1077
Pseudo-Hurler I	2070	0.07	1.49	285	980
	2502	< 0.02	0.92	233	1106
Variant	GM3391	0.08	0.84	209	738
Pseudo-Hurler II	GM3392	0.05	1.39	247	1215

(complementation group I) when α -methyl mannoside was employed as an acceptor. On the other hand, fibroblasts from patients with variant forms of pseudo-Hurler polydystrophy (complementation group II) had normal activity toward this monosaccharide substrate. Among cell lines from patients with the classical forms, the activity toward α -methyl mannoside correlated with the severity of the disease as indicated by higher residual activity in pseudo-Hurler polydystrophy lines as compared with I-cell disease lines. Except for two I-cell-disease lines (1225 and GM3066) that had very diminished activity toward the monosaccharide acceptor, the activity levels in all other mutant cell lines were suitable for molecular size estimation and other characterization studies.

Radiation inactivation of mutant and normal GlcNAc-PO₄ transferase

A linear inverse relationship between the log of the remaining GlcNAc-PO₄ transferase activity toward α -methyl mannoside and the radiation dose was observed in membrane preparations from all the cell lines examined, indicating a single-hit single-target response. The results for representative mutant cell lines and a normal control are shown in Fig. 1 as slopes of linear regression plots. The rate of inactivation of this activity in enzyme preparation from I-cell-disease fibroblasts was significantly lower ($P < 0.01$) than that of the normal but essentially indistinguishable from that of classical pseudo-Hurler polydystrophy fibroblasts. This indicated a smaller target size for the mutant GlcNAc-PO₄ transferase in the classical forms of both disorders. In contrast, molecular sizes larger than the normal were found for the mutant enzyme in cell lines from patients with the variant forms of pseudo-Hurler polydystrophy. This was

indicated by rates of inactivation which were significantly higher ($P < 0.01$) than that of the normal enzyme activity. The extent of the increased decay of enzyme activity as a function of the radiation dose was about 1.4 times the normal in cell lines from patients of two unrelated families (variant pseudo-Hurler polydystrophy I), and about 2.1 times the normal in cell lines from patients of a third family (variant pseudo-Hurler polydystrophy II). This suggests a heterogeneity within the variants' complementation group.

$D_{37,38}$ values (the radiation dose that inactivates the enzyme to 37% of its initial activity at 38 °C) and the molecular sizes of the normal and mutant GlcNAc-PO₄ transferases (Table 2) were obtained from the rate of inactivation of α -methyl mannoside phosphorylation, and thus reflect the minimal size of enzymic protein that is necessary for carrying out this reaction or destructive energy transfer between associated subunits (Beauregard *et al.*, 1987). The molecular size of the mutant enzyme in I-cell disease and classical pseudo-Hurler-polydystrophy cell lines was about two-thirds of the normal size, and those of pseudo-Hurler polydystrophy variants I and II were about 1.4 and 2.1 times the normal, respectively. These findings suggest that an enzyme component that is normally involved in the catalytic function may be altered or missing in the classical forms of these disorders, and that the mutations responsible for the variant forms probably cause aggregation of the enzyme which does not affect its catalytic activity but interferes with its binding to the natural lysosomal glycoprotein substrates.

DISCUSSION

Our previous results on the large molecular size of normal GlcNAc-PO₄ transferase from human fibroblasts

Fig. 1. Radiation inactivation of normal and mutant GlcNAc-PO₄ transferase

Freeze-dried Golgi-rich membrane preparations from cultured skin fibroblasts of five normal controls (○) and three patients with I-cell disease (▲), two with classical pseudo-Hurler polydystrophy (▼), three with variant pseudo-Hurler polydystrophy I (□), and two with variant pseudo-Hurler polydystrophy II (■), were irradiated and the remaining activity toward α -methyl mannoside acceptor was determined as described in the Materials and methods section. The results represent the means $\pm$ s.d. of the log of the percentage remaining activities for three experiments. Curve fitting was achieved by linear regression.

Table 2. Molecular size of normal and mutant GlcNAc-PO₄ transferase

Determined *in vivo* in fibroblast Golgi-rich membranes by radiation inactivation as described in the Materials and methods section. D_{37} is the radiation dose in Mrad that inactivates the enzyme to 37% of its initial activity.

Cell type	Number	D_{37} (Mrad)	Molecular size (kDa)
Normal control	5	2.30–2.84	225–278
I-cell disease	3	3.79–4.24	151–169
Classical pseudo-Hurler polydystrophy	3	3.68–4.05	158–174
Variant I pseudo-Hurler polydystrophy	3	1.80–1.99	321–356
Variant II pseudo-Hurler polydystrophy	2	1.17, 1.21	528, 547

and placenta (> 200 kDa) have suggested a multimeric structure for this enzyme (Ben-Yoseph *et al.*, 1986b). If it is a hetero-oligomer, the distinct complementation groups among patients with I-cell disease and pseudo-Hurler polydystrophy would be consistent with non-allelic mutations affecting different subunits of the same enzyme. However, one cannot rule out the possible involvement of mutations at loci other than those encoding for the enzyme or its subunits. Such mutations may affect an activator protein, a stabilizer protein, or other cofactors which are necessary for the proper processing and functioning of GlcNAc-PO₄ transferase. The results for the molecular size of the mutant enzyme in the various cell lines examined (Fig. 1 and Table 2) are consistent with other characteristics which distinguish between the cell lines from the two major complementation groups. Although an abnormal molecular size of GlcNAc-PO₄ transferase was found in all cases, the values obtained for I-cell disease and classical pseudo-

Hurler-polydystrophy fibroblasts were essentially identical to each other but significantly smaller than that of the normal enzyme, whereas those of variant cell lines were significantly larger than the normal. The difference in the extent of the increased molecular size among cell lines of the latter group is probably the result of allelic mutations occurring within the locus responsible for this complementation group. Molecular size changes were also observed in Gaucher disease by using the same technique (Maret *et al.*, 1983; Choy *et al.*, 1986).

The observed molecular size alterations suggest that the mutations responsible for the classical forms cause a reduction in the size of GlcNAc-PO₄ transferase, which may be due to a missing enzyme component, improper processing or increased susceptibility to proteolysis. These mutations render the enzyme catalytically inefficient toward both natural and artificial acceptor substrates and thus may be localized to a catalytic subunit. In contrast, the mutations responsible for the variant forms probably cause aggregation of the enzyme, which does not affect its catalytic activity toward artificial substrates. The mutant enzyme in this group of patients is incapable of binding natural acceptor substrate, and these mutations are probably occurring at a gene that encodes a lysosomal protein-recognizing subunit.

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