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Identification of a Rat Liver α-N-Acetylglucosaminyl Phosphodiesterase Capable of Removing "Blocking" α-N-Acetylglucosamine Residues from Phosphorylated High Mannose Oligosaccharides of Lysosomal Enzymes*

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We recently reported that the high mannose-type oligosaccharides of the biosynthetic intermediates of β-glucuronidase contain phosphate groups in diester linkage between mannose residues and outer α-linked N-acetylglucosamine residues (Tabas, I., and Kornfeld, S. (1980) *J. Biol. Chem.* 255, 6633-6639). We now describe an α-N-acetylglucosaminyl phosphodiesterase from rat liver that is capable of removing the N-acetylglucosamine residues, leaving phosphomonoester groups on the high mannose oligosaccharide units. This activity is greatly enriched in smooth membrane preparations. It can be distinguished from a lysosomal α-N-acetylglucosaminidase by several criteria, including subcellular localization and differential inhibition by amino sugars. In addition, human fibroblasts with mutations which lead to a deficiency of the lysosomal activity have normal levels of the α-N-acetylglucosaminyl phosphodiesterase.

This enzyme may be involved in the "unmasking" of the phosphomannosyl recognition marker on newly synthesized acid hydrolases which could then direct the targeting of these enzymes to lysosomes.

There is now considerable evidence which indicates that phosphomannosyl residues present on high mannose-type oligosaccharide units of acid hydrolases serve as the recognition marker that mediates enzyme uptake by various cell types and targeting to lysosomes (1-10). The finding that alkaline phosphatase treatment of secreted acid hydrolases abolishes their high affinity cellular uptake indicates that the phosphate is present as a phosphomonoester group in these molecules (1, 2, 4, 10). However, when we analyzed the biosynthetic intermediates of the acid hydrolase β-glucuronidase, we found that they primarily contain phosphate groups in diester linkage between mannose residues of the underlying oligosaccharide and outer α-linked N-acetylglucosamine residues (11). Only about 7% of the labeled phosphorylated oligosaccharides contained phosphomonoester groups following a 3-h labeling period with [2-^{3}H]mannose. Similarly, approximately 75% of the phosphorylated oligosaccharides derived from the total glycopeptide material of mouse lymphoma cells labeled for 3 h with [2-^{3}H]mannose contained phosphodiester groups and only 25% contained phosphomonoester groups.[1] In addition, the underlying high mannose oligosaccharides of the molecules with phosphomonoester moieties contained fewer mannose residues consistent with their being the "oldest" molecules which had undergone oligosaccharide processing.[1] For these various reasons, it appears likely that during the maturation of the acid hydrolases the covering N-acetylglucosamine residues are removed to unmask the targeting function of the phosphate residues. If this is the case, one must postulate the presence of an enzyme capable of cleaving the α-linked N-acetylglucosamine residues from these molecules. In this paper, we demonstrate that rat liver smooth membrane preparations contain an enzyme that is capable of catalyzing this reaction.

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EXPERIMENTAL PROCEDURES

Materials—Sprague-Dawley rats were obtained from Harlan Industries, Inc. D-[2-^{3}H]Mannose (10 Ci/mmol) and the formula 963 scintillation mixture were from New England Nuclear. p-Nitrophenyl-α-N-acetylglucosaminide was from Calbiochem. Triton X-100 was from Research Products International, Elk Grove, IL. Quaternary aminoethyl (QAE)-Sephadex, N-acetyl-D-glucosamine, and N-acetyl-D-mannosamine were from Sigma Chemical Co. All other chemicals were of reagent grade and were obtained from commercial sources.

Enzymes—Homogeneous *E. coli* alkaline phosphatase was a gift from Dr. M. Schlesinger, Washington University. Pig liver α-N-acetylglucosaminidase (0.024 unit/ml) was prepared as previously described (12).

Preparation of the Oligosaccharide Substrate Containing a Single Phosphodiester Group—To assay for the proposed α-N-acetylglucosaminyl phosphodiesterase, we used as substrate a mixture of [^{3}H]mannose-labeled high mannose-type oligosaccharides, each of which contained a single phosphate residue in diester linkage in one of three possible positions (see Fig. 1). The isolation and detailed characterization of this oligosaccharide fraction (termed IIC_{10}) are described elsewhere.[1]

Assay of the α-N-Acetylglucosaminyl Phosphodiesterase—The assay takes advantage of the fact that conversion of the phosphodiester moiety of fraction IIC_{10} to a phosphomonoester by the removal of the α-linked N-acetylglucosamine residue results in an increase in the net negative charge of the molecule. Thus, while the original IIC_{10} fraction is eluted from QAE-Sephadex with 10 to 20 mM NaCl, the species with the phosphomonoester group requires at least 50 mM NaCl. The standard reaction mixture contained 1000 cpm of the [^{3}H]mannose-labeled substrate IIC_{10}, 0.5% Triton X-100, 50 mM Tris/maleate, pH 6.8, and cell extract in a final volume of 0.03 ml. Following incubation at 37°C for the indicated times, the reaction was quenched by the addition of 2 ml of 2 mM Tris base. The diluted sample was applied to a column (0.5 × 3.0 cm) of QAE-Sephadex equilibrated with 2 mM Tris base. The column was washed with an additional 2.5 ml of 2 mM Tris base and then eluted stepwise with 6 ml of 20 mM NaCl in 2 mM Tris base followed by 4.5 ml of 200 mM NaCl in 2 mM Tris base. The unmodified IIC_{10} material eluted with the 20 mM NaCl, whereas the "uncovered" material containing a phosphomonoester group eluted with the 200 mM NaCl. The total radioactivity in each fraction was determined and the amount of substrate eluting at 200 mM NaCl was expressed as a percentage of the total radioactivity eluting at 20 and 200 mM NaCl.

The oligosaccharide which eluted from the column with 200 mM NaCl was totally converted to a neutral species by alkaline phospha-

[1] A. Varki and S. Kornfeld (1980) *J. Biol. Chem.* **255,** in press.

tase, demonstrating that it contained a phosphomonoester group. The original phosphorylated oligosaccharides in fraction IIC_{10} were totally resistant to alkaline phosphatase.

Assay of p-Nitrophenyl-α-N-acetylglucosaminidase—The reaction mixture contained 2.5 mM *p*-nitrophenyl-α-*N*-acetylglucosaminide, 0.5% Triton X-100, 50 mM citrate buffer, pH 4.5, and cell extract in a final volume of 0.03 ml. Following incubation at 37°C for the indicated times, the reaction was terminated by the addition of 0.22 ml of 0.2 M Na_2CO_3. The sample was centrifuged at 12,000 × *g* for 5 min and the absorbance of the supernatant at 410 nm was determined.

Other Enzyme Assays—β-Hexosaminidase activity was determined in the identical fashion as the *p*-nitrophenyl-α-*N*-acetylglucosaminidase except that the substrate was *p*-nitrophenyl-β-*N*-acetylglucosaminide. UDP-galactose:*N*-acetylglucosamine galactosyltransferase was assayed as previously described (13).

Subcellular Fractionation of Rat Liver—The subcellular fractionation and the preparations of the Golgi-enriched smooth membranes was performed as previously described (14) using a modification of the method of Leelevathi *et al.* (15).

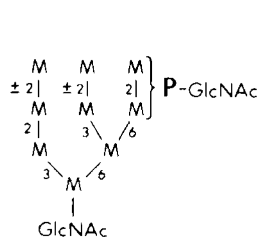
Protein Determination—Protein was measured by the method of Lowry *et al.* (16).

Cell Lines—Human diploid fibroblasts, obtained from Dr. Gene Bauer, Washington University, were grown as monolayers in α minimal essential medium supplemented with 10% fetal calf serum, glutamine, penicillin, and streptomycin. The I-cell disease fibroblasts (GM 2273) and the San Fillippo syndrome, type B fibroblasts (GM 2552), were obtained from the Human Genetic Mutant Cell Repository, N. J., and grown in the same medium as the normal fibroblasts.

RESULTS

Assay of Rat Liver Subcellular Fractions for α-N-Acetylglucosaminyl Phosphodiesterase Activity—In our previous studies, we demonstrated that the nonspecific lysosomal α-*N*-acetylglucosaminidase was able to remove the outer *N*-acetylglucosamine residues from the substrate IIC_{10} (11).[1] However, we postulated the existence of a separate, more specific enzyme that would be capable of removing the outer *N*-acetylglucosamine residues in preparation for the targeting of newly synthesized acid hydrolases to lysosomes. We therefore assayed the various subcellular fractions of rat liver for such an activity. The distribution of the subcellular organelles in the fractionation scheme which we used has been well characterized previously with both ultrastructural and marker enzyme studies (15). While we examined all of the fractions obtained, only the results from the relevant fractions will be described. As shown in Table I, the α-*N*-acetylglucosaminyl phosphodiesterase activity co-fractionated with the Golgi marker galactosyltransferase and was most enriched in Fraction D. This enzymatic activity was clearly separated from the *p*-nitrophenyl-α-*N*-acetylglucosaminidase activity which was most enriched in fraction B along with the acid hydrolase β-hexosaminidase.

General Properties of the Enzyme—The rate of cleavage of α-linked *N*-acetylglucosamine residues from the substrate was linear with respect to time and proportional to the amount of enzyme added (Fig. 2). This enzymatic activity was present over a broad range of pH, with activity at pH 4.5 and 8.8 being greater than 50% of the activity at pH 6.8. The α-*N*-acetylglucosaminyl phosphodiesterase activity was strongly inhibited by *N*-acetylglucosamine and only weakly inhibited by *N*-acetylmannosamine (Fig. 3, *left panel*). In contrast, the *p*-nitrophenyl-α-*N*-acetylglucosaminidase activity was more in-



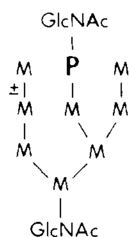


FIG. 1. **Proposed structures of the isomers present in oligosaccharide fraction IIC_{10}.** The isolation and the structural analysis of this fraction are described elsewhere.[1] *M*, mannose; *GlcNAc*, *N*-acetylglucosamine.

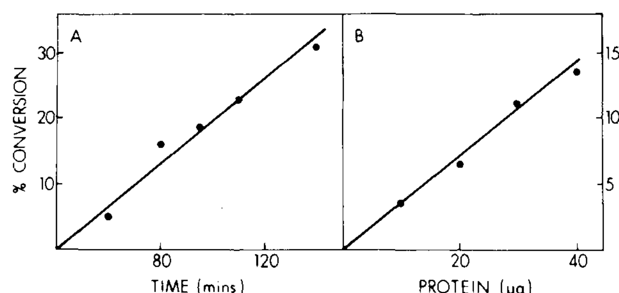
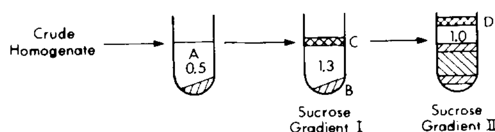


FIG. 2. **Effect of time and protein concentration upon release of *N*-acetylglucosamine from the oligosaccharides in Fraction IIC_{10}.** Membranes from Fraction D (Golgi-enriched) were used as the source of enzyme. All incubations were performed in 50 mM Tris/maleate, pH 6.8, at 37°C. *Panel A* shows the effect of increasing time upon the conversion of GlcNAc-P-Man-R (IIC_{10}) to P-Man-R. Each incubation contained 40 μg of protein. *Panel B* shows the effect of changing the protein concentration. These reactions were carried out for 90 min.

TABLE I

Subcellular distribution of α-N-acetylglucosaminyl phosphodiesterase and p-nitrophenyl-α-N-acetylglucosaminidase activity in rat liver

The procedure used for the subcellular fractionation and the details of the enzyme assays are described under "Experimental Procedures." The diagram shows the source of the fractions. Each fraction was dialyzed against 7.5 mM Na maleate, pH 6.4, 0.5% Triton X-100 prior to the enzyme assays. The enzyme activities in the various fractions are expressed relative to the values obtained with the postnuclear supernatant.



Fraction	Major content	Galactosyltransferase		α-N-Acetylglucosaminylphosphodiesterase		p-Nitrophenyl-α-N-acetylglucosaminidase		β-Hexosaminidase	
		Purity	Yield	Purity	Yield	Purity	Yield	Purity	Yield
		-fold	%	-fold	%	-fold	%	-fold	%
A	Postnuclear supernatant	1	100	1	100	1	100	1	100
B	Lysosomes, mitochondria, rough endoplasmic reticulum	0.2	4	0.17	4	3.1	72	2.0	48
C	Crude smooth membranes	4.1	79	2.8	54	0.2	4	0.5	10
D	Golgi-enriched smooth membranes	18.2	9.0	18.6	9.0	1.3	0.6	1.0	0.5

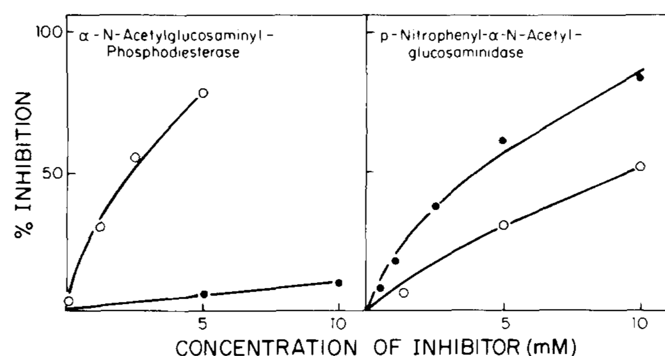


FIG. 3. **Differential inhibition of the α-N-acetylglucosaminyl phosphodiesterase and p-nitrophenyl-α-N-acetylglucosaminidase activities by amino sugars.** The assays for α-N-acetylglucosaminyl phosphodiesterase (*left panel*) and p-nitrophenyl-α-N-acetylglucosaminidase (*right panel*) were carried out as described under "Experimental Procedures" using 40 μg of protein from Fraction C. The reactions were incubated at 37°C for 90 min. The inhibitors used were N-acetylglucosamine (○——○) and N-acetylmannosamine (●——●).

TABLE II

Comparison of α-N-acetylglucosaminyl phosphodiesterase and p-nitrophenyl-α-N-acetylglucosaminidase activities in normal and mutant fibroblasts

Fibroblasts in monolayer cultures were grown to confluence in α minimal essential medium with 10% fetal calf serum. The cells were harvested, washed twice with phosphate-buffered saline, and sonicated three times (15 s each time) in 5 ml of 10 mM Na maleate, pH 6.4, using the microprobe of a Biosonik IV sonicator with the setting at position 1. The sonicate was centrifuged at 160,000 × g × 30 min and the pellet was homogenized in 10 mM Na maleate, pH 6.4, containing 0.5% Triton X-100. The enzyme assays were performed as described under "Experimental Procedures."

Cell type	α-N-Acetylglucosaminyl phosphodiesterase	p-Nitrophenyl-α-N-acetylglucosaminidase
	% substrate converted/mg protein/h	*ΔA_{410}/mg protein/h*
Normal human diploid fibroblasts	14.6 (100%)	1.8 (100%)
I-cell disease fibroblasts	10.3 (71%)	0.01 (<5%)
San Fillippo syndrome type B fibroblasts	17.0 (116%)	0 (0%)

hibited by N-acetylmannosamine than by N-acetylglucosamine (Fig. 3, *right panel*), providing further evidence that the two activities represent different enzymes.

Other inhibitors of the enzyme were UDP-N-acetylglucosamine (72% at 5 mM) and UDP-glucose (25% at 5 mM). Mannose, mannose 6-phosphate, Ca^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, and EDTA (all at 5 mM) had no effect on enzymatic activity. The cyclic AMP phosphodiesterase inhibitors, theophylline (1 mM) and 1-methyl isobutylxanthine (0.1 mM), had no effect on the activity. The Golgi-enriched fraction also cleaved N-acetylglucosamine residues from phosphorylated oligosaccharides that contained two phosphodiester-linked N-acetylglucosamine residues (data not shown).

Activity in Normal and Mutant Human Fibroblasts—Fibroblasts obtained from patients with I-cell disease and the San Fillippo syndrome are known to be deficient in p-nitrophenyl-α-N-acetylglucosaminidase activity (17). As shown in Table II, we confirmed this finding and could demonstrate that both of these cell types have α-N-acetylglucosaminyl phosphodiesterase activities comparable to that found in normal human diploid fibroblasts.

DISCUSSION

These data demonstrate that rat liver, as well as human diploid fibroblasts, contain an enzyme capable of cleaving α-linked N-acetylglucosamine residues which are in phosphodiester linkage with mannose residues of high mannose-type oligosaccharides. This enzyme activity can be distinguished from the lysosomal α-N-acetylglucosaminidase activity by several criteria. Firstly, the subcellular fractionation resulted in a physical separation of the two activities. Secondly, the two activities were inhibited very differently by the amino sugars N-acetylglucosamine and N-acetylmannosamine. Finally, fibroblasts from patients with I-cell disease and the San Fillippo syndrome, which are markedly deficient in the lysosomal α-N-acetylglucosaminidase activity, have essentially normal levels of α-N-acetylglucosaminyl phosphodiesterase activity.

The α-N-acetylglucosaminyl phosphodiesterase clearly copurified with the Golgi marker enzyme, galactosyltransferase. However, this same fraction was also slightly enriched for the lysosomal marker β-hexosaminidase, although the yield was much lower than that found in the dense lysosomal pellet (see Table I). This raises the possibility that the Golgi-enriched fraction may also contain the fraction of light lysosomes which form part of the network originally described by Novikoff as GERL (18, 19). Accordingly, we cannot be certain about the precise subcellular localization of the α-N-acetylglucosaminyl phosphodiesterase.

We propose that the α-N-acetylglucosaminyl phosphodiesterase functions in the processing of newly synthesized acid hydrolases. We have postulated that the acid hydrolases are phosphorylated by the transfer of α-N-acetylglucosamine 1-phosphate residues to one or more mannose residues of the high mannose oligosaccharides present in these proteins (11). At a later time, perhaps in the Golgi or the GERL region of the cell, the outer N-acetylglucosamine residues would be removed by the α-N-acetylglucosaminyl phosphodiesterase. This would expose the phosphate groups which act as the recognition marker for the selective sequestration of this group of enzymes into lysosomes (1). The presence of the α-N-acetylglucosaminyl phosphodiesterase provides an explanation for the finding that the biosynthetic intermediates of β-glucuronidase contain phosphate in diester linkage whereas the more mature forms of the acid hydrolases have phosphomonoester groups.

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