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BINDING OF LYSOSOMAL HYDROLASES BY THE MEMBRANES OF RAT LIVER LYSOSOMES: EVIDENCE FOR PHOSPHOMANNOSYL RECOGNITION MARKERS

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SUMMARY

Most of the soluble hydrolase activity of broken lysosomes was found to be bound to lysosomal membranes. However, this soluble activity could be released from the membranes by the addition of sugar phosphates. Mannose-6-phosphate was found to displace N-acetyl-β-D-glucosaminidase (NAβGase) from the membrane in a concentration dependent manner. In addition, fructose-6-phosphate and AMP were also effective. The binding of β-glucuronidase was similarly affected by sugar phosphates. The glycosyl specificity of the lysosomal membrane receptor appears to be similar to that of the plasma membrane receptor in cultured fibroblasts, as previously reported (Kaplan, A., Achord, D. T., and Sly, W. S. (1977) Proc. Natl. Acad. Sci. 74, 2026-2030). These results indicate that phosphomannosyl receptors for the lysosomal hydrolases may exist in the lysosomal membrane as well as in the plasma membrane.

Phosphomannosyl residues present in the carbohydrate moiety of lysosomal enzymes are thought to be involved in the uptake of lysosomal enzymes (1). As a result of studies on cultured fibroblasts exhibiting deficiencies in lysosomal enzymes, two mechanisms involving these residues have been proposed. The secretion-recapture hypothesis of Neufeld (2) states that newly synthesized hydrolytic enzymes are secreted and recaptured by receptor mediated endocytosis and then packaged into lysosomes. A plasma membrane receptor recognizes the phosphomannosyl residues and mediates the uptake of the enzymes. More recent studies (3,4,5) suggest that secretion is not obligatory, but that the phosphomannosyl recognition markers are the means by which the lysosomal enzymes remain bound to the plasma membrane and are retained during the extensive membrane flow taking place during endo- and exocytosis.

Although a plasma membrane receptor has been indirectly demonstrated (6), the presence of such a receptor on lysosomal membranes has not been reported.

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We have previously found that in pure lysosomes isolated from rat liver, soluble enzymes remain bound to the membranes, and high salt at an alkaline pH must be used to displace the enzymes (7). We now present evidence that this binding may be similar to the specific phosphomannosyl mediated binding reported to be present on fibroblast plasma membranes.

MATERIALS AND METHODS

Male rats were obtained from Charles River, Wilmington, MA; chemicals were obtained through Sigma Chemical Co., St. Louis, MO.

Livers were removed immediately following decapitation and chilled in ice cold 0.25 M sucrose. After homogenization, a lysosome enriched fraction (L fraction) was prepared by differential centrifugation, as described previously (8). The impure lysosomes were enriched at least 15X for the lysosomal enzyme N-acetyl-β-D-glucosaminidase (NAβGase). Enzyme activity was measured using p-nitrophenyl-N-acetyl-β-D-glucosaminide as described (9).

Prior to incubation with the various sugars and nucleotides, the L fraction pellet was resuspended in distilled water and frozen and thawed to insure breakage of the lysosomes. All incubations were carried out on ice in 10 mM 2-(N-morpholino)ethane sulfonic acid (MES) at pH 6, at a protein concentration of 200 μg/ml. The soluble and membranous fractions were separated by centrifugation at 35,000 rpm for 40 min in a 50Ti Spinco rotor (g_{max}=110,000). The pellets (membranes) were resuspended in ice cold distilled water by bath sonication.

RESULTS

Most soluble lysosomal enzymes remain associated with the lysosomal membrane under conditions of low ionic strength. Figure 1 shows that the soluble enzyme NAβGase can be removed from the membranes by mannose-6-phosphate, in a concentration dependent manner. This release is not due to the rupture of sealed lysosomal vesicles; the fraction was frozen prior to use to insure breakage of all vesicles. In addition, freezing and thawing in the presence of the phosphomannose did not significantly enhance release of the enzyme. Also, mannose-6-phosphate was found to have similar effects on the release of NAβGase from the membranes of the lysosomes purified by the Triton WR-1339 technique. Table I compares the ability of various hexose phosphates to displace NAβGase from the membranes. Both mannose-6-phosphate and fructose-6-phosphate were effective; glucose-6-phosphate was much less effective. This would suggest that the binding of NAβGase to the lysosomal

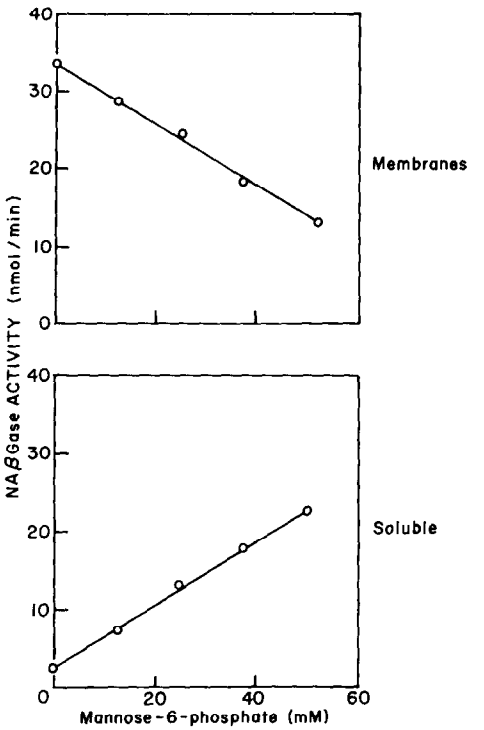

Fig. 1. Effect of Mannose-6-Phosphate Concentration on Binding of NAβGase to Lysosomal Membranes.

A lysosome-enriched fraction (200 µg/ml), prepared as described in Methods, was incubated in 10 mM MES, pH 6, 5 mM $MgCl_2$, and mannose-6-phosphate at 0°C for 10 min. The soluble and membranous fractions were separated by ultracentrifugation at 110,000 xg for 35 min.

Table I. Effect of Sugar Phosphates on Binding of NAβGase to Lysosomal Membranes

	NAβGase Activity (nmol/min)		(%)
Addition	soluble	membranous	soluble
None	1.9	32.9	5.5
Mannose-6-phosphate, 50 mM	22.5	14.0	62
Fructose-6-phosphate, 50 mM	16.8	16.0	51
Glucose-6-phosphate, 50 mM	10.2	26.5	28

Experiments were conducted as described in Fig. 1. The pH of stock solutions of the sugar phosphates was adjusted to 6.

Table II. Effect of Sugars on Binding of NAβGase to Lysosomal Membranes

Addition	NAβGase Activity (nmol/min) soluble	membranous	(%) soluble
None	11.0	26.7	29
Mannose, 100 mM	8.0	27.2	23
Glucose, 100 mM	9.0	25.3	26
Galactose, 100 mM	9.9	26.2	27
N-Acetyl-galactosamine	1.6	29.1	5.2

A lysosome-enriched fraction (200 µg/ml) was incubated at 0° for 10 min in 10 mM MES, pH 6, and 100 mM sugar. Membranous and soluble fractions were prepared as described in Fig. 1.

membrane is mediated by a phosphohexosyl moiety. Nonphosphorylated sugars were significantly less effective in displacing the enzyme from the membrane (Table II).

Parallel results were obtained with another lysosomal enzyme, β-glucuronidase; hence, several soluble enzymes may share a common recognition marker.

As shown in Table III, inorganic phosphate alone displaced relatively little enzyme from the membrane; however the phosphate group seems to be an

Table III. Effect of Nucleotides on Binding of NAβGase to Lysosomal Membranes

Addition	NAβGase Activity (nmol/min) soluble	membranous	(%) soluble
None	2.1	27.8	7.0
ATP, 10 mM	2.4	31.7	7.0
ADP, 10 mM	5.7	28.4	17
AMP, 10 mM	10.1	20.0	34
Pyrophosphate, 10 mM	7.1	25.7	22
Phosphate, 10 mM	4.2	31.8	12

Experiments were performed as described in Fig. 1. The pH of the added compounds was adjusted to 6 prior to addition.

essential component of the binding of the lysosomal enzymes because several nucleotides were able to promote the release of the soluble enzymes from the membrane. It should be pointed out that AMP may be more effective than mannose-6-phosphate: at a concentration of 10 mM, 34% of the enzyme can be released by AMP, 10 mM mannose-6-phosphate released only 18%. Whether AMP and mannose-6-phosphate are acting at the same site or at different sites will be discerned in subsequent studies.

DISCUSSION

The data presented here indicate that receptors on rat liver lysosomal membranes may bind lysosomal enzymes by way of phosphohexosyl moieties. Mannose-6-phosphate is the most effective sugar phosphate in displacing the soluble enzymes from the membrane (Figure 1); fructose-6-phosphate is almost as effective and glucose-6-phosphate is minimally effective (Table I). The phosphoryl group seems to be essential, as nonphosphorylated sugars are relatively ineffective in releasing lysosomal enzymes. We have also found that AMP is also effective in displacing bound enzymes from the lysosomal membranes; the effect of AMP on the plasma membrane receptor has not been reported.

These results are similar to those reported by Kaplan and coworkers (1,3,6) in which sugar phosphates, especially mannose-6-phosphate, were found to inhibit pinocytosis of lysosomal enzymes by fibroblasts. Presumably the sugar phosphates and lysosomal enzymes compete for a membrane bound receptor recognizing phosphohexosyl moieties. In a similar manner mannose-6-phosphate and the acidic hydrolases could compete for binding sites on the lysosomal membranes. Although a substantial difference in effective concentration of mannose-6-phosphate exists between their system and ours, this could be due to differences in conditions (i.e. ionic strength) or a difference in affinities.

The presence of such receptors on the membrane of the lysosome may be vitally necessary to insure the integrity of the lysosomal system during

repeated fusion events. Alternately, these receptors could play a role in segregating lysosomal enzymes from secretory proteins during the early events in the biosynthesis of the lysosome.

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