FIBROBLAST RECEPTOR FOR LYSOSOMAL ENZYMES MEDIATES PINOCYTOSIS OF MULTIVALENT PHOSPHOMANNAN FRAGMENT

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ABSTRACT

Mild acid hydrolysis of phosphomannan secreted by the yeast *Hansenula holstii* (NRRL Y-2448) produces two phosphomannosyl fragments which differ strikingly in their potency as inhibitors of pinocytosis of human β-glucuronidase by human fibroblasts. The larger molecular weight polyphosphomonoester fragment is 100,000-fold more potent an inhibitor of enzyme uptake than the smaller pentamannosyl-monophosphate fragment. Binding to attached fibroblasts at 3°C was much greater with the polyphosphomonoester fragment than with the pentamannosyl-monophosphate. The larger molecular weight fragment was also subject to adsorptive pinocytosis and was taken up by fibroblasts at a rate 30-fold greater than the rate of uptake of pentamannosyl-monophosphate. Evidence that the polyphosphomonoester fragment is taken up by the phosphomannosyl-recognition system that mediates uptake of lysosomal enzymes includes: (*a*) its pinocytosis is inhibited by the same compounds that competitively inhibit enzyme pinocytosis (mannose-6-phosphate and phosphomannan from *Saccharomyces cerevisiae* mutant mnn-1); (*b*) alkaline phosphatase treatment greatly reduces its susceptibility to pinocytosis; (*c*) its pinocytosis is competitively inhibited by high-uptake human β-glucuronidase; and (*d*) this inhibition by high-uptake enzyme is dramatically reduced by prior treatment of the enzyme with alkaline phosphatase or endoglycosidase-H.

Endoglycosidase-H treatment of human β-glucuronidase dramatically reduced its susceptibility to pinocytosis by fibroblasts. The phosphomannosyl components of high-uptake enzyme released by endoglycosidase-H treatment were much less effective inhibitors of polyphosphomonoester pinocytosis than when present on the phosphomannosyl-enzyme. These results suggest that high-uptake acid hydrolases may be polyvalent ligands analogous to the polyphosphomonoester mannan fragment whose pinocytosis depends on interaction of more than one phosphomannosyl recognition marker with pinocytosis receptors on fibroblasts.

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Many lysosomal acid hydrolases are subject to adsorptive pinocytosis by fibroblasts, i.e., their uptake displays the saturability and selectivity expected of a receptor-mediated process (3, 13, 20, 26). Hickman and Neufeld (10) suggested that many acid hydrolases share a common recognition marker for uptake, and suggested further (11) that the recognition marker may reside in the carbohydrate portion of the glycoprotein hydrolases. Glaser et al. (8) reported that the fraction of a given acid hydrolase which was subject to adsorptive pinocytosis by fibroblasts corresponded to a subpopulation of enzyme molecules that was relatively acidic, and showed that this "high-uptake" form of the enzyme was converted to less acidic "low-uptake" forms after pinocytosis by fibroblasts.

That phosphomannose is the acidic group in the common recognition marker for uptake by fibroblasts was first suggested by Kaplan, Achord, and Sly (13) on the basis of studies with purified human platelet β-glucuronidase. The presence of phosphate on the enzyme and its role in enzyme pinocytosis were inferred from the finding that alkaline phosphatase treatment of "high-uptake" human platelet β-glucuronidase altered its electrophoretic properties and destroyed its ability to be taken up by fibroblasts. Linkage of the phosphate to a mannose-type carbohydrate was inferred from the potent competitive inhibition of enzyme pinocytosis by 6-phosphomannose. Mannose-6-phosphate was 1,000-fold more potent an inhibitor of enzyme pinocytosis than mannose, which had been reported earlier to inhibit enzyme pinocytosis (12).

These observations were extended by studies from this laboratory (14) to human β-glucuronidase from four other tissue sources, to two additional platelet enzymes (β-galactosidase and β-hexosaminidase), and to fibroblast secretion hexosaminidase. Sando and Neufeld (21) extended these results to human α-L-iduronidase, the original Hurler-corrective factor (19), and Ullrich et al. (25) confirmed the evidence for phosphomannosyl involvement in the pinocytosis of a number of the enzymes mentioned above and extended these observations to human urinary arylsulfatase A,*N*-acetyl-α-D-glucosaminidase, and to a pig kidney α-mannosidase.

Part of the initial evidence implicating 6-phosphomannose recognition in the uptake of acid hydrolases by fibroblasts was the competitive inhibition of uptake by phosphomannans containing 6-phosphomannose (13). We subsequently reported on the correlation of structural features of phosphomannans and phosphomannan hydrolysis fragments with their ability to inhibit enzyme pinocytosis (15). These results indicated (*a*) that mannose-6-phosphate in some macromolecules is more inhibitory than free mannose-6-phosphate, (*b*) that phosphate in monoester linkage is a more potent inhibitor than phosphate in diester linkage, (*c*) that a large molecular weight polyphosphomonoester (PPME) fragment of the phosphomannan from the yeast *Hansenula holstii* (NRRL Y-2448) is a very potent inhibitor of enzyme pinocytosis, and (*d*) that alkaline phosphatase treatment of this phosphomonoester fragment reduces its inhibitory potency in proportion to the phosphate released.

In the present paper, we report studies examining products of acid hydrolysis of *H. holstii* phosphomannans for their potency as inhibitors of enzyme pinocytosis, and for their susceptibility to pinocytosis by fibroblasts. These studies show (*a*) that the multivalent, large molecular weight PPME fragment from *H. holstii* phosphomannan is a much more potent inhibitor of enzyme pinocytosis than a monovalent pentamannosyl-monophosphate fragment from the same yeast phosphomannan, (*b*) that the multivalent, large molecular weight PPME fragment is subject to adsorptive pinocytosis by fibroblasts while the smaller fragment is not, (*c*) that the uptake of the large PPME fragment is competitively inhibited by purified high-uptake β-glucuronidase, and (*d*) that the inhibition of phosphomannan pinocytosis by β-glucuronidase is destroyed by prior treatment of the enzyme with either alkaline phosphatase or endoglycosidase-H. These results suggest that the large molecular weight phosphomannan fragment is pinocytosed by the previously reported system for receptor-mediated uptake of acid hydrolases and illustrate how phosphomannan fragments may be useful as ligands for studies of the fibroblast pinocytosis receptor for lysosomal hydrolases.[1]

[1] Preliminary portions of the present work were presented as an abstract at the meeting of the Federation of American Societies For Experimental Biology in Dallas, Texas, April 1-5, 1979.

MATERIALS AND METHODS

Most reagents were purchased from Sigma Chemical Co., St. Louis, Mo. *H. holstii* mannan and phosphomannan were gifts of Dr. M. Slodki. Uniformly labeled *H. holstii* [^{14}C]phosphomannan (~3.0×10^{13} cpm/mol organic phosphate) was obtained as previously described (5). *Saccharomyces cerevisiae* phosphomannan (X2180-mnn1) was the gift of Dr. C. E. Ballou. Homogeneous *Escherichia coli* alkaline phosphatase was a gift of Dr. M. Schlesinger. Cultured fibroblasts were established from skin biopsies obtained from patient J. E. with β-glucuronidase deficiency mucopolysaccharidosis (available as cell strain GM-151 from the Human Mutant Cell Repository, Camden, N. J.) and patient T. M. with I-cell disease (Division of Medical Genetics, University of Texas Health Science Center, Dallas, Tex.).

Hydrolysis of Phosphomannan

A solution of native *H. holstii* phosphomannan was hydrolyzed in 0.1 N HCl for 20 min at 100°C. A pentamannosylmonophosphate fragment and a large molecular weight PPME fragment were subsequently separated by column chromatography on Bio-Gel-P2 (Bio-Rad Laboratories, Richmond, Calif.). ^{14}C-labeled phosphomannan fragments were separated as previously described (5).

Purification of Human Spleen β-Glucuronidase

High-uptake human spleen β-glucuronidase was isolated from homogenates of human spleen by sequential applications of a heat step, ammonium sulfate precipitation, concanavalin A (Con-A) column chromatography, gel filtration, and two successive CM-Sephadex columns. The details of this procedure are published elsewhere (18). The β-glucuronidase obtained appeared pure on sodium dodecyl sulfate (SDS) slab gels. The properties of the high-uptake enzyme purified from this source were no different than those reported for high-uptake enzyme purified from platelets (13).

Alkaline Phosphatase Treatment

Most alkaline phosphatase treatments were carried out as previously described (13) with *E. coli* alkaline phosphatase. Treatments of the PPME were done for 24 h at 37°C with 40 μg of alkaline phosphatase/100 μg of PPME. This treatment removed >90% of the total organic phosphate on the PPME fragment.

Endoglycosidase-H Treatment

Endoglycosidase-H treatments were carried out for 48 h at 37°C in 50 mM Na-acetate, 0.2 M citrate-PO_4 buffer, pH 5.5, with a total of 20 mU of endoglycosidase-H (Miles Laboratories, Inc., Elkhart, Ind.). The protein was subsequently precipitated with 80% ice-cold ethanol, and the released oligosaccharides were recovered from the supernate.

Pinocytosis Measurements

Fibroblasts were grown in Eagle's minimum essential medium with Earle's salts (KC Biological) supplemented with 15% heat-inactivated fetal bovine serum and 3 mM glutamine. Pinocytosis rates for β-glucuronidase and [^{14}C]phosphomannan were determined in duplicate 35-mm petri dishes (Falcon Labware, Div. Becton, Dickinson Co., Oxnard, Calif.) containing ~2.5×10^5 cells at confluence (~0.2 mg protein/dish). The cells were exposed to the indicated concentrations of enzyme or phosphomannan (± inhibitors) in 1.0 ml of this medium and incubated at 37°C for 2.0 h (enzyme) or 24 h (phosphomannan). The cells were then washed six times with 3-ml portions of ice-cold, PBS and lysed with 0.5 ml of 2% sodium deoxycholate.

Assays

Phosphate was determined by the Ames method (1). β-Glucuronidase was assayed as previously described using 10 mM 4-methylumbelliferyl-glucuronide (9). 1 U of enzyme activity is defined as the activity which releases 1 nmol of 4-methylumbelliferone/h. Radioactivity was counted in 10 ml of Biofluor (New England Nuclear, Boston, Mass.). Protein was determined by the method of Lowry et al. (17).

RESULTS

"Mild acid-hydrolysis conditions" (0.01–0.1 N HCl at 100°C for 15–20 min) selectively cleave phosphodiester bonds in yeast cell-wall phosphomannans (4). The 6-phosphomonoester groups exposed by this treatment are far more resistant to acid hydrolysis. In fact, the $t_{1/2}$ for 6-phosphomannose monoester bonds is 1,034 min in 1.0 N HCl at 100°C (16). Thus, the 6-phosphomannose monoester phosphate largely resists acid hydrolysis conditions strong enough to hydrolyze mannans completely to monosaccharides.

Mild acid hydrolysis of native extracellular *H. holstii* phosphomannan produces two predominant phosphomannosyl species. One is a highly branched, large molecular weight PPME fragment composed solely of mannose and phosphate in a molar ratio of ~5.7:1 (23). The other is a smaller molecular weight pentamannosyl monophosphate, $(Man)_5$-P, having the structure P-6-Man$\alpha(1 \rightarrow 3)$ Man$\alpha(1 \rightarrow 3)$ Man$\alpha(1 \rightarrow 3)$ Man$\alpha(1 \rightarrow 2)$ Man (5), and which can be viewed as a monovalent ligand with a single 6-phosphomonoester of mannose at the nonreducing end of the molecule. By comparison of the number of reducing groups per mole of phosphate on the PPME and $(Man)_5$-P using ^{3}H-$NaBH_4$ reduction (7), the average size of the PPME fragment was estimated to be 1,000 times larger than that of $(Man)_5$-P (Fischer and Sly, unpublished observations). This suggests a molecular weight of ~1×10^6 for the PPME fragment which can thus be viewed as a multivalent ligand containing nearly 1,000 phosphate groups/molecule.

Since nearly all (90–95%) of the phosphate present in the PPME is released by prolonged treatment with alkaline phosphatase, and since mannose-6-phosphate is the only phosphorylated sugar produced by strong acid hydrolysis of the PPME

(22), all of the phosphate present in the PPME fragment is thought to be present as the 6-phosphomonoester of mannose. Slodki et al. (23) have shown by periodate oxidation analysis that $\alpha(1 \rightarrow 2)$ and $\alpha(1 \rightarrow 6)$-linked mannose residues are present in the PPME. The same group has more recently shown by methylation analysis that $\alpha(1 \rightarrow 3)$-linked residues are also present and that a significant number of nonreducing end groups results from 1,2,6-tri-O-substituted points of branching (Slodki, personal communication). As acetolysis, which selectively cleaves $(1 \rightarrow 6)$ linkages, of dephosphorylated PPME produced only short oligosaccharides of mannose (Monaghan and Bretthauer, unpublished observations), the structure of the PPME could consist of a linear $\alpha(1 \rightarrow 6)$-linked polymannose backbone with short oligosaccharide side chains linked $\alpha(1 \rightarrow 2)$ to the main chain which contain $\alpha(1 \rightarrow 2)$ and $\alpha(1 \rightarrow 3)$-linked mannose residues, some of which are phosphorylated at the 6-position. Further experimentation is required to more precisely define the structure of the PPME.

Fig. 1 shows the relative potency of $(Man)_5$-P and the PPME fragment as inhibitors of β-glucuronidase pinocytosis by fibroblasts. While the monovalent $(Man)_5$-P displays nearly the same inhibitory potency previously described for mannose-6-phosphate (14), the large molecular weight, multivalent PPME is in excess of 100-fold more potent per mole of organic phosphate (or 100,000-fold more potent per molecule) as an inhibitor of enzyme pinocytosis than is $(Man)_5$-P.

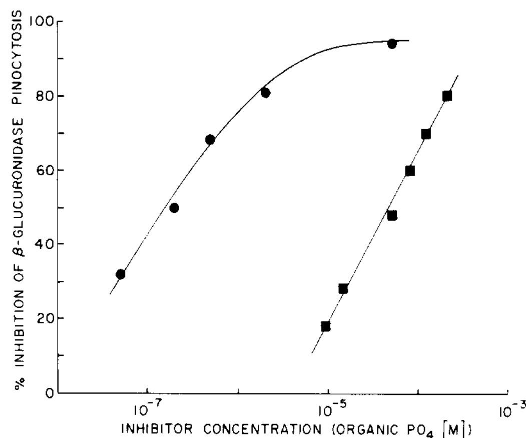

FIGURE 1 Concentration dependence of inhibition of enzyme pinocytosis by *H. holstii* phosphomannan hydrolysis fragments. Pinocytosis was measured with 1,000 U of human spleen β-glucuronidase as described under Materials and Methods. Phosphate in the abscissa refers to concentration of organic phosphate in the fragments. Cells were confluent 35-mm dishes of β-glucuronidase-deficient fibroblasts. (●) Large molecular weight PPME fragment, (■) pentamannosyl-monophosphate fragment.

Strong acid hydrolysis reduces yeast mannans to monosaccharides. In the case of *H. holstii* phosphomannan, the products of strong acid hydrolysis are mannose and mannose-6-phosphate (22). Fig. 2 displays the effect of strong acid hydrolysis of the PPME on its ability to inhibit β-glucuronidase pinocytosis. Fig. 2*A* shows the time and concentration dependence of loss of inhibitory potency on acid hydrolysis. Nearly all of the inhibitory capacity is destroyed by hydrolysis in 1.0 N HCl at 100°C for 1.0 h. Fig. 2*B* indicates that with acid hydrolysis conditions under which the glycosidic bonds are hydrolyzed but the phosphate monoester bond is relatively stable (16), the inhibitory capacity of the PPME is rapidly reduced to the level of potency for free mannose-6-phosphate. Further hydrolysis is very slow and comparable to the rate of hydrolysis of mannose 6-phosphate. These results suggest that the large initial loss in potency is not due to hydrolysis of the phosphate bond but rather to destruction of some secondary feature of the PPME molecule.

Data comparing the relative abilities of the PPME and $(Man)_5$-P fragments from ^{14}C-labeled *H. holstii* phosphomannan to bind to and be taken up by human I-cell disease fibroblasts are presented in Fig. 3. Fig. 3*A* demonstrates that the binding, per mole of phosphate, of the large, multivalent PPME at 3°C is much more efficient than that of the small, monovalent $(Man)_5$-P. A Scatchard analysis of these data (not shown) indicates that the affinity constants for these two ligands are nearly equal to their inhibitory constants for enzyme uptake and suggest that there are ~6,000 PPME binding sites on fibroblasts. Fig. 3*B* shows that the multivalent PPME is also taken up much more efficiently than monovalent $(Man)_5$-P after incubation with fibroblasts for 24 h at 37°C. Even at a concentration 100-fold greater than the highest shown in Fig. 3*B* (i.e., concentrations where $(Man)_5$-P is an effective inhibitor of enzyme pinocytosis), uptake of $(Man)_5$-P was no greater than that explainable by nonspecific, fluid endocytosis (0.3 μl/mg/h). Trypsinization of fibroblasts for 5 min at 37°C with 0.05% trypsin after binding of PPME for 2 h at 3°C released ~90% of the cell-associated radioactivity, while nearly all of the radioactivity associated with cells incubated with

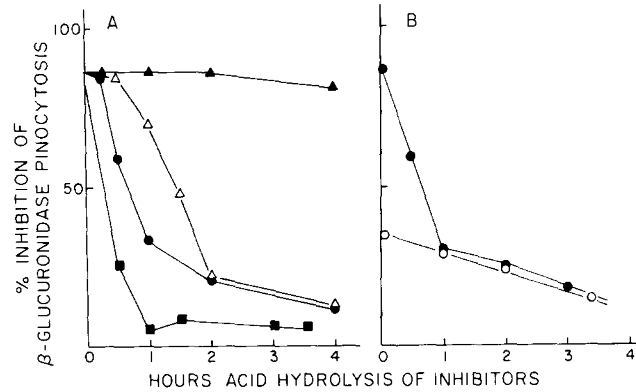

FIGURE 2 Effect of acid hydrolysis at 100°C on inhibitory potency of *H. holstii* phosphomannan PPME fragment for β-glucuronidase pinocytosis. Pinocytosis was measured as described under Materials and Methods with β-glucuronidase-deficient fibroblasts. Phosphomannan and Man-6-P concentrations were 5×10^{-5} M organic phosphate. (*A*) Acid normality, (▲) 0.1 N HCl, (△) 0.25 N HCl, (●) 0.5 N HCl, (■) 1.0 N HCl, (*B*) Hydrolysis of PPME and Man-6-P with 0.5 N HCl. (●) 5×10^{-5} M (PO_4) PPME, (○) 5×10^{-5} M Man-6-P.

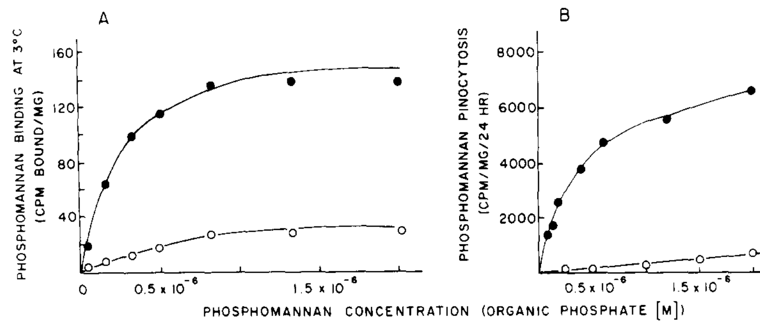

FIGURE 3 Binding and pinocytosis by I-cell disease fibroblasts of large molecular weight PPME fragment and pentamannosyl-monophosphate from *H. holstii* by I-cell disease fibroblasts. Both fragments isolated from [^{14}C]mannose-labeled *H. holstii* phosphomannan (~30,000 cpm/nmol organic PO_4). (*A*) 3°C binding of phosphomannan fragments. Binding was carried out on ice in a cold room in a total volume of 1 ml/ petri dish for 2 h. Concentration is expressed as organic phosphate concentration. Cpm-bound refers to the amount of radioactivity which becomes cell-associated at 3°C and remains cell associated after six washes with PBS at 4°C. 90% of the cpm bound were released by trypsin treatment (see text). Specific binding refers to the amount of binding by ^{14}C-labeled phosphomannan fragments which is inhibited by a 100-fold excess of unlabeled ligand. (*B*) Pinocytosis measurement was as described in Materials and Methods. Phosphomannan fragment concentration is expressed as organic phosphate concentration. (●) PPME, (○) $(Man)_5$-P.

the PPME for 24 h at 37°C was insensitive to release by trypsin and assumed to be internalized (data not shown).

I-Cell disease fibroblasts, which are deficient for multiple acid hydrolases, were chosen for these studies to minimize degradation of the ligands after pinocytosis. The data in Fig. 4 indicate that under our assay conditions for PPME pinocytosis by human fibroblasts, uptake over 24 h approximates linearity, and little of the material internalized over the first 24 h by I-cell disease fibroblasts was lost to the medium over the next 24 h. Although $(Man)_5$-P was internalized at a much slower rate than PPME, its stability, once taken up by I-cell fibroblasts, was similar to that of PPME. Less than 5% of the ^{14}C-$(Man)_5$-P accumulated by I-cell fibroblasts during a 24-h incubation was lost to the medium over the next 24 h (data not shown).

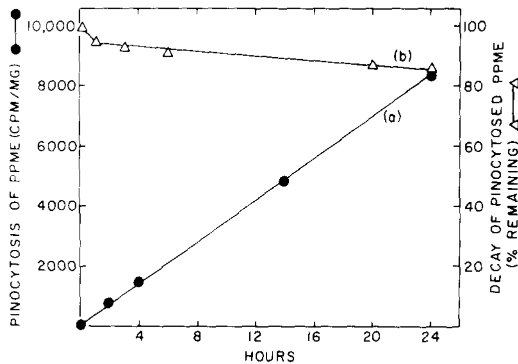

FIGURE 4 Time course of ^{14}C-PPME uptake and disappearance in I-cell disease fibroblasts. (*A*) Pinocytosis measured as in Materials and Methods. PPME concentration was 1.0×10^{-6} M organic phosphate (~30,000 cpm). (*B*) Disappearance of pinocytosed PPME. Cells were preloaded for 24 h with PPME as described. Plates were then washed four times with uptake medium, fresh medium was added, and the cells were incubated at 37°C for the indicated times. Plates were then washed and assayed as described in Materials and Methods.

If the greater efficiency of the larger molecular weight ligand is a consequence of its ability to interact as a multivalent ligand, one might expect to see inhibition of its pinocytosis at high ligand concentrations analogous to the inhibition of antibody precipitin reactions in the region of high antibody excess. Fig. 5 demonstrates such an inhibition of PPME pinocytosis at high ligand concentrations. We interpret this as specific "substrate inhibition" of pinocytosis since greater than tenfold higher concentrations of other polyanions (chondroitin sulfate, heparin sulfate, polyglutamic acid, and sodium phosphate glasses) had no effect on PPME pinocytosis (data not shown).

It was previously reported (15) that alkaline phosphatase treatment of the PPME diminished its potency as an inhibitor of β-glucuronidase pinocytosis. Table I shows that alkaline phosphatase treatment of the PPME also reduces its ability to be pinocytosed by fibroblasts. The effect of the alkaline phosphatase is sensitive to inhibition by inorganic phosphate and was completely inhibitable by 1 mM Pi. Table I also presents data showing that two potent inhibitors (13) of lysosomal enzyme pinocytosis, mannose 6-phosphate and mild acid hydrolyzed *S. cerevisiae* X2180-mnn-1 mannan (4), also inhibit PPME pinocytosis. A nonphosphorylated mannan produced by *H. holstii* grown in phosphate-free media (22) is noninhibitory for PPME uptake, however. Fig. 6 presents data showing inhibition of PPME pinocytosis by two compounds which were previously demonstrated to be competitive inhibitors of β-glucuronidase pinocytosis (13). These data suggest a common binding site for these inhibitors and the PPME fragment and a common mechanism for pinocytosis of lysosomal hydrolases and the PPME.

The possibility that PPME and β-glucuronidase are pinocytosed by a common fibroblast receptor was examined in an experiment in which PPME uptake was inhibited by the addition of purified human spleen β-glucuronidase to the uptake medium. Fig. 7*A* shows the effect of increasing concentration of added β-glucuronidase on the inhibition of PPME uptake by fibroblasts. The data presented in Fig. 7*B* show that the inhibition of PPME pinocytosis by the added enzyme is competitive, as would be expected if pinocytosis of both ligands required binding to the same receptor.

Alkaline phosphatase treatment of spleen β-glucuronidase destroys its ability to be pinocytosed by fibroblasts (14). The data in Table II demonstrate this previously reported effect of alkaline phosphatase treatment on the uptake properties of human spleen enzyme, and show, in addition, that this treatment also reduces the ability of the enzyme to inhibit the pinocytosis of PPME. These

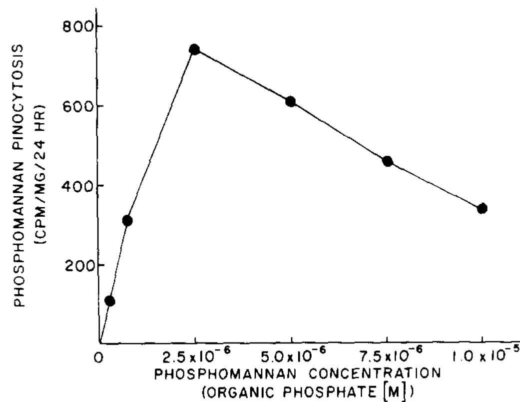

FIGURE 5 Inhibition of PPME pinocytosis at high substrate concentrations. ^{14}C-PPME was diluted tenfold with unlabeled PPME and pinocytosis was measured as described in Materials and Methods. The phosphomannan concentrations, expressed as organic phosphate concentration, indicate the total concentration of PPME in the incubation mixture and a constant ratio of radiolabeled to unlabeled PPME at each point.

results are in accord with the previously suggested role of a 6-phosphomannose group in the recognition marker for lysosomal hydrolase uptake by fibroblasts, and suggest a similar role for 6-phosphomannose in the recognition and pinocytosis of PPME by fibroblasts.

Finally, Natowicz et al. have shown that endoglycosidase-H treatment of spleen β-glucuronidase quantitatively released oligosaccharide chains containing 6-phosphomannose (18). High-uptake enzyme contained up to 4.4 mol of Man-6-P/mol of enzyme, and release of the Man-6-P by endoglycosidase-H destroyed the susceptibility of the enzyme to pinocytosis by fibroblasts (18). We reasoned that if high-uptake enzyme containing multiple 6-phosphomannose groups/enzyme molecule interacts as a multivalent ligand with fibroblast pinocytosis receptors, its effectiveness as an inhibitor of PPME pinocytosis should depend on the presence of the 6-phosphomannose in a multivalent form (i.e., on the enzyme). 6-Phosphomannose containing oligosaccharides released by endoglycosidase-H would be expected to be much less effective inhibitors of PPME uptake, analogous to the 100-fold loss of inhibitory potency for enzyme pinocytosis found in conversion of PPME to mannose-6-phosphate. The data in Table III demonstrate the effect of endoglycosidase-H treatment on the uptake properties of human spleen β-glucuronidase, and show in addition that this treatment also reduces the ability of the enzyme to inhibit pinocytosis of PPME. Moreover, the 6-phosphomannose containing oligosaccharides of spleen β-glucuronidase are much less inhibitory for PPME uptake when released by endoglycosidase-H than when present on the enzyme.

TABLE I

Effect of Prior Alkaline Phosphatase Treatment and Enzyme Pinocytosis Inhibitors on ^{14}C-PPME Uptake

	Pinocytosis of PPME*	
Prior Treatment	cpm/mg/ 24 h	% Inhibition
None	6,145	—
Alkaline phosphatase	1,997	67.5
Alkaline phosphatase + 1 mM Pi	6,149	0.0
	Pinocytosis of PPME*	
Added Inhibitor	cpm/mg/ 24 h	% Inhibition
None	6,145	—
1 mM Man-6-P	2,382	61.2
0.5 mg/ml Phosphomannan‡	2,975	51.6
0.5 mg/ml Mannan§	6,133	0.2

* Pinocytosis measured as in Materials and Methods. PPME concentration = 1.0×10^{-6} M organic phosphate.

‡ Phosphomamman from *S. cerevisiae* mutant X2180-mnn 1 hydrolyzed for 15 min at 100°C in 0.1 N HCl.

§ Nonphosphorylated mannan from *H. holstii* grown in phophate deficient media.

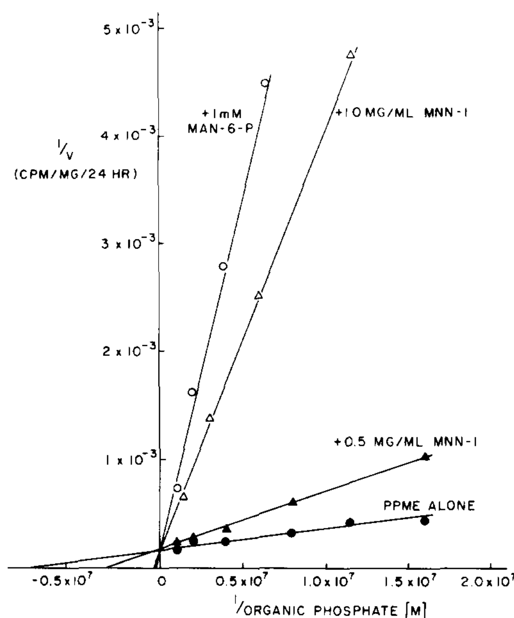

FIGURE 6 Double reciprocal plot of the effect of ^{14}C-PPME concentration on ^{14}C-PPME pinocytosis in the absence and presence of inhibitors. Pinocytosis was measured as described in Materials and Methods except that the indicated concentrations of PPME and inhibitor were used. (●) no additions, (▲) +0.5 mg/ml acid hydrolyzed (20 min at 100°C with 0.1 N HCl) X2180-mnn-1 Mannan, (△) +1.0 mg/ml acid hydrolyzed X2180-mnn-1-mannan, (○) +1.0 mM Man-6-P.

DISCUSSION

Strong acid hydrolysis reduces *H. holstii* phosphomannan to mannose and 6-phosphomannose (22). The potency of the strong acid hydrolysate as an inhibitor of enzyme pinocytosis was comparable to that expected from its 6-phosphomannose content. Hydrolysates produced by mild acid treatment, however, had far greater inhibitory potency than that expected from their 6-phosphomannose content. The two principal products in mild acid hydrolysates, the monovalent pentamannosylmonophosphate, $(Man)_5$-P and the large molecular

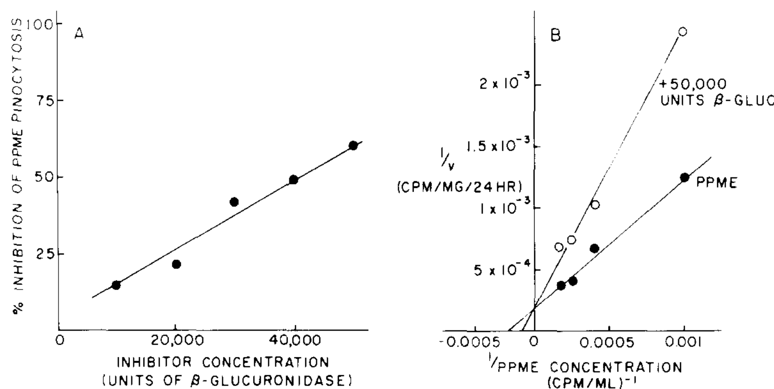

FIGURE 7 Inhibition of ^{14}C-PPME pinocytosis by purified spleen β-glucuronidase. Pinocytosis measurements were as described in Materials and Methods. (*A*) Concentration dependence of the inhibition of PPME pinocytosis by β-glucuronidase. PPME concentration equivalent to 0.1×10^{-6} M organic phosphate (~3,000 cpm). (*B*) Double reciprocal plot of effect of PPME concentration on phosphomannan pinocytosis by human I-cell disease fibroblasts in presence and absence of human spleen β-glucuronidase. (●) no addition, (○) +50,000 U/ml spleen β-glucuronidase.

TABLE II

Effect of Prior Alkaline Phosphatase Treatment on the Pinocytosis Rate and on the Inhibitory Potency of Spleen β-Glucuronidase for ^{14}C-PPME Pinocytosis

	Pinocytosis of β-glucuronidase*	
Treatment	U/mg/h	% Inhibition
None	77.0	—
+Alkaline phosphatase	16.5	79
	Pinocytosis of PPME‡	
Inhibitor	cpm/mg/24 h	% Inhibition
None	1,146	—
+50,000 U β-glucuronidase	417	63.6
+50,000 U alkaline phosphatase treated β-glucuronidase	1,142	0.3

* Measured as described in Materials and Methods with 1,000 U/ml β-glucuronidase.

‡ Measured as described in Materials and Methods with PPME concentration of 0.1×10^{-6} M organic phosphate.

TABLE III

Effect of Prior Endoglycosidase-H Treatment on Pinocytosis Rate and on the Inhibitory Potency of Spleen β-Glucuronidase for ^{14}C-PPME Pinocytosis

	Pinocytosis of β-glucuronidase*	
Treatment	U/mg/h	% Inhibition
None	87.6	—
+Endoglycosidase-H	2.1	97.6
	Pinocytosis of PPME‡	
Inhibitor	cpm/mg/24 h	% Inhibition
None	4,529	—
+60,000 U spleen β-glucuronidase	2,544	43.8
+60,000 U endoglycosidase-H treated spleen β-glucuronidase	4,144	8.5
+Oligosaccharides released by endoglycosidase-H from 60,000 U spleen β-glucuronidase	4,489	0.9

* Measured as described in Materials and Methods with 1,000 U/ml β-glucuronidase.

‡ Measured as described in Materials and Methods with PPME concentration of 0.25×10^{-6} M organic phosphate.

weight, multivalent PPME, were separated by gel chromatography and shown to be quite different from each other in their potency as pinocytosis inhibitors. The large molecular weight PPME fragment, which contained many exposed 6-phosphomannose groups, was nearly 100-fold more potent as an inhibitor per mole of organic phosphate (100,000-fold more potent per molecule).

Although $(Man)_5$-P and PPME both showed saturable binding to fibroblasts at 3°C, the binding per mole of phosphate of the large, multivalent PPME was much more efficient that that of the small, monovalent pentamannosyl-monophos-

phate. These two phosphomannan fragments also differed in their susceptibility to pinocytosis by fibroblasts. The rate and kinetics of uptake of the pentamannosyl-monophosphate suggest that it is taken up only by nonspecific fluid-phase endocytosis. Its rate of uptake is comparable to that for ^{125}I-albumin and is less than threefold the rate of [^{3}H]methoxydextran uptake (14), two markers for fluid-phase endocytosis which gave rate estimates of 0.5 and 0.15 μl/mg/h, respectively, for this process in fibroblasts. As with these nonspecific fluid-phase pinocytosis markers, the rate of $(Man)_5$-P uptake was linearly dependent on its concentration in the medium. Thus, although $(Man)_5$-P is an inhibitor of adsorptive pinocytosis of acid hydrolases, it is very poorly taken up by the process it inhibits. In contrast, the large molecular weight PPME fragment appears to be taken up by adsorptive pinocytosis. The PPME uptake process is saturable. At low ligand concentrations, its rate of uptake is at least 30-fold greater than can be explained by the rate of fluid-phase endocytosis as estimated with ^{125}I-albumin. As seen in Fig. 5, concentrations of PPME significantly above that required for saturation of binding actually inhibited pinocytosis. The apparent substrate inhibition of PPME uptake at high concentrations is consistent with pinocytosis of a multivalent ligand.

The simplest interpretation for the differences in inhibitory potency and susceptibility to pinocytosis between pentamannosyl-monophosphate and PPME is that the PPME is a multivalent ligand which interacts with multiple receptors on the surface of fibroblasts. If this is indeed the fundamental difference between these two ligands, one might then infer that a monovalent interaction with 6-phosphomannose or pentamannosylmonophosphate does not lead to internalization of these ligands, whereas a multivalent interaction of the PPME with one or many pinocytosis receptors does induce pinocytosis of the ligand.

Multivalent ligands could be taken up more rapidly for two reasons. First, multivalency could confer higher affinity of binding leading to a greater fraction of occupied receptors at low ligand concentrations. Secondly, multivalent ligands might actually stimulate adsorptive pinocytosis by cross-linking more than one receptor.

There are several precedents for multivalency in the recognition and uptake of ligands by cell surface receptors. Ash and Singer (2) have shown that the tetravalent lectin Con A induces a clustering of bound cell surface receptors which are then collected into a few large patches or a single "cap" on the cell surface. During and after the capping process, the bound ligands are internalized by endocytosis of the capped regions of the membrane. If divalent succinylated-Con A is added to cells, however, the clustering and capping of bound receptors fails to occur. In this case, capping can subsequently be induced if the cells that have been reacted with succinylated-Con A are further reacted with antibodies to the lectin. Similarly, the effective capping of Fab fragments specific for the T25 antigen and the H2 antigen on lymphocytes requires a second cross-linking antibody (24).

The evidence that uptake of the multivalent PPME ligand depends on the same receptors that mediate internalization of acid hydrolases is considerable. The uptake of both the PPME and acid hydrolases is diminished by prior treatment of these ligands with alkaline phosphatase. Similarly, the uptake of both the PPME and acid hydrolases is competitively inhibited by mannose-6-phosphate and mild acid-hydrolyzed phosphomannan from *S. cerevisiae* mutant X2180 mnn-1, but is not inhibited by a nonphosphorylated *H. holstii* mannan. Moreover, just as the PPME competitively inhibits the pinocytosis of purified high-uptake human β-glucuronidase by fibroblasts, so also is the pinocytosis of the PPME fragment competitively inhibited by the high-uptake enzyme.

The suggestion that the same pinocytosis receptors are involved in pinocytosis of lysosomal enzymes and the multivalent PPME phosphomannan fragment of *H. holstii* raises an interesting question concerning the pinocytosis of lysosomal hydrolases, i.e., are acid hydrolases multivalent ligands which induce their own pinocytosis by binding to multiple cell surface receptors? One can easily visualize how an acid hydrolase such as human β-glucuronidase could be a multivalent ligand. The enzyme is a glycoprotein and appears to be a tetramer of identical 75,000-mol wt subunits (6). As such, a multivalent interaction with several cell surface receptors could result from any of several possibilities: (*a*) through an interaction of single 6-phosphomannose groups on different protein subunits; (*b*) through each subunit having more than one oligosaccharide chain, each of which could bear a 6-phosphomannose moiety; (*c*) multiple 6-phosphomannose moieties could be present on a single oligosaccharide chain. We have demonstrated up to 4.4 mol of 6-phosphomannose/mol high-uptake enzyme, and a direct correlation between the 6-phosphomannose content

and the susceptibility to pinocytosis of different fractions of β-glucuronidase isolated from spleen (18).

While it is clear that acid hydrolases could be multivalent ligands and that their pinocytosis could depend on an interaction with multiple pinocytosis receptors, an alternate possibility is that high-uptake acid hydrolases are monovalent ligands which bind to pinocytosis receptors with much higher affinity than 6-phosphomannose due to some other structural feature of the recognition marker. In this case, pinocytosis of the multivalent PPME phosphomannan fragment could simply result from its binding to and cross-linking multiple acid hydrolase receptors. However, the observations that the potency of high-uptake enzyme as an inhibitor of PPME pinocytosis is dramatically reduced by an endoglycosidase-H treatment that releases the phosphomannosyl-containing oligosaccharides from the enzyme is certainly compatible with the idea that high-uptake enzyme acts as a multivalent ligand.

Since multivalent *H. holstii* PPME fragments are pinocytosed by the same receptors that mediate uptake of acid hydrolases by fibroblasts, they may prove useful ligands in the analysis of the fibroblast pinocytosis receptor for lysosomal hydrolases. Moreover, they can probably be covalently attached to enzymes and other agents to target these compounds for uptake by cell types which express the phosphomannosyl-enzyme pinocytosis receptors.

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