Journal of Cellular Biochemistry 22:69–86 (1983)
Protein Transport and Secretion 15–32

Comparative Kinetics of Phosphomannosyl Receptor-Mediated Pinocytosis of Fibroblast Secretion Acid Hydrolases and Glycopeptides Prepared From Them

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In a previous report we demonstrated that phosphorylated oligosaccharides isolated from acid hydrolases were subject to pinocytosis by phosphomannosyl receptors present on the cell surface of human fibroblasts [9]. However, limiting quantities of oligosaccharides precluded detailed comparison of the kinetics of pinocytosis of these phosphorylated oligosaccharides to those of the acid hydrolases from which they were derived. In this report we present studies comparing the kinetics of pinocytosis of acid hydrolases from NH_4Cl-induced fibroblast secretions with those of concanavalin A-binding glycopeptides prepared from them by pronase digestion. The uptake of both secretion acid hydrolases and ^{125}I-labeled glycopeptides was linear for at least 3 hr, saturable, inhibited competitively by mannose 6-phosphate, and destroyed by prior treatment of the ligand with alkaline phosphatase. The inhibition constants of excess unlabeled glycopeptide for the uptake of ^{125}I-labeled glycopeptides (K_i of 1.5×10^{-6} M) and for the uptake of secretion acid hydrolases (K_i of 2.2×10^{-6} M) were remarkably similar. Furthermore, the K_i for mannose 6-phosphate inhibition of pinocytosis of glycopeptide uptake (3×10^{-5} M) compares closely to that previously determined for the pinocytosis of intact "high-uptake" acid hydrolases ($3–6 \times 10^{-5}$ M).

"High-uptake" fractions of both ligands were prepared and quantified by affinity chromatography on immobilized phosphomannosyl receptors purified from bovine liver. Only 10% of the concanavalin A-binding glycopeptides bound to the immobilized phosphomannosyl receptors, while 80% of the acid hydrolases from which they were prepared bound and were eluted with 10 mM mannose 6-phosphate. However, the fraction of each type of ligand that binds to the immobilized phosphomannosyl receptors accounts for all the uptake activity of that ligand.

Abbreviations used: Man 6-P, mannose 6-phosphate; Con A, concanavalin A; PBS, phosphate-buffered saline

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Received April 30, 1983; revised and accepted June 17, 1983.

The pinocytosis rates (% of added ligand internalized/mg protein/hr) of the "high-uptake" fraction of both intact acid hydrolase (12%/mg/hr) and glycopeptide (6%/mg/hr) differed by only twofold. The apparent K_{uptake} for both ligands was of the same order of magnitude. The similarity in the kinetics of pinocytosis of the secreted acid hydrolases and of the phosphomannose-bearing glycopeptides prepared from them suggests that the structural information which confers high-affinity binding to the phosphomannosyl receptor is contained in the glycopeptide units themselves. No additional information from the intact protein backbone appears essential for phosphomannosyl receptor-mediated pinocytosis.

Key words: phosphomannosyl receptor, pinocytosis, fibroblast secretions, glycopeptides, acid hydrolases, lysosomotropic amines, β-hexosaminidase B

Pinocytosis and intracellular transport of acid hydrolases to lysosomes is mediated through phosphomannosyl receptors on membranes and on phosphomannosyl moieties on the oligosaccharide chains of the enzymes [reviewed in 1–3]. Structural studies have shown that phosphomannose groups are located on high mannose-type oligosaccharide chains that contain one or two 6-phosphomannosyl groups in monoester or diester linkage to any one of five of the nine mannoses on the oligosaccharide chain [4–6]. Phosphomannosyl oligosaccharides released from β-glucuronidase [7,8] or from acid hydrolases secreted by fibroblasts [6,9] contain a heterogeneous mixture of oligosaccharides that vary in charge, number of mannose residues, and number of phosphates in either monoester linkage or in diester linkage to an α-linked N-acetylglucosamine residue.

Phosphomannosyl recognition in pinocytosis of acid hydrolases was inferred initially from the kinetics of inhibition of enzyme uptake by compounds containing Man 6-P, and from the loss of the susceptibility to pinocytosis when enzymes were treated with phosphatase [10–13]. Although considerable evidence [10–15] suggested that 6-phosphomonoester moieties of mannose are essential components of the recognition marker on acid hydrolases, the exact structural features of the ligand that is recognized by the receptor and subsequently endocytosed are still being characterized. As an approach to determining the structural features of the phosphomannosyl recognition marker required for receptor recognition we recently studied the pinocytosis and binding properties of phosphorylated oligosaccharides isolated from fibroblast secretion glycoproteins [9,16]. We found that oligosaccharides bearing two phosphomonoesters of mannose were pinocytosed by fibroblasts at a rate about 30-fold greater than that observed for oligosaccharides containing a single phosphomonoester or two phosphodiesters [9]. Furthermore, only those oligosaccharides containing two phosphomonoesters were bound to immobilized phosphomannosyl receptors and specifically eluted with Man 6-P [16]. Although these studies clearly showed that oligosaccharide chains containing two phosphomonoesters were much better ligands for the phosphomannosyl receptor than those bearing either a single phosphate or two phosphates in diester linkage, limiting material precluded any detailed kinetic comparison of the interaction of these oligosaccharides with phosphomannosyl receptors to those of the acid hydrolases from which they were isolated.

Gonzales-Noriega et al [17] and Hasilik and Neufeld [18] have shown that lysosomotropic amines cause normal fibroblast lines to secrete most newly synthesized lysosomal enzyme precursors into the extracellular medium as high-uptake enzyme forms. In the studies reported here we took advantage of the effect of lysosomotropic amines on enzyme secretion to produce ligands of fibroblast origin in

sufficient quantity for kinetic studies of the phosphomannosyl receptor system. Ammonium chloride-stimulated fibroblast secretion acid hydrolases were collected and glycopeptides prepared from them by pronase digestion. We then used β-hexosaminidase B as a "marker" enzyme of the several acid hydrolases present in the ammonium chloride-stimulated secretions, and the glycopeptides generated from the secretions, to compare several kinetic parameters of pinocytosis by the phosphomannosyl receptor present on the cell surface of fibroblasts. This comparison depends on the assumption that the pinocytosis of the β-hexosaminidase activity present in the secretions accurately reflects the pinocytosis and binding properties of the other phosphomannosyl-containing acid hydrolases present in the secretion mixture from which the glycopeptides were generated. Previous results showing similar uptake properties of several different phosphomannosyl enzyme activities [1–3,10–13] suggest that β-hexosaminidase is a valid marker for the uptake of enzymes present in secretion glycoproteins. Our results demonstrate (1) that secretion acid hydrolases and glycopeptides prepared from them are taken up by fibroblasts with similar kinetics, suggesting that the glycopeptide units themselves contain the structural information required for pinocytosis; (2) that uncovered phosphates on secretion β-hexosaminidase B and glycopeptides are important for pinocytosis and binding to immobilized phosphomannosyl receptors; (3) that the high-uptake properties of acid hydrolases is conferred by a minority population of the total oligosaccharide chains; and (4) that an immobilized phosphomannosyl receptor column can be used for the separation of high- and low-uptake fractions of lysosomal enzymes and glycopeptides prepared from them.

EXPERIMENTAL PROCEDURES

Materials

Several reagents, including fluorescamine, were purchased from Sigma Chemical Co, St Louis, MO. Fluorometric substrates were obtained from Research Products International, Elk Grove, IL. Concanavalin A (Con A)-Sepharose was from Pharmacia. Carrier-free ^{125}I was obtained from New England Nuclear. Alkaline phosphatase from Escherichia coli (P-4252) was from Sigma Chemical Co, and pronase from Boehringer Mannheim. Bio-Gel P-2 (200–400 mesh)was from Bio-Rad Laboratories. N-succinimidyl-3-(4-hydroxyphenyl) propionate was from Pierce Chemical Co, Rockford, IL.

Assays

β-Hexosaminidase activity was determined fluorometrically using the synthetic substrate 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside as described previously [19]. One unit of activity is the amount of enzyme which catalyzes the release of 1 nmole of 4-methylumbelliferone hr^{-1}. Protein was measured by the method of Lowry et al [20].

Collection of Fibroblast Secretions

Tay-Sachs disease fibroblasts (GM-502) were obtained from the Human Mutant Cell Repository, Camden, NJ. Cultures were maintained at 37°C in minimal essential medium (MEM Eagle Medium, Gibco), supplemented with 15% heat-inactivated fetal calf serum (Gibco), 1 mM sodium pyruvate, 100 units/ml penicillin, and 100 mg/ml

streptomycin sulfate. Addition of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (15 mM) maintained pH at 7.6. Cells were grown to confluency in 490-cm^2 roller bottles (Corning), washed with 50 ml of 0.9% NaCl, and maintained in 50 ml per roller bottle of serum-free Waymouth media (KC Biological) containing 10 mM NH_4Cl. The cells were maintained at 37°C for 24 hr, at which time the media was changed and the secretion-containing media stored at -20°C.

Preparation of Glycoproteins and Glycopeptides From Fibroblast Secretions

Secretions were collected from Tay-Sachs disease fibroblasts (GM-502) maintained in serum-free Waymouth medium (KC Biological) containing 10 mM NH_4Cl and stored at −20°C. A total of 8 liters of media containing 22 × 10^6 units of β-hexosaminidase B was thawed, filtered first by vacuum through a 70–100 μm fritted disc-filtering funnel, then through a Millipore borosilicate microfiber glass prefilter, and finally through a membrane filter (Gelman, 0.45 μm). The secretions were then concentrated 30-fold by ultrafiltration using a PM 10 membrane filter (Amicon). The concentrated secretions were then applied to a column of Con A-Sepharose (5.0 ml of packed beads) equilibrated with phosphate-buffered saline (PBS) at 4°C. The column was then washed with 35 ml of PBS and eluted at room temperature with 35 ml of PBS containing 0.75 M α-methylmannoside. The eluted glycoproteins were dialyzed extensively against 10 mM TRIS, pH 8.0. A small fraction of the dialyzed material (~2%) was saved and stored at −20°C while the rest (containing 20 × 10^6 units of β-hexosaminidase B) was frozen and lyophilized. The lyophilized Con A-Sepharose-binding secretions were dissolved in 3.0 ml of buffer containing 200 mM glucose 6-posphate, 50 mM N-acetylglucosamine, and 20 mM $CaCl_2$. The pH was adjusted to 8.0 with 1.0 M TRIS giving a final TRIS concentration of 0.25 M. Pronase was added to this suspension to generate glycopeptides by the procedure previously described [9]. Following pronase digestion, the mixture was diluted to 30 ml with PBS and added to a 5.0-ml column of Con A-Sepharose equlibrated with PBS at 4°C. The column was washed with PBS (25 ml) and eluted at room temperature with PBS (25 ml) containing 0.5 α-methylmannoside. The glycopeptides eluted with α-methylmannoside were then lyophilized to reduce the volume and desalted on a Bio-Gel P-2 column in water. The void volume containing glycopeptides was lyophilized. solubilized in 2.0 ml of 10 mM sodium phosphate, pH 7.0, and stored at −20°C.

Determination of Primary Amino Groups

The concentration of glycopeptides was estimated by assaying for primary amino groups. Primary amino groups were measured by adding with vigorous mixing 0.5 ml of a solution containing 12 mg of fluorescamine per 50 ml of dry acetone to samples containing up to 10 nmoles of primary amino group in 1.5 ml of 0.2 M sodium borate, pH 8.5. Fluorescence of samples was measured and compared to alanine standards.

Iodination of Glycopeptides

Iodinated glycopeptides were prepared by first coupling the succinimide ester of 4-hydroxyphenyl propionic acid to the amino terminus of the glycopeptide using a tenfold molar excess of the ester in 250 μl of 50 mM sodium phosphate, pH 8.5, at 4°C overnight. This mixture was desalted on a Bio-Gel P-2 column and iodinated by

the chloramine T procedure [21]. Free ^{125}I was removed by desalting on a column of Bio-Gel P-2 and the void volume containing the ^{125}I-labeled glycopeptides was added to a column of (0.5 × 2.0 cm) of Con A-Sepharose equilibrated in PBS. The Con A-Sepharose column was washed with 10 ml of PBS and the ^{125}I-labeled glycopeptides containing high mannose-type oligosaccharide chains were eluted with 3.0 ml of 0.5 M α-methylmannoside in PBS, heated to 60°C. The α-methylmannoside was removed by desalting on a column of Bio-Gel P-2. Specific activity of glycopeptides ranged from 1,000 to 10,000 cpm/pmoles.

Pinocytosis Measurements

Measurements of β-hexosaminidase pinocytosis by β-hexosaminidase-deficient Sandhoff disease fibroblasts (available from the Repository for Mutant Human Cell Strains, Montreal Children's Hospital, Montreal, Canada, cell strain WG98) were carried out by the method described previously [10,11] with the indicated concentrations of enzyme in 1.0 ml of uptake medium in the presence or absence of 2 mM Man 6-P or other indicated inhibitors. Enzyme internalized over 2 hr was measured in cells disrupted by the addition of 1.0 ml of distilled water followed by freezing 20 min at −20°C and then thawing. Aliquots of the disrupted cells were then assayed for enzyme and protein, and the amount of enzyme internalized corrected for the level of endogenous cell enzyme activity. Measurements of pinocytosis of ^{125}I-labeled glycopeptides was carried out in duplicate 35-mm petri dishes containing I-cell disease fibroblasts (available from the Human Mutant Cell Repository, Camden, NJ). The indicated concentration of radiolabeled glycopeptide was added to the cells in 1.0 ml of medium. After 2 hr at 37°C, the uptake was terminated by chilling the cells on ice, removing the uptake medium and carefully washing the cells six times with 3-ml portions of ice-cold PBS. The cells were then lysed with 1.0 ml of 1% sodium deoxycholate and radioactivity and protein determined in the lysate. The uptake of radiolabeled glycopeptides was inhibited at least 95% by 2 mM Man 6-P. Sandhoff disease fibroblasts were chosen for measurements of enzyme pinocytosis because of their low levels of endogenous enzyme. I-cell disease fibroblasts were chosen for glycopeptide uptake because they are deficient intracellularly for many acid hydrolases and would be expected to degrade accumulated, low molecular weight ligands more slowly than other cell types. We have shown previously that at low ligand concentrations, the two different cell lines accumulate enzyme at equivalent rates with identical K_{uptake} values [our unpublished data]. Kinetically, the cell lines differ in uptake primarily at high ligand concentrations at which the uptake rate approachs the V_{max}.

Preparation and Immobilization of Phosphomannosyl Receptors

Bovine liver phosphomannosyl receptors were purified on a Dictyostelium discoideum glycoprotein secretion-Affi Gel conjugate and immoblized by covalent coupling to Affi-Gel as described previously [16].

Affinity Chromatography of Phosphorylated Secretion Enzymes and Glycopeptides on Immobilized Phosphomannosyl Receptors

Fibroblast secretion enzyme was applied in 0.5 ml of 50 mM TRIS-HCl, 5 mM sodium phosphate, 0.05% Triton X-100, 0.025% sodium azide (pH 7.0) at a flow rate of 6.0 ml/hr to a column of (0.7 × 26 cm) of Affi-Gel-phosphomannosyl receptor

euqilibrated with the same buffer. The column was then washed at the same flow rate with 32 ml of buffer followed by 32 ml of buffer containing 10 mM Man 6-P. Fractions (1.6 ml) were collected and assayed for enzyme activity. Radiolabeled glycopeptides prepared from fibroblast secretions were applied in 0.25 ml of 5 mM sodium phosphate, 0.05% Triton X-100, 0.025% sodium azide (pH 7.0) at a flow rate of 3.0 ml/hr to a column (0.7 × 6.5 cm) of Affi-Gel-phosphomannosyl receptor equilibrated with the same buffer at 4°C. After the sample had loaded, the column was turned off overnight and then washed at a flow rate of 3.0 ml/hr with 10 ml of 5 mM sodium phosphate, 0.05% Triton X-100, 0.025% sodium azide (pH 7.0), followed by 6 ml of the same buffer containing 10 mM Man 6-P. Fractions of 0.4 ml were collected and radioactivity determined in a Packard auto-gamma scintillation spectrometer.

Alkaline Phosphatase Treatment of Enzyme and Glycopeptides

Fibroblast secretion β-hexosaminidase activity was treated with alkaline phosphatase as folows. Aliquots of the enzyme were digested with E coli alkaline phosphatase (10 units) in 0.5 ml of 0.15 M TRIS-HCl buffer, pH 8.0. After incubation for 2 hr at 37°C, the reaction mixture was transferred to a dialysis bag and the incubation continued for 2 additional hr with dialysis against 4 l of 0.15 M TRIS-HCl buffer, pH 8.0, maintained at 37°C. Glycopeptides were digested with E coli alkaline phosphatase (1 unit) in 0.025 ml of 0.15 M TRIS-HCl buffer, pH 8.0, for 3 hr at 37°C.

Mild Acid Hydrolysis of Glycopeptides

Glycopeptides in 0.2 ml of 0.01 N HCl were heated at 100°C for 30 min, cooled on ice, and neutralized with 1.0 M TRIS-HCl.

RESULTS

Characterization of the β-Hexosaminidase B Activity Present in Ammonium Chloride-Stimulated Fibroblast Secretion Glycoproteins

The addition of lysosomotropic amines, such as ammonium chloride, to the growth medium of cultured cells disrupts the normal receptor-mediated process by which newly synthesized acid hydrolases are segregated from other products of the endoplasmic reticulum. Failure to segregate the acid hydrolases for delivery to lysosomes allows them to be secreted as high-uptake precursor enzyme forms instead [17,18]. We previously showed that β-hexosaminidase B prepared from ammonium chloride-stimulated fibroblast secretions was susceptible to Man 6-P inhibitable pinocytosis by human fibroblasts and was enriched in high-uptake enzyme forms [17]. Also, as we have demonstrated previously for β-glucuronidase purified from human spleen [22], β-hexosaminidase B present in ammonium chloride-stimulated fibroblast secretions exhibits saturable, Man 6-P inhibitable, binding to fibroblast membranes (data not shown). Furthermore, the binding is destroyed by prior treatment of the β-hexosaminidase B with alkaline phosphatase or endoglycosidase H [data not shown]—treatments which also destroy the binding of purified human spleen β-glucuronidase to fibroblast membranes [22] and the susceptibility of high-uptake enzyme forms to pinocytosis by fibroblasts [10,23]. Therefore, the β-hexosaminidase B activity present in ammonium chloride-stimulated fibroblast secretions has all the properties of phosphomannosyl-acid hydrolases purified from several tissue sources [10–13] and should

provide a good preparative source of ligand for studies of the phosphomannosyl enzyme receptor pinocytosis system. In fact, for reasons addressed in the discussion, secretion acid hydrolases have several advantages in studies of the phosphomannosyl recognition system over acid hydrolases purified from tissue homogenates.

Pinocytosis Properties of Secreted Acid Hydrolases and Glycopeptides Prepared From Them

Secretions were colllected from Tay-Sachs disease fibroblasts maintained in the presence of 10 mM amonium chloride, concentrated by ultrafiltration, and a glycoprotein-rich fraction purified from the concentrated secretions by chromatography on Con A-Sepharose. Radiolabeled glycopeptides containing high mannose-type oligosaccharide chains were prepared from secretion acid hydrolases by exhaustive pronase digestion, chromatography on Con A-Sepharose, and iodination with ^{125}I. To compare the pinocytosis properties of ^{125}I-labeled glycopeptides prepared from fibroblast secretions with the uptake properties of the intact acid hydrolases from which they were prepared we chose β-hexosaminidase B as a marker for the uptake of acid hydrolases present in the secretions. A typical time course of β-hexosaminidase B pinocytosis by fibroblasts is shown in Figure 1A. Enzyme uptake was linear for at least 3 hr. The time course for pinocytosis of ^{125}I-glycopeptides was similar (Fig. 1B).

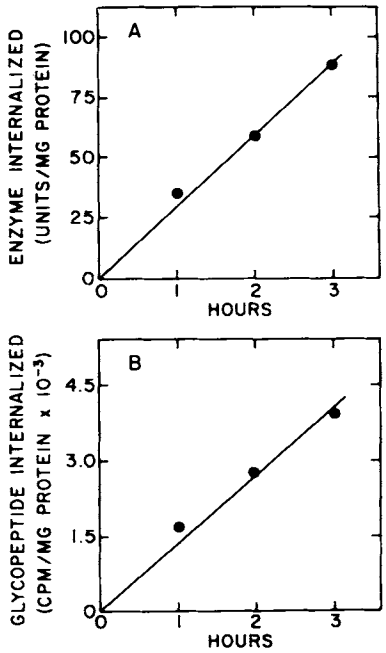

Fig. 1. Time course of enzyme and glycopeptide uptake. A. Fibroblast secretion glycoproteins were prepared as described under Experimental Procedures and 300 units of β-hexosaminidase was incubated with confluent 35-mm dishes of β-hexosaminidase deficient fibroblasts for the indicated times. Internalization of added enzyme was determined as described under Experimental Procedures. B. Radiolabeled glycopeptides (230 pmoles, 265,000 cpm) prepared from fibroblast secretions were incubated for the indicated times with confluent 35-mm dishes of I-cell disease fibroblasts. Internalization of added glycopeptide was determined as described under Experimental Procedures.

Figure 2A shows the kinetics of enzyme pinocytosis by fibroblasts. The process was saturable with increasing enzyme concentration. The apparent K_{uptake} of β-hexosaminidase, which is one of several acid hydrolases present in this mixture, was calculated from a double reciprocal plot (not shown) of the data in Figure 2A. The apparent K_{uptake} for β-hexosaminidase in the mixture was 572 units/ml. This value must be regarded as an estimate since other acid hydrolases present in fibroblast secretions would act as competitive inhibitors of the uptake of β-hexosaminidase. As shown in Figure 2B, the uptake of ^{125}I-glycopeptide was also saturable with increasing ligand concentration and displayed a K_{uptake} of 1.5×10^{-6} M. Taken together, the data in Figures 1 and 2 demonstrate that glycopeptides are subject to pinocytosis by fibroblasts with uptake properties grossly similar to those of the intact acid hydrolases from which they were generated.

We next compared the ability of excess unlabeled glycopeptides to inhibit the uptake of ^{125}I-glycopeptide and secretion acid hydrolases. In the experiment shown in Figure 3A, increasing amounts of unlabeled glycopeptide were added to a fixed

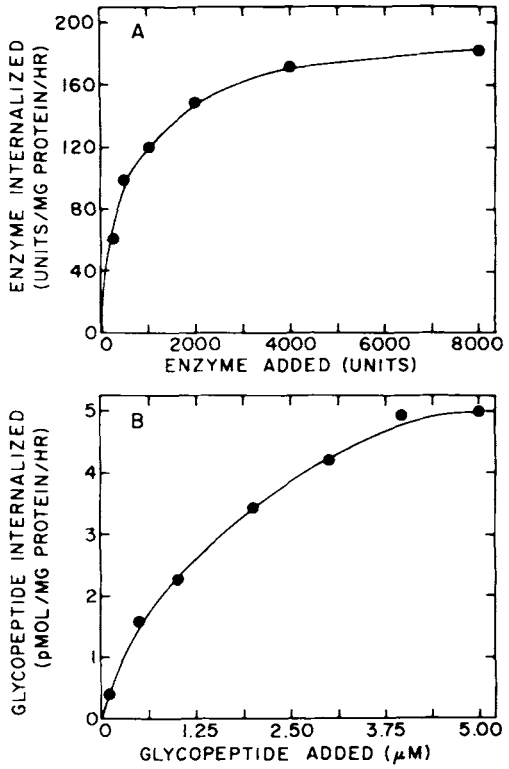

Fig. 2. Kinetics of pinocytosis of enzyme and glycopeptides prepared from fibroblast secretions. A, Fibroblast secretion glycoproteins were prepared as described under Experimental Procedures. Aliquots of this glycoprotein preparation containing the indicated amounts of β-hexosaminidase B activity were then added to β-hexosaminidase deficient fibroblasts and the internalization of enzyme determined as described under Experimental Procedures. B. Glycopeptides were prepared from fibroblast secretion glycoproteins and radiolabeled as described under Experimental Procedures. The indicated amounts of glycopeptide were then added to I-cell disease fibroblasts and pinocytosis determined as described under Experimental Procedures.

concentration of ^{125}I-glycopeptide and the rates of pinocytosis by fibroblasts were measured. In this experiment, the unlabeled ligand acts as a competitive inhibitor whose K_i equals the K_{uptake}. From the plot of 1/v vs glycopeptide concentration shown in Figure 3A, one can calculate that the K_i (and therefore K_{uptake}) of the glycopeptides is 1.5×10^{-6} M. Figure 3B shows a similar experiment in which the pinocytosis of two fixed amounts of enzyme was measured in the presence of increasing amounts of unlabeled glycopeptide. Again, the glycopeptide acted as a competitive-type inhibitor of enzyme uptake with a K_i of 2.2×10^{-6} M very similar to that for uptake of glycopeptides themselves. A similar experiment (not shown) using Man 6-P as the uptake inhibitor displayed a K_i of 3×10^{-5} M for inhibition of glycopeptide uptake. This compares with previously reported K_is of $3–6 \times 10^{-5}$ M for Man 6-P inhibition of pinocytosis of intact high-uptake acid hydrolases [10,12].

Binding of Glycopeptides and Fibroblast Secretion Enzymes to Immobilized Phosphomannosyl Receptors

We previously showed a correlation between the binding of phosphorylated oligosaccharides to immoblized phosphomannosyl receptors and their ability to be

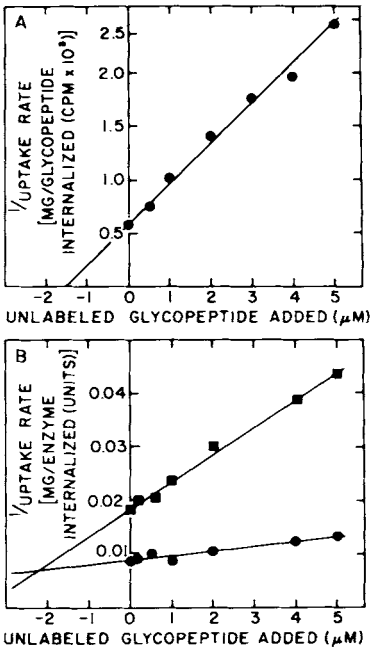

Fig. 3. Inhibition of fibroblast secretion enzyme and radiolabeled glycopeptide pinocytosis by excess, unlabeled glycopeptide prepared from fibroblast secretion glycoproteins. A. The indicated concentrations of unlabeled glycopeptide were added to 0.025 μM radiolabeled glycopeptide and the pinocytosis of the radiolabeled ligand in the mixture was determined as described under Experimental Procedures. The X-intercept is equal to -(K_{uptake} plus the concentration of radiolabeled glycopeptide). B. The indicated concentrations of unlabeled glycopeptide were added to aliquots of fibroblast secretion glycoproteins containing 250 (■) and 5,000 (●) units of β-hexosaminidase in 1.0 ml of uptake medium. Pinocytosis of the enzyme activity was determined as described under Experimental Procedures. The value of the X-axis at the point where the two different enzyme concentration lines intersect is equal to the K_i of the glycopeptides for enzyme pinocytosis.

pinocytosed by fibroblasts [9,16]. Figure 4A shows the profile obtained when ^{125}I-glycopeptides prepared from fibroblast secretion glycoproteins were chromatographed on purified bovine liver phosphomannosyl receptors immobilized on an agarose gel bead support. Approximately 90% of the Con A-Sepharose binding glycopeptides prepared from fibroblast secretions failed to be retained by the column; they eluted as a large, sharp peak at the void volume of the column. The remainder of the glycopeptides ($\sim$ 10%) were bound by the column, and eluted as a single peak by 10 mM Man 6-P. Figure 4B shows that after prior treatment of the glycopeptides with alkaline phosphatase, essentially all of the added glycopeptide ($<$99%) failed to bind to the immobilized receptors, indicating that the binding of glycopeptides in this preparation to the receptor column ocurred through phosphomonoesters of mannose.

Figure 5A shows the strikingly different elution profile obtained when intact, fibroblast secretion acid hydrolases were chromatographed on the immoblized receptor column. In contrast to the glycopeptides, most of the β-hexosaminidase B activity (80%) bound to the column and was eluted by 10 mM Man 6-P. Only 20% failed to

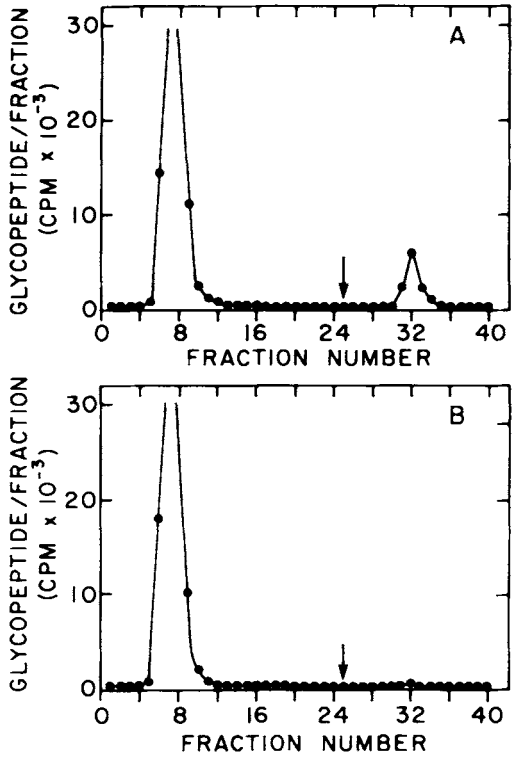

Fig. 4. Affinity chromatography of glycopeptides prepared from fibroblast secretions on immobilized phosphomannosyl receptors. A. ^{125}I-labeled glycopeptides (133,000 cpm) were chromatographed on immobilized phosphomannosyl receptors as described under Experimental Procedures and the amount of radioactivity present in each fraction was determined. B. Same as A except that the glycopeptides were pretreated with alkaline phosphatase as described under Experimental Procedures. (The arrows indicate where 10m Man 6-P was added to the column elution buffer; the first fraction containing Man 6-P was 31.)

bind. Thus, even though the glycopeptide species which can bind to the phosphomannosyl receptors represent only a small portion of the total oligosaccharide population present on secretion glycoproteins, they are apparently distributed in such a way that the majority of the enzyme present in the secretions have at least one of the phosphorylated oligosaccharide species necessary for receptor binding. Figure 5B shows that, as with glycopeptides, prior treatment of β-hexosaminidase B enzyme with alkaline phosphatase destroys its ability to bind to the phosphomannosyl receptor column.

In Table I we compare the uptake properties of the species of enzyme and glycopeptides which either bound to or failed to bind to the immobilized phosphomannosyl receptors. The uptake rate for the total secretion enzyme was ~10%/mg/hr. However, the enzyme which failed to bind to the immobilized receptor was taken up at only a fraction of that rate. By contrast, the enzyme which was retained from the column and eluted by Man 6-P was enriched in high-uptake enzyme (its uptake was 20% greater than the original enzyme pool). Prior treatment of the total enzyme with

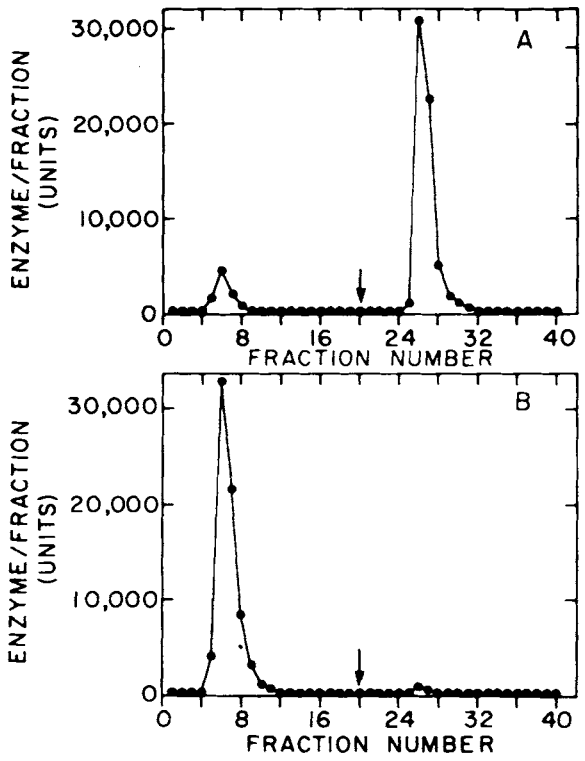

Fig. 5. Affinity chromatography of fibroblast secretion enzyme on immmobilized phosphomannosyl receptors. Glycoproteins were prepared from fibroblast secretions as described under Experimental Procedures. A. An aliquot of fibroblast secretion glycoproteins containing 75,000 units of β-hexosaminidase was chromatographed on immobilized phosphomannosyl receptors as described under Experimental Procedures and the amount of enzyme present in each fraction was determined. B. Same as A except that the glycoprotein secretions were pretreated with alkaline phosphatase as described under Experimental Procedures. (The arrows indicate where 10 m Man 6-P was added to the column elution buffer; the first fraction containing Man 6-P was 5.)

TABLE I. Pinocytosis of High and Low-Uptake Fractions of Acid Hydrolases and Glycopeptides Prepared by Affinity Chromatography on Immoblized Phosphomannosyl Receptors*

Ligand	Susceptibility to pinocytosis (% of added ligand internalized per mg protein per hr)
Acid hydrolase[a]	
Total	9.95
Total (phosphatase treated)	0.60
Unbound (20% of total)	0.40
Bound (80% of total)	12.11
Glycopeptide[b]	
Total	0.67
Total (phosphatase treated)	0.01
Unbound (90% of total)	<0.01
Bound (10% of total)	6.25

*Fibroblast secretion glycoproteins were prepared as described under Experimental Procedures.

[a]Aliquots of the secretion acid hydrolase preparation were either treated with phosphatase or subjected to affinity chromatography on immobilized phosphomannosyl receptors. Fractions containing untreated, phosphatase treated, or untreated acid hydrolase which had bound or failed to interact (unbound) with the immobilized phosphomannosyl receptors were then tested for their susceptibility to pinocytosis as described under Experimental Procedures. Each uptake mixture contained ~1,000 units of β-hexosaminidase activity per ml.

[b]Glycopeptides were prepared from fibroblast secretion acid hydrolases and labeled with ^{125}I as described under Experimental Procedures. The radiolabeled glycopeptides were then chromatographed on immobilized phosphomannosyl receptors or treated with alkaline phosphatase and the different fractions were tested for susceptibility to pinocytosis as described under Experimental Procedures.

alkaline phosphatase, which abolished binding to the immobilized receptors, also destroyed susceptibility of the enzyme to pinocytosis.

A similar pattern of susceptibility to pinocytosis was seen for the glycopeptides as well. The uptake rate for the total glycopeptide pool was 0.67%/mg/hr and this uptake was abolished by prior treatment with alkaline phosphatase (Table I). As was the case with secretion enzyme, glycopeptides which failed to bind the column were not susceptible to pinocytosis. The fraction of this ligand which bound to the immobilized receptor column and was eluted with Man 6-P was enriched nearly tenfold in its uptake rate. This high rate of uptake for specifically bound glycopeptides approximates the rate seen with high-uptake acid hydrolases. These results demonstrate that for both secretion enzyme and glycopeptides prepared from these enzymes, there is a striking correlation between susceptibility to pinocytosis by the phosphomannosyl recognition system and the ability to bind to the immobilized phosphomanosyl receptors.

We have shown previously that oligosaccharide chains bearing two phosphates in diester linkage are neither pinocytosed by fibroblasts [9] nor bound to immobilized phosphomannosyl receptors [16] unless they are first treated with mild acid to remove the α-N-acetylglucosamine residues covering the phosphomannosyl groups. A similar result can be demonstrated using the glycopeptides prepared from fibroblast secretions. Prior treatment of total glycopeptides with mild acid results in a 25% increase in their rate of uptake (not shown), suggesting that the total pool of glycopeptides contains a population bearing phosphomannosyl residues which are initially covered, and which are able to interact with phosphomannosyl receptors only after treatment

with mild acid. To verify this interpretation, we took glycopeptides that failed to bind to immobilized receptors and which were not subject to pinocytosis (Table I) and treated them with mild acid under conditions which would remove α-N-acetylglucosamine residues covering the phosphomannosyl groups. Following mild acid treatment, these glycopeptides were subject to pinocytosis with an initial rate of 0.17%/mg/hr (data not shown). Figure 6 shows the effect of mild acid treatment on the binding properties of glycopeptides that initially failed to bind the immobilized receptor column. Figure 6A is a control showing that untreated glycopeptides which failed to bind on the first column run failed to bind on the second pass over the column. However, following treatment with mild acid (Fig. 6B) 2.6% of the glycopeptides which had originally failed to bind to the phosphomannosyl receptor column were now retained by the immobilized receptors and eluted only after the addition of Man 6-P. Thus, the total glycopeptide population includes a fraction which initially will not bind to immobilized phosphomannosyl receptors, but which acquire the ability to bind after treatment with mild acid. This result suggests that the increase in the uptake rate of the mild acid-treated glycopeptides is due to uncovering of phosphate residues, allowing a new population of glycopeptides to interact with the immobilized receptors sufficiently to be retained, and also to interact with cell surface receptors productively leading to their pinocytosis.

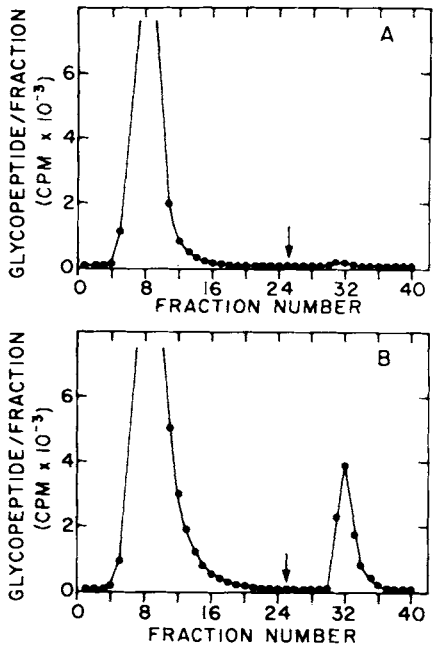

Fig. 6. Affinity chromatography of mild acid-treated glycopeptides on immobilized phosphomannosyl receptors. Radiolabeled glycopeptides were subjected to affinity chromatography on immobilized phosphomannosyl receptors. That fraction which failed to interact (unbound) was pooled and an aliquot (380,000 cpm) rechromatographed (A) or treated with mild acid (0.01 N HCl at 100°C for 30 min) and rechromatographed (B) on immobilized phosphomannosyl receptors as described under Experimental Procedures. (The arrows indicate where 10 m Man 6-P was added to the column elution buffer; the first fraction containing Man 6-P was 31.)

Comparative Kinetics of Pinocytosis of Secretion Acid Hydrolases and Glycopeptides Prepared From Them

As shown in Table I there exists a direct correlation between pinocytosis of intact acid hydrolases and glycopeptides prepared from them with the ability of both ligands to bind to immobilized phosphomannosyl receptors. We can thus compare the kinetics of pinocytosis of these ligands. We calculated from Figure 2A that the apparent K_{uptake} for β-hexosaminidase was 572 units/ml. As shown in Figure 5A, 80% of this enzyme binds to the immobilized receptors. The following calculations assume that the same fraction of the other acid hydrolases present in the secretions would have similar binding properties. Since the population of enzyme which binds to the column accounts for all of the "uptake activity" of the enzymes (Table I), we can conclude that the fraction of the total enzyme pool which actually is susceptible to pinocytosis is about 80%. On this basis, one can calculate the K_{uptake} as follows: 572 units/ml (measured apparent K_{uptake}) × 0.8 (the fraction of ligand susceptible to pinocytosis) equals 457 units/ml (K_{uptake} for enzyme susceptible to pinocytosis). A similar argument can be made to estimate the K_{uptake} of glycopeptides. As calculated from Figure 2B, the apparent K_{uptake} for the glycopeptides is 1.5×10^{-6} M. Figure 4A and Table I demonstrate that the uptake susceptible fraction of the glycopeptide pool is 10%. Thus, the measured K_{uptake} can be corrected similarly: 1.5×10^{-6} M (measured apparent K_{uptake}) × 0.1 (the fraction of ligand susceptible to pinocytosis) equals 1.5×10^{-7} M (K_{uptake} for glycopeptide susceptible to pinocytosis). Thus, the corrected apparent $K_{uptakes}$ are 457 units/ml for acid hydrolase, and 1.5×10^{-10} moles/ml for glycopeptide. For each unit of β-hexosaminidase present in the fibroblast secretions, 2.18×10^{-13} moles of Con A-binding glycopeptide was produced. Thus, the concentration of free glycopeptides at the K_{uptake} corresponded to an enzyme equivalent of 688 units/ml, which compares closely with the apparent K_{uptake} of hexosaminidase in the fibroblast secretions from which the glycopeptides were prepared. These results suggest that the phosphorylated glycopeptides themselves contain the structural information needed for enzyme binding to and uptake by phosphomannosyl receptors and display a similar affinity for cell surface phosphomannosyl receptors as the secretion acid hydrolases from which they were prepared.

DISCUSSION

Many useful findings concerning the phosphomannosyl receptor system have come from earlier studies using acid hydrolases from several different tissue sources [1–3,8,10–15,23]. However, detailed kinetic studies of this system have been limited by the great variability [10,11,23] in the percentage of high-uptake ligand present in preparations of enzyme from tissue homogenates. In the studies reported here we have exploited two different tools to mitigate this limitation and to answer several outstanding questions concerning the phosphomannosyl recognition system in fibroblasts. First, we took advantage of the effect of lysosomotropic amines on enzyme secretions to produce ligands of fibroblast origin which are free from at least some of the problems of ligands prepared from tissue homogenates. Second, we used immobilized phosphomannosyl receptors as an analytical tool for studying ligands for this system. Our results demonstrate that (1) glycopeptides prepared from fibroblast secretions are themselves subject to pinocytosis with kinetics similar to those of intact phosphoglycoproteins, (2) the distribution and state (monoester vs diester) of phosphomannosyl groups may be at least as important as the total phosphomannose content

in the pinocytosis of acid hydrolases, and (3) the predominant phosphorylated oligosaccharide species on an enzyme is not necessarily the one that confers the high-uptake property.

Acid hydrolases prepared from ammonium chloride-stimulated secretions of human fibroblasts more faithfully reflect the state of newly-synthesized enzymes enroute to lysosomes than enzymes prepared from tissue homogenates. Tissue enzyme represents a "steady-state" [8] distribution of acid hydrolases in several different cell types and organelles (including lysosomes) and includes ligand which has been delivered to lysosomes and subject to the many different hydrolytic processing activities in this organelle. Variability in the extent of processing which has occurred prior to enzyme isolation could explain the different uptake properties of separate acid hydrolase preparations from tissue homogenates [11,23]. By contrast, acid hydrolases produced in amine-induced secretions of fibroblasts are the product of a single cell type and appear to represent only newly synthesized enzyme precursors [17,18]. Rather than being delivered to lysosomes, they have been diverted from lysosomes to the extracellular medium and have not experienced variable degrees of intralysosomal processing. The secreted enzyme is produced in quantities sufficient for both biochemical and kinetic studies and has the characteristics of a high-uptake ligand for the phosphomannosyl receptor. At least 80% of the secreted hexosaminidase B is subject to pinocytosis by fibroblasts while only 25% of the β-glucuronidase prepared from human spleen homogenates binds to immobilized phosphomannosyl receptors and is subject to pinocytosis [24].

Results presented here show that glycopeptides prepared from fibroblast secretion acid hydrolases are subject to pinocytosis. Thus, an intact protein backbone is not necessary for receptor recognition. In addition, the kinetics of pinocytosis of the isolated glycopeptides and of intact acid hydrolases show several similarities. First, pinocytosis of both glycopeptides and intact enzyme is saturable and inhibited by Man 6-P. Second, the inhibition constant of excess unlabeled glycopeptides for pinocytosis of both enzyme and radiolabeled glycopeptides are similar. Third, the K_i for Man 6-P inhibition of glycopeptide uptake compares closely with previously reported K_i's for Man 6-P inhibition of pinocytosis of intact acid hydrolases [10,12]. Fourth, the pinocytosis rate (%/mg/hr) of the high-uptake fractions of both intact enzyme and glycopeptides are similar; ie, the uptake rate for glycopeptides which bound to the immobilized receptors differed by less than twofold from the rate of pinocytosis of enzyme which also bound to the receptor column. Finally, when the apparent K_{uptake} of glycopeptide and intact enzyme are corrected for the fraction of high-uptake ligand each contains, the calculated K_{uptake} for both types of ligand are of the same order of magnitude. Thus, the information required for uptake by the phosphomannosyl receptor is contained in the glycopeptides themselves. However, we cannot exclude the possibility that other, as yet undefined, features of the protein may modify to some extent the rate of pinocytosis and the effectiveness of binding to the receptor.

The immobilized phosphomannosyl receptors used here bind only the fractions of ligands that interact with the pinocytosis receptors. The fraction of each ligand which fails to interact with the immobilized receptor was very poorly pinocytosed, if at all. Thus, immobilized phosphomannosyl receptors may be exploited in at least three ways. First, they provide a preparative tool for purification of high-uptake enzyme essentially devoid of low-uptake forms. Second, immobilized receptors may be used as an analytic tool to estimate the fraction of high-uptake forms in a ligand preparation. Third, they can be used to analyze what structural features of high-uptake ligands are essential for recognition by the enzyme transport receptors.

We recently reported that oligosaccharides containing only phosphates in diester linkage fail to bind to immobilized receptors and are very poorly taken up, if at all [9,16]. These findings were confirmed and extended by two recent reports [25,26]. The data reported here also support these findings. Phosphatase treatment destroyed the pinocytosis of both glycopeptides and secretion β-hexosaminidase B, and converted both ligands into forms which no longer bound to immobilized receptors. This suggested that the structural feature responsible for both kinds of receptor interaction contains phosphatase-sensitive phosphomonoesters, ie, uncovered phosphates. Thus, efficient interaction of glycopeptides and secretion β-hexosaminidase B-bearing phosphodiesters can occur only after the covering α-N-acetylglucosamines are removed. Additional evidence supporting this conclusion consists of the facts that (1) mild acid treatment enhanced the uptake of total glycopeptides and that (2) mild acid converted a fraction of glycopeptides that initially failed to bind the immobilized phosphomannosyl receptors into forms capable of binding to the receptor. The latter result indicated that the total pool of glycopeptides prepared from fibroblast secretions included a fraction bearing phosphates in diester linkage which did not interact efficiently with receptor until they were first uncovered by mild acid hydrolysis. In contrast, Talkad and Sly [24] recently showed that small but significant amounts of β-glucuronidase binding and uptake can be mediated by phosphatase-resistant recognition markers. Thus, while it is possible for acid hydrolases to be taken up efficiently by fibroblasts as the result of an interaction with a single competent uncovered diphosphorylated recognition marker, it appears likely also that some acid hydrolases may be taken up via receptor interaction with multiple copies of covered recognition markers, each with too low an affinity for the receptor individually to demonstrate binding, but which cooperatively interact to provide the multivalent ligand with an affinity great enough to both bind to immobilized receptor and be pinocytosed by fibroblasts.

An initially unexpected result was the large difference in the proportion of total glycopeptides and enzyme that bound to the immobilized receptors. Only 10% of the glycopeptides bound, while 80% of the β-hexosaminidase bound. Thus, although ammonium chloride-induced enzyme secretions contain predominantly high-uptake enzymes, the oligosaccharides bearing a proper recognition marker for uptake are a minority of the total oligosaccharides present on these enzymes. Since many lysosomal enzymes are multimeric, and each monomer can contain three or more oligosaccharide chains [7], processing only a minority of the oligosaccharide chains into recognition markers may be sufficient to target most of the acid hydrolases to lysosomes, and to explain the degree of enzyme binding observed. Another possible contribution to the larger fraction of enzyme than glycopeptides retained by the column is the possible cooperative interaction in the multivalent ligand (the enzyme) of singly phosphorylated oligosaccharides that individually bind with too low an affinity to be retained by the column.

Prior work from this laboratory [23] demonstrated a direct correlation between the total Man 6-P content and the susceptibility to pinocytosis of different column fractions of human β-glucuronidase purified from spleen homogenates and fractionated on the basis of charge. Two pieces of evidence now make it clear that such a direct correlation between Man 6-P content and uptake of acid hydrolases need not exist. First, Creek and Sly [9] demonstrated that oligosaccharides containing two phosphomonoesters are pinocytosed many times more efficiently than those with two

phosphodiesters, and also many times more efficiently than those with a single phosphomonoester. Thus, the correlation of Man 6-P content and uptake of isolated oligosaccharides is clearly not a direct one. Furthermore, the studies reported here show that only 10% of the Con A binding glycopeptides may contain the critical recognition marker for uptake. However, the other 90% could contribute more to the average Man 6-P content. Thus, one can conclude that the Man 6-P content of an enzyme need not correlate directly with its susceptibility to pinocytosis, and also that the predominant phosphorylated oligosaccharide on an enzyme is not necessarily the one that confers the high-uptake property.

Recent findings of Varki and Kornfeld [26] indicate that the generation of the physiological phosphomannosyl recognition marker on lysosomal enzymes involves the removal of blocking N-acetylglucosamine residues, trimming of certain mannose residues, and the correct positioning of phosphomonoesters. These studies greatly advanced our understanding of the phosphomannosyl recognition system but several important questions still remain unanswered. First, of the five mannose residues which may be phosphorylated on high mannose-type oligosaccharides, are some sites preferred? Second, what determines the specificity by which only the high mannose chains on lysosomal enzymes are selectively phosphorylated? Third, what determines which of the oligosaccharide chains on lysosomal enzymes are phosphorylated? Fourth, can intact enzyme be bound and pinocytosed in different ways? For example, the data to date suggest that a single oligosaccharide chain bearing two phosphomonoesters is sufficient (in fact preferred) for pinocytosis. However, neoglycoproteins have been constructed by covalently coupling multiple oligosaccharides, each with a single phosphomonoester, to a protein backbone [27]. These ligands are pinocytosed by fibroblasts. Answers to these and other questions will depend on sufficient quantities of ligand to allow detailed structural, biochemical, and kinetic studies. The results presented here lay the groundwork for approaching these questions and suggest that ligands produced by amine-induced fibroblast secretions and fractionated by affinity chromatography on immobilized phosphomannosyl receptors can provide useful tools for further analysis of the phosphomannosyl recognition system.

ACKNOWLEDGMENTS

We sincerely thank Drs Venugopal Talkad and Michael Merion for many helpful discussions during the course of this work; Sarah Thomas, Jeffrey Grubb, and Joann Nielsen for able technical assistance; and Mrs Sabra Lovejoy for expert typing. We also thank Dr Alfonso Gonzalez-Noriega, whose work in this laboratory provided the inspiration for use of fibroblast secretions as ligands for the phosphomannosyl receptor system.

This research was supported by United States Public Health Service grant GM 21096 and the Ranken Jordan Trust Fund for the Crippling Diseases of Children.

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