Proc. Natl. Acad. Sci. USA
Vol. 78, No. 7, pp. 4289–4293, July 1981
Biochemistry

Characterization of a membrane-associated receptor from bovine liver that binds phosphomannosyl residues of bovine testicular β-galactosidase

(lysosomal enzymes/adsorptive endocytosis/recognition marker)

G. GARY SAHAGIAN*, JACK DISTLER, AND GEORGE W. JOURDIAN†

Rackham Arthritis Research Unit and Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan 48109

Communicated by Elizabeth F. Neufeld, April 16, 1981

ABSTRACT **A receptor that binds the phosphomannosyl recognition marker of bovine testicular β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) was isolated from bovine liver membranes. The receptor was extracted from crude plasma membrane preparations with Triton X-100 and immunoprecipitated as a receptor–β-galactosidase complex with anti-β-galactosidase. The receptor was dissociated from the precipitate with mannose 6-phosphate, labeled with ^{125}I, and purified on a β-galactosidase-Sepharose 4B affinity matrix. A quantitative binding assay employing anti-β-galactosidase and IgGsorb (formalin-fixed *Staphylococcus aureus*) was devised to study the binding of ^{125}I-labeled receptor to β-galactosidase. Maximal binding of receptor to enzyme occurred at pH values between 5.7 and 6.5. Divalent cations were not required for binding. The values of the dissociation constant obtained for β-galactosidase varied between 200 nM observed with "lower uptake" forms and 20 nM for "higher uptake" forms of the enzyme. A number of phosphorylated monosaccharides were tested as inhibitors of binding of enzyme to receptor; mannose 6-phosphate and fructose 1-phosphate served as inhibitors and exhibited K_i values of 0.064 mM and 0.24 mM, respectively. The receptor has a subunit molecular weight of 215,000. Similar receptors were also demonstrated in Triton X-100 extracts of human skin fibroblasts, Chinese hamster ovary cells, and rat hepatocytes. These cell types are known to assimilate lysosomal enzymes containing covalently bound mannose 6-phosphate residues.**

Certain lysosomal enzymes are selectively and efficiently taken up by cultured human fibroblasts (1). This process is thought to be mediated by a specific cell surface receptor that recognizes phosphomannosyl residues on oligosaccharide chains of the enzymes (2–5). Recognition of lysosomal enzymes by a phosphomannosyl receptor has been proposed as an essential step for the delivery of newly synthesized lysosomal enzymes to lysosomes (6–8). Direct evidence for the existence of phosphomannosyl receptors has been obtained by demonstration of the reversible binding of α-L-iduronidase to the cell surface of human skin fibroblasts (9) and by the binding of β-glucuronidase to fibroblast cell membranes (10).

Phosphomannosyl receptors also occur in other mammalian cell types and tissues. Phosphomannosyl-dependent uptake or binding of lysosomal enzymes has been observed in Chinese hamster ovary cells (11), normal rat kidney cells (12), a rat liver epithelial cell line (13), rat hepatocytes (12, 14), and Swarm rat chondrosarcoma (15). Binding studies using β-hexosaminidase suggest the presence of the phosphomannosyl receptor in all major tissues of the rat and in several rat liver subcellular fractions (16).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

In previous studies we demonstrated the presence of mannose 6-phosphate residues in β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) (17, 18) and showed that the enzyme is subject to endocytosis by the phosphomannosyl uptake system in human skin fibroblasts (5). We now report the isolation and partial characterization of a phosphomannosyl receptor from bovine liver that binds β-galactosidase. A preliminary report of this work has been presented (19).

MATERIALS AND METHODS

Alkaline phosphatase (*Escherichia coli*) was obtained from Sigma; IgGsorb (formalin-fixed *Staphylococcus aureus*, Cowan strain A) from the New England Enzyme Center (Boston, MA); endoglucosaminidase H from Miles; and carrier-free $Na^{125}I$ from Amersham. *N*-Acetylglucosamine 6-phosphate was prepared by the method of Distler *et al.* (20). All other reagents were obtained from commercial sources and were of the highest grade available.

Protein content was estimated by the procedure of Lowry *et al.* (21). Polyacrylamide gel electrophoresis was conducted under reducing conditions as described by Laemmli (22) in a slab gel containing 7.5% acrylamide. ^{125}I-Labeled compounds were detected by autoradiography with Kodak X-Omat film in x-ray cassettes containing Du Pont Cronex Hi Speed intensifying screens (23).

Conditions for the culture of Chinese hamster ovary cells [CHO, KI (24)] and human diploid skin fibroblasts (25) have been described. Rat hepatocytes were isolated and cultured as described by Kilberg *et al.* (26), except the cells were plated and maintained in an arginine-deficient medium to prevent growth of nonparenchymal cells (27).

Bovine testicular β-galactosidase was purified and assayed as described (28). One unit hydrolyzes 1 μmol of *p*-nitrophenyl β-D-galactopyranoside per min under the conditions of the assay. Fractions containing isozymes of β-galactosidase were obtained by chromatography on DEAE-Sephacel (Pharmacia) (17). β-Galactosidase was labeled with ^{125}I by the method of Bolton and Hunter (29). Bovine testicular inhibitor glycoproteins that possess the phosphomannosyl recognition marker were obtained by chromatography on concanavalin A-Sepharose (Pharmacia) (18). β-Galactosidase was coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia) by the method of Cuatrecasas (30). The substituted Sepharose contained approximately 1 unit of β-galactosidase per ml of Sepharose.

* Present address: Genetics and Biochemistry Branch, National Institute of Arthritis, Metabolism and Digestive Diseases, Bethesda, MD 20205.

† To whom reprint requests should be addressed at: 4633 Kresge Medical Research I, University of Michigan, Ann Arbor, MI 48109.

Plasma membranes were prepared from bovine liver by the procedure of Ray (31), except that the discontinuous sucrose gradient step was omitted.

Anti-β-galactosidase was raised in New Zealand White rabbits (3–4 kg). Each animal was injected with 1 ml of a mixture containing 0.5 ml of Freund's complete adjuvant and 0.5 ml of physiological saline containing 250 μg of β-galactosidase. The antibodies were concentrated 3-fold from the serum by precipitation with $(NH_4)_2SO_4$ at 50% saturation.

Purification of ^{125}I-Labeled Receptor from Bovine Liver Membranes. All steps were performed at 4°C. Receptor was extracted from bovine liver membranes (26 mg of protein) by extraction for 1 hr with 2 ml of a Krebs–Ringer phosphate buffer (pH 6.8) containing 1% Triton X-100. The mixture was centrifuged for 10 min at 10,000 × *g* and the supernatant, containing receptor, was diluted with an equal volume of solution A (a mixture composed of 1 vol of Krebs–Ringer phosphate buffer and 1 vol of physiological saline containing 0.1% Triton X-100). β-Galactosidase (0.4 unit) was added and the mixture was incubated for 90 min to form a receptor–enzyme complex. Anti-β-galactosidase was added and incubation was continued for an additional 60 min.‡ The resulting immunoprecipitate was pelleted by centrifugation, washed twice with a total of 8 ml of solution A, and incubated for 2 hr in 200 μl of Krebs–Ringer phosphate buffer containing 5 mM mannose 6-phosphate. The mixture was centrifuged and the supernatant containing the receptor was radioiodinated with 1 mCi (3.7×10^7 becquerels) of carrier-free Na^{125}I, using Enzymobeads as described by Bio-Rad. The labeled protein fraction containing receptor was separated from Na^{125}I by gel filtration on Sephadex G-25 equilibrated and eluted with a mixture composed of 1 vol of Krebs–Ringer phosphate buffer and 1 vol of physiological saline containing 0.1% Triton X-100 and bovine serum albumin at 1 mg/ml (solution B).

Resolution of receptor from other ^{125}I-labeled components was achieved by affinity chromatography on a column consisting of 1 ml of packed β-galactosidase-coupled Sepharose 4B equilibrated with solution B. The ^{125}I-labeled receptor fraction was applied to the column and the column was washed with 20 vol of solution B. The receptor (approximately 5×10^7 cpm) was eluted from the column with 4 vol of solution B containing 5 mM mannose 6-phosphate.

Binding Assay Using ^{125}I-Labeled Receptor. The assay mixture contained the following components: 100 μl of solution B; 100 μl of physiological saline containing β-galactosidase (2.5–to 25 milliunits); 15 μl of ^{125}I-labeled receptor (approximately 20,000 cpm); and 10 μl of saline with or without added inhibitor. Reaction mixtures were incubated for 90 min at 0°C and 25 μl of a mixture containing anti-β-galactosidase‡ and IgGsorb (20% cell suspension) was added to each tube. The reaction mixtures were incubated for an additional 20 min, 1 ml of solution B was added, the immunoprecipitates were pelleted, and the pellet-associated radioactivity was quantitated in a γ-counter.

RESULTS

Binding of ^{125}I-labeled β-galactosidase to bovine liver cell membranes is shown in Fig. 1. Binding is inhibited by an inhibitor glycoprotein fraction and mannose 6-phosphate but not by glucose 6-phosphate, suggesting that binding is mediated by a phosphomannosyl receptor. The binding capacity of the membranes was 0.27 milliunit of β-galactosidase per mg of membrane protein.

‡ The amount of anti-galactosidase used was that required for quantitative precipitation of β-galactosidase activity.

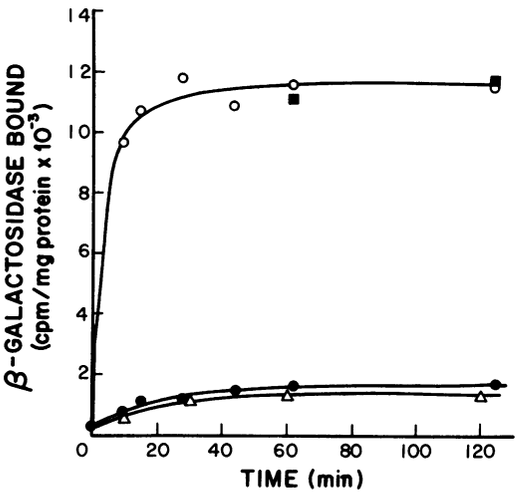

FIG. 1. Binding of ^{125}I-labeled β-galactosidase to bovine liver membranes as a function of time. Each assay mixture (250 μl) contained 1.5 mg of membrane protein and 2.5×10^5 cpm of β-galactosidase in Krebs–Ringer phosphate buffer. After incubation at 4°C, 200 μl of each assay mixture was layered over 3 ml of Krebs–Ringer phosphate buffer containing bovine serum albumin at 100 mg/ml and centrifuged for 3 min at 2200 × *g*. The supernatant was aspirated off and the membranes were resuspended in 3 ml of Krebs–Ringer phosphate buffer. The suspension was again centrifuged and the radioactivity contained in the pellet was determined. ○, No additions; ●, incubation mixtures containing 5 mM mannose 6-phosphate; △, bovine testicular inhibitor glycoproteins at 100 μg/ml; ■, 5 mM glucose 6-phosphate.

After extraction of the membrane preparation with 1% Triton X-100, 55% of the binding activity was lost from the membranes, suggesting that the receptor had been extracted. The receptor was demonstrated in the Triton X-100 extract in the following manner. β-Galactosidase was added to the Triton X-100 extract and the resulting receptor–β-galactosidase complex was immunoprecipitated with anti-β-galactosidase. The receptor was dissociated from the immunoprecipitate with mannose 6-phosphate and labeled with ^{125}I. The labeled receptor fraction was further purified by affinity chromatography on β-galactosidase-Sepharose 4B (Fig. 2). Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate indicated that the purified receptor was homogeneous and had an apparent molecular weight of 215,000 (Fig. 2). The intact receptor was not sedimented when centrifuged at 105,000 × *g* for 1 hr.

Properties of the Binding of ^{125}I-Labeled Receptor to β-Galactosidase. In order to characterize the purified ^{125}I-labeled receptor, a binding assay employing β-galactosidase and anti-β-galactosidase was used. The labeled receptor was allowed to react with β-galactosidase and the resulting receptor–enzyme complex was precipitated quantitatively by addition of anti-β-galactosidase and IgGsorb. Radioactivity contained in the precipitate was measured. Under the conditions of the assay, maximal binding of ^{125}I-labeled receptor to β-galactosidase occurred within 90 min at 4°C. Concentrations of Triton X-100 between 0.05% and 0.55% had no effect on the binding of receptor to enzyme.

Previous studies revealed that preparations of β-galactosidase are composed of a mixture of isozymes that display differing rates of uptake by skin fibroblasts. The isozymes were partially resolved into fractions by chromatography on DEAE-Sephacel (17). As may be seen in Table 1, binding of the purified ^{125}I-labeled receptor to the enzyme fractions paralleled their uptake by fibroblasts. Binding of the receptor to each enzyme fraction was inhibited greater than 93% by mannose 6-phosphate (data not shown). Dissociation constants for β-galactosidase fractions

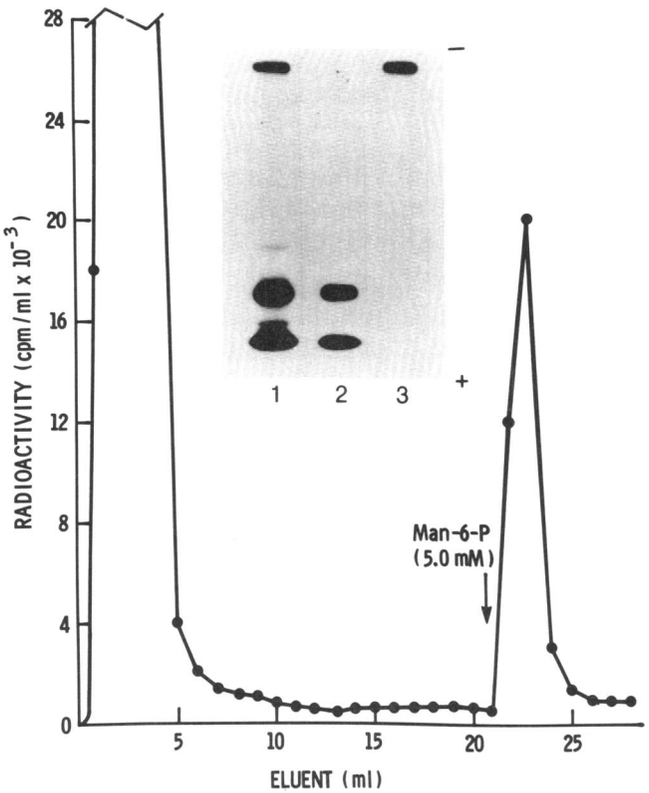

FIG. 2. Affinity chromatography of the ^{125}I-labeled receptor fraction from bovine liver membranes on β-galactosidase-Sepharose 4B. Polyacrylamide gel electrophoresis of the fractions is shown in the *Inset*: lane 1, crude ^{125}I-labeled receptor before affinity chromatography; lane 2, affinity column wash fraction; and lane 3, material eluted with mannose 6-phosphate. ^{125}I-Labeled compounds were detected by autoradiography.

varied between 200 nM for a "lower uptake" fraction (0.01–0.03 M NaCl) to 20 nM for a "higher uptake" fraction (0.09–0.12 M NaCl). Removal of the phosphomannosyl recognition marker from the "higher uptake" fraction with alkaline phosphatase or endoglucosaminidase H decreased binding by 98% and 90%, respectively.

Several sugar phosphates were studied for their ability to inhibit binding of ^{125}I-labeled receptor to β-galactosidase. Inhibition constants for these compounds were determined as described by Dixon (32). Mannose 6-phosphate and fructose 1-phosphate were strong inhibitors of binding, with K_i values of 0.064 and 0.240 mM, respectively; noninhibitory compounds (K_i > 4.0 mM) included glucose 1-phosphate, glucose 6-phosphate, mannose 1-phosphate, fructose 6-phosphate, and *N*-acetylglucosamine 6-phosphate. These values are in close agreement with the K_i values obtained when these compounds were tested as inhibitors of lysosomal enzyme uptake in fibroblasts (3).

The effect of pH on the binding of the receptor to the enzyme is shown in Fig. 3. Greater than 95% of the β-galactosidase activity was immunoprecipitated at each pH value tested. Maximal binding of the labeled receptor to the enzyme occurred between pH values of 5.7 and 6.5. A precipitous drop in binding was observed between pH values of 5.7 and 5.0. Substitution of EDTA/phosphate buffer for Krebs–Ringer phosphate buffer did not affect binding of the receptor to the enzyme, suggesting that divalent cations are not required for binding. These results closely parallel the results obtained for binding of lysosomal enzymes to cultured skin fibroblasts (9, 33).

Phosphomannosyl-dependent uptake of lysosomal enzymes

Table 1. Binding of the ^{125}I-labeled receptor to β-galactosidase isozymes

β-Galactosidase fraction,* NaCl concentration	Uptake by fibroblasts,† milliunits/hr per mg cell protein	^{125}I-Labeled receptor bound,‡ cpm
0 (column wash)	0.22	3,990
0.01–0.03 M	0.37	6,710
0.03–0.06 M	0.66	7,640
0.06–0.09 M	0.82	9,110
0.09–0.12 M	1.11	16,450
0.09–0.12 M (treated with alkaline phosphatase)§	<0.13	1,620
0.09–0.12 M (treated with endoglucosaminidase H)¶	<0.13	430

* β-Galactosidase (50 units) was applied to a 2 × 15 cm column of DEAE-Sephacel in 0.05 M Tris·HCl, pH 7.5, and eluted with 800 ml of a linear salt gradient from 0 to 0.2 M NaCl in the same Tris buffer (15).

† β-Galactosidase uptake studies were performed with generalized gangliosidosis skin fibroblasts at a concentration of 0.025 unit/ml as described (23).

‡ The binding assay was performed with 40,000 cpm of ^{125}I-labeled receptor and 0.010 unit of β-galactosidase.

§ β-Galactosidase (1.6 units) was treated with 0.3 unit of alkaline phosphatase at 25°C for 24 hr. The mixtures were dialyzed against 0.05 M Tris·HCl, pH 7.5, during the reaction.

¶ β-Galactosidase (1.6 units) was treated with 0.025 unit of endoglucosaminidase H at 37°C for 5 hr in 0.05 M sodium acetate, pH 5.0.

has been reported to occur in human skin fibroblasts (2–5), Chinese hamster ovary cells (9), and rat hepatocytes (12, 13). The demonstration of phosphomannosyl receptors in these cell types is shown in Fig. 4. The receptors present in extracts of the cells were enriched and labeled as described in *Materials and Methods* for the purification of ^{125}I-labeled bovine liver receptor. An ^{125}I-labeled material with a molecular weight similar to that of the purified bovine liver receptor (215,000) was found in each case (lane 1). The specificity of the binding of each ^{125}I-labeled receptor to β-galactosidase was established by immunoprecipitation of receptor–enzyme complexes in the presence and absence of inhibitors known to prevent binding of enzyme

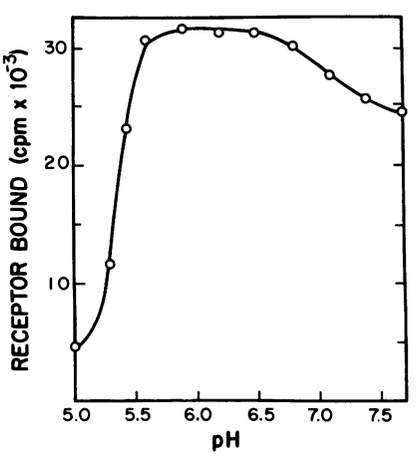

FIG. 3. Effect of pH on the binding of ^{125}I-labeled receptor to β-galactosidase. Binding assays were performed as described in *Materials and Methods* except that solution B contained McIlvain citrate/phosphate buffer at the indicated pH values in place of the Krebs–Ringer phosphate buffer.

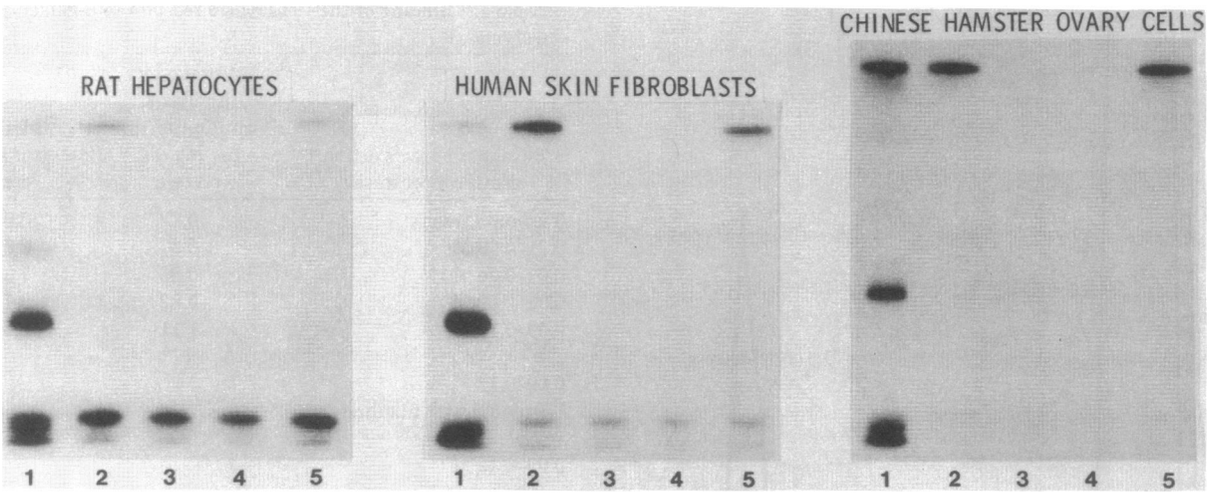

FIG. 4. Demonstration of ^{125}I-labeled phosphomannosyl receptor in mammalian cells. Approximately 1×10^7 cells of each cell type were extracted at 4°C for 1 hr with 2 ml of Krebs–Ringer phosphate buffer containing 1% Triton X-100. Phosphomannosyl receptor of each cell type was enriched and labeled as described for the bovine liver receptor. Each ^{125}I-labeled receptor fraction was characterized in the following manner. β-Galactosidase was added to samples of each fraction and the resulting complex was immunoprecipitated with anti-β-galactosidase in the presence or absence of potential inhibitors. The resulting immunoprecipitates were subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and the ^{125}I-labeled compounds were detected by autoradiography. Lane 1 shows results from the enriched, labeled fraction from each cell type; lane 2, the corresponding immunoprecipitates obtained with no additions; lane 3, with added bovine testicular inhibitor (100 μg/ml); lane 4, with added mannose 6-phosphate (5 mM); and lane 5, with added glucose 6-phosphate (5 mM).

to receptor. Binding was inhibited by the bovine testicular inhibitor glycoprotein fraction and mannose 6-phosphate as evidenced by the absence of the ^{125}I-labeled band (215,000) in lanes 3 and 4, respectively. As expected, glucose 6-phosphate was noninhibitory (lane 5).

DISCUSSION

A membrane protein that specifically binds the phosphomannosyl recognition marker of lysosomal β-galactosidase has been isolated from Triton X-100 extracts of bovine liver plasma membranes. The ^{125}I-labeled receptor was isolated by immunoprecipitation of the enzyme–receptor complex, dissociation of the receptor from the complex with mannose 6-phosphate, and finally purification by affinity chromatography on β-galactosidase-Sepharose 4B.

Properties of the purified binding protein are similar to those of the phosphomannosyl receptor that mediates endocytosis of lysosomal enzymes in fibroblasts (2–5) and other mammalian cells (11–15). The purified protein binds "higher uptake" isozymes of β-galactosidase more effectively than "lower uptake" isozymes. Binding of β-galactosidase to the purified protein is abolished when the enzyme is pretreated with alkaline phosphatase or endohexosaminidase H, treatments that alter or remove the phosphomannosyl recognition marker of lysosomal enzymes (2–5, 34, 35). Binding is specifically inhibited by mannose 6-phosphate and fructose 1-phosphate, inhibitors of the uptake of lysosomal enzymes by fibroblasts (2, 3). The pH profile for the binding of β-galactosidase to the receptor is strikingly similar to that observed for binding of β-glucuronidase to fibroblast cell surface phosphomannosyl receptors (33). As in the case of lysosomal enzyme binding to fibroblasts (9), divalent cations are not required. Phosphomannosyl receptors of the same molecular weight and binding specificity as the purified bovine liver receptor were demonstrated in human skin fibroblasts, Chinese hamster ovary cells, and rat hepatocytes. We conclude from these results that the binding protein isolated from bovine liver membranes is the phosphomannosyl receptor thought to be involved in the absorptive endocytosis of extracellular lysosomal enzymes (2–5, 11–15) and in the delivery of newly synthesized lysosomal enzymes to lysosomes (6–8).

This work was supported in part by Grant AM-10531 from the National Institute of Arthritis, Metabolism and Digestive Diseases, The National Foundation–March of Dimes, and the Arthritis Foundation, Michigan Chapter. G.S. was supported in part by Training Grant AM-5026 from the U. S. Public Health Service and a predoctoral fellowship from the Horace H. Rackham School of Graduate Studies, University of Michigan.

1. Neufeld, E. F., Lim, W. T. & Shapiro, L. J. (1975) *Annu. Rev. Biochem.* **44,** 375–376.
2. Kaplan, A., Fischer, D., Achord, D. & Sly, W. (1977) *J. Clin. Invest.* **60,** 1088–1093.
3. Sando, G. N. & Neufeld, E. F. (1977) *Cell* **12,** 619–627.
4. Ullrich, K., Mersmann, G., Weber, E. & von Figura, K. (1978) *Biochem. J.* **170,** 643–650.
5. Distler, J., Hieber, W., Schmickel, R. & Jourdian, G. W. (1979) in *Carbohydrate-Protein Interaction,* American Chemical Society Symposium Series, ed. Goldstein, I. (Am. Chem. Soc., Washington, DC), pp. 163–180.
6. Neufeld, E. F., Sando, G. N., Garvin, A. J. & Rome, L. H. (1977) *J. Supramol. Struct.* **6,** 95–101.
7. von Figura, K. & Weber, E. (1978) *Biochem. J.* **176,** 943–950.
8. Sly, W. S. & Stahl, P. (1978) in *Transport of Macromolecules in Cellular Systems,* Life Science Research Report II, ed. Silverstein, S. (Dahlem Konferenzen, Berlin), p. 229.
9. Rome, L. H., Weissmann, B. & Neufeld, E. F. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 2331–2334.
10. Fischer, H. D., Gonzalez-Noriega, A. & Sly, W. S. (1980) *J. Biol. Chem.* **255,** 5069–5074.
11. Robbins, A. R. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 1911–1915.
12. Sahagian, G. G. (1981) Dissertation (Univ. Michigan, Ann Arbor, MI).
13. Ullrich, K., Mersmann, G., Fleischer, M. & von Figura, K. (1978) *Hoppe-Seyler's Z. Physiol. Chem.* **359,** 1591–1598.
14. Ullrich, K., Basner, R., Gieselmann, V. & von Figura, K. (1979) *Biochem. J.* **180,** 413–419.
15. Rome, L. H. & Miller, J. (1980) *Biochem. Biophys. Res. Commun.* **92,** 986–992.
16. Fischer, H. D., Gonzalez-Noriega, A., Sly, W. S. & Morré, J. D. (1980) *J. Biol. Chem.* **255,** 9608–9615.
17. Sahagian, G., Distler, J., Hieber, V., Schmickel, R. & Jourdian, G. W. (1979) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **38,** 467 (abstr.).

18. Distler, J., Hieber, V., Sahagian, G., Schmickel, R. & Jourdian, G. W. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4235–4239.
19. Sahagian, G., Distler, J. & Jourdian, G. W. (1980) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **39,** 1968.
20. Distler, J. J., Merrick, J. M. & Roseman, S. (1958) *J. Biol. Chem.* **230,** 497–509.
21. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
22. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
23. Swanstrom, R. & Shank, P. R. (1978) *Anal. Biochem.* **86,** 184–192.
24. Stanners, C. P., Eliceiri, G. L. & Green, H. (1971) *Nature (London) New Biol.* **230,** 52–54.
25. Hieber, V., Distler, J., Myerowitz, R., Schmickel, R. D. & Jourdian, G. W. (1980) *J. Clin. Invest.* **65,** 879–884.
26. Kilberg, M. S., Handlogten, M. E. & Christensen, H. N. (1980) *J. Biol. Chem.* **255,** 4011–4019.
27. Acosta, D., Anuforo, D. C. & Smith, R. V. (1978) *In Vitro* **14,** 28–30.
28. Distler, J. J. & Jourdian, G. W. (1978) *Methods Enzymol.* **50,** 514–523.
29. Bolton, A. E. & Hunter, W. M. (1973) *Biochem. J.* **133,** 529–539.
30. Cuatrecasas, P. (1970) *J. Biol. Chem.* **245,** 3059–3065.
31. Ray, T. K. (1970) *Biochim. Biophys. Acta* **196,** 1–9.
32. Dixon, M. (1953) *Biochem. J.* **55,** 170–171.
33. Gonzalez-Noriega, A., Grubb, J. H., Talkad, V. & Sly, W. S. (1980) *J. Cell Biol.* **85,** 839–844.
34. Natowicz, M. R., Chi, M. M.-Y., Lowry, O. H. & Sly, W. S. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4322–4326.
35. von Figura, K. & Klein, U. (1979) *Eur. J. Biochem.* **94,** 347–354.