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Identification and characterization of cells deficient in the mannose 6-phosphate receptor: Evidence for an alternate pathway for lysosomal enzyme targeting

(oligosaccharides/lysosomes/phosphorylation)

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ABSTRACT **Newly synthesized lysosomal enzymes acquire phosphomannosyl units, which allow binding of the enzymes to the mannose 6-phosphate receptor and subsequent translocation to lysosomes. In some cell types, this sequence of events is necessary for the delivery of these enzymes to lysosomes. Using a slime mold lysosomal hydrolase as a probe, we have identified three murine cell lines that lack the receptor and one line that contains very low (3%) receptor activity. Each of these lines synthesizes the mannose 6-phosphate recognition marker on its lysosomal enzymes, but, unlike cell lines with high levels of receptor, the cells accumulate oligosaccharides containing phosphomonoesters. The receptor-deficient lines possess high levels of intracellular acid hydrolase activity, which is contained in dense granules characteristic of lysosomes. The data suggest that intracellular mechanisms independent of the mannose 6-phosphate receptor must exist in some cells for the delivery of acid hydrolases to lysosomal organelles.**

Recent investigations have demonstrated that many cells sequester newly synthesized lysosomal enzymes into lysosomes via a unique series of specific recognition events (reviewed in ref. 1). Lysosomal hydrolases, like many secretory and membrane glycoproteins, contain asparagine residues that are glycosylated by the *en bloc* transfer of a preformed high-mannose oligosaccharide from a lipid-linked intermediate to the nascent polypeptide. The newly synthesized lysosomal enzymes then move from the rough endoplasmic reticulum to the Golgi complex, where specific mannose residues of the oligosaccharides are phosphorylated (2–5). Acid hydrolases containing the phosphomannosyl recognition marker bind with high affinity to the mannose 6-phosphate (Man-6-*P*) receptor and are then translocated to lysosomes, where the low pH causes dissociation and completes the delivery process (6, 7). After arrival of the acid hydrolases in the lysosomes, the phosphate moieties are cleaved, presumably by the action of an acid phosphatase (8).

The Man-6-*P* receptor is, therefore, an important component of the targeting pathway. The receptor has been purified from bovine liver (9) and a rat chondrosarcoma (10) and appears to be a single protein with an apparent molecular weight of 215,000. Using human fibroblasts, Fischer *et al.* (6) found that over 80% of the Man-6-*P* receptor is located intracellularly, supporting its role in lysosomal enzyme targeting. In addition, many different rat tissues possess Man-6-*P* receptor activity, indicating that the Man-6-*P* recognition signal is a general mechanism for lysosomal enzyme targeting (11).

In a previous study (12) we observed that mouse $P388D_1$ macrophages are deficient in membrane-associated phosphorylated oligosaccharides, and we suggested that these cells may lack the Man-6-*P* receptor. In this report we provide direct evidence that this cell line is deficient in Man-6-*P* receptor activity. We also identify several additional cell lines that lack receptor activity yet possess high levels of intracellular hydrolase activity. These data indicate that some cells possess pathways independent of the Man-6-*P* receptor for the intracellular transport of acid hydrolases to lysosomes.

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MATERIALS AND METHODS

Cells. BW5147 mouse lymphoma, $P388D_1$ mouse macrophage, J774.2 mouse macrophage, mouse L cells, MOPC 315 mouse myeloma, Chinese hamster ovary (CHO), and (human) HeLa cells were grown in suspension culture in α minimal essential medium containing 10% fetal calf serum; human and Swiss 3T3 fibroblasts and baby hamster kidney (BHK) cells were maintained as monolayers in α minimal essential medium containing 10% fetal calf serum; human U937 (monocytes) and HL-60 (promyelocytes) cells were grown in RPMI 1640 medium supplemented with glutamine and 10% fetal calf serum in suspension culture. Cells were harvested as they neared maximum density (or confluency).

Isolation of Slime Mold β-Hexosaminidase. *Dictyostelium discoideum* A_3 cells growing in HL-5 medium were collected by centrifugation and resuspended in differentiation buffer (13) to a final concentration of 10^7 cells per ml. After 6 hr of differentiation, the cells were removed by centrifugation and the medium was applied to a column of DE-52 DEAE-cellulose (2.7 × 10 cm) equilibrated in 20 mM sodium phosphate, pH 6.5; all of the β-hexosaminidase activity bound to the column and was eluted with 20 mM sodium phosphate containing 1 M NaCl. The 1 M NaCl eluate was dialyzed against 50 mM sodium citrate, pH 4.8, and applied to a *p*-aminophenyl-β-*N*-acetylglucosaminide-Sepharose column (0.7 × 18 cm); 90% of the β-hexosaminidase activity was recovered by eluting with 10 mM sodium phosphate, pH 6.5/0.2 M NaCl. The enzyme was purified further by a combination of DE-52 DEAE-cellulose chromatography (using a linear NaCl gradient from 0 to 500 mM) and Sephacryl S-200 chromatography. The final enzyme preparation was purified 30-fold over the initial 1 M NaCl eluate and had a specific activity of 7.4×10^4 units/mg, assayed as described (14). The enzyme was radioiodinated with ^{125}I by using the Chloramine-T method (15).

Membrane Preparation and Man-6-*P* Receptor Assay. Cells ($1–2 \times 10^8$) were collected by centrifugation and washed twice with 10 mM sodium phosphate, pH 7/150 mM NaCl (phosphate-buffered saline); monolayers were washed with the same buffer and then harvested by using a rubber policeman. The washed cells were resuspended in 3 ml of 50 mM Tris·HCl, pH 7.5, containing bovine serum albumin at 1 mg/ml, 5 mM so-

Abbreviations: Man-6-*P*, mannose 6-phosphate; TIU, trypsin inhibitor unit.

dium phosphate, and Trasylol (Sigma) at 0.17 trypsin inhibitor unit (TIU)/ml and ruptured by three 10-sec bursts (100-W) of a Bronwill sonicator. The cell lysates were centrifuged at 850 × *g* for 10 min and the resulting supernatants were recentrifuged at 40,000 × *g* for 30 min. The membrane pellets were suspended by homogenization in 3 ml of 25 mM Hepes, pH 7/0.1 M NaCl/5 mM sodium phosphate/0.34 TIU of Trasylol per ml/0.5% saponin (membrane buffer), collected by centrifugation, then resuspended in membrane buffer containing 10 mM Man-6-*P* and incubated at 22°C for 30 min. The membranes were collected by centrifugation and washed twice with membrane buffer; the final pellets were suspended in 1 ml of membrane buffer. The combined Man-6-*P* and saponin washings remove all endogenous phosphorylated oligosaccharides from the membrane preparations (12). Protein concentrations were determined by precipitating an aliquot of each membrane suspension with acetone and assaying the precipitate with a modified Lowry assay (16).

Man-6-*P* receptor activity was assayed by modification of a previous procedure (11). Aliquots of membranes were diluted with membrane buffer (with or without 10 mM Man-6-*P*) to a final volume of 0.25 ml in Eppendorf centrifuge tubes. ^{125}I-Labeled β-hexosaminidase (^{125}I-β-hexosaminidase) was added to initiate the assay and the tubes were incubated at 4°C. After 30 min, the reaction mixtures were diluted with 1 ml of 25 mM Hepes, pH 7/50 mM NaCl/5 mM sodium phosphate/0.17 TIU of Trasylol per ml/0.1% saponin (wash buffer) and centrifuged for 15 min at 4°C in an Eppendorf microcentrifuge, and the pellets were washed twice with 1 ml of wash buffer by repeated centrifugations. The tips of the plastic tubes (containing the membrane pellets) were cut off and their radioactivities were measured in a gamma counter.

[2-^{3}H]Mannose Labeling and Oligosaccharide Isolation. Cells were collected by centrifugation and suspended at a density of 1 × 10^7 per ml in glucose/bicarbonate-free minimal essential medium containing 20 mM Hepes, pH 7, 10% dialyzed horse serum, and [2-^{3}H]mannose at 0.1 mCi/ml (1 Ci = 3.7 × 10^{10} Bq). After 20 min at 37°C, the mixtures were adjusted to 10 mM in unlabeled mannose and glucose and diluted with a 10-fold excess of complete minimal essential medium containing 10% fetal calf serum to initiate the chase. After the indicated time, cells were collected by centrifugation and washed with cold phosphate-buffered saline, and the cell pellets were frozen.

To isolate phosphorylated oligosaccharides, the cell pellets were digested with Pronase, the glycopeptides were fractionated on concanavalin A-Sepharose, and the high-mannose glycopeptides were digested with endo-β-*N*-acetylglucosaminidase H (Endo H) as described (12). The Endo H digests (0.05 ml) were diluted with 2 ml of 2 mM ammonium acetate, pH 5.4, and applied to QAE-Sephadex columns (0.7 × 15 cm) equilibrated in 2 mM ammonium acetate. Phosphorylated oligosaccharides were eluted by using a 200-ml linear ammonium acetate (pH 5.4) gradient from 2 to 350 mM (in acetate); 2-ml fractions were collected. Secreted phosphorylated oligosaccharides were isolated by precipitation with 10% trichloroacetic acid; the precipitates were washed twice with 95% (vol/vol) ethanol and once with diethyl ether and then digested with Pronase and fractionated as described above.

Colloidal Silica Density Gradient Centrifugation. Cells (2 × 10^8) were washed twice with 10 mM Hepes, pH 7/150 mM NaCl and suspended in 6 ml of 0.25 M sucrose containing 1 mM EDTA, 10 mM acetic acid, and 10 mM triethanolamine, pH 7. The cells were ruptured by nitrogen cavitation (BW5147, 750 pounds/inch^2 for 15 min; MOPC 315, 50 pounds/inch^2 for 15 min; 1 pound/inch^2 = 6.9 kPa) and the lysates were centrifuged at 850 × *g* for 10 min. The supernatants (5 ml) were layered onto 30 ml of a colloidal silica/polyvinylpyrrolidone suspension and centrifuged as described (17). After centrifugation, fractions were collected and assayed for β-glucuronidase.

Enzyme Assays. β-Hexosaminidase and β-glucuronidase were assayed at 37°C using the appropriate *p*-nitrophenyl or 4-methylumbelliferyl derivative (1 mM) in 0.1 M sodium acetate, pH 4.6, containing 0.5% Triton X-100; 1 unit of β-glucuronidase is equal to the release of 1 nmol of *p*-nitrophenol per min.

RESULTS

Identification of Man-6-*P* Receptor-Deficient Cell Lines. β-Hexosaminidase isolated from the differentiation medium of *D. discoideum* cells was used as a probe for the Man-6-*P* receptor because slime mold lysosomal enzymes serve as ligands for human fibroblast cell surface Man-6-*P* receptors (18) and the differentiation medium represents a convenient source of the enzymes (13). In our initial studies, we found that BW5147 mouse lymphoma cells, unlike human fibroblasts (19, 20) and CHO cells (21), lacked cell surface receptor activity as measured by the lack of enzyme binding and uptake into intact cells. However, total cellular membranes isolated from BW5147 cells contained demonstrable activity; therefore, all of our receptor assays assess binding of slime mold β-hexosaminidase to total cellular membranes rather than to intact cells.

Fig. 1 shows the binding of ^{125}I-β-hexosaminidase to BW5147 and mouse myeloma MOPC 315 cell membranes. Ligand binding to BW5147 membranes is dependent upon both the concentration of ligand (Fig. 1*A*) and the amount of membrane (Fig. 1*B*) and is inhibited by the presence of 10 mM Man-6-*P*. Thus, binding to the Man-6-*P* receptor is presumed to be occurring. In contrast, only a small amount of ^{125}I-β-hexosaminidase binds to MOPC 315 membranes, and this is mostly unaffected by the presence of Man-6-*P* (Fig. 1 *C* and *D*). At a saturating concentration of ligand, the Man-6-*P*-inhibitable binding of ^{125}I-β-hexosaminidase to MOPC 315 membranes is only 3% of the value obtained with BW5147 membranes.

Two types of experiments were performed to exclude the possibility that the observed receptor deficiency results from

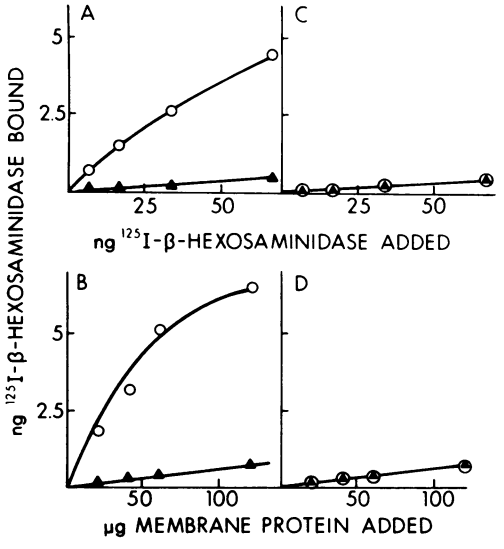

FIG. 1. Binding of ^{125}I-β-hexosaminidase to total cellular membranes prepared from BW5147 (*A* and *B*) or MOPC 315 (*C* and *D*) cells in the absence (○) or presence (▲) of 10 mM Man-6-*P*. In *A* and *C*, 50 μg of membrane was utilized for each determination, and in *B* and *D* 70 ng of ^{125}I-β-hexosaminidase (1.5 × 10^7 cpm) was added at each point.

an inhibitor or inactivator of the Man-6-*P* receptor. First, detergent extracts of two receptor-deficient lines, MOPC 315 and $P388D_1$, were incubated at 22°C for 4 hr with purified ^{125}I-labeled mouse liver Man-6-*P* receptor (22) and then the state of the receptor was analyzed by sodium dodecyl sulfate-gel electrophoresis and autoradiography. There was no change in the migration of the ^{125}I-labeled Man-6-*P* receptor, and no appearance of proteolytic fragments (data not shown). These experiments demonstrate that the receptor-deficient lines do not degrade the receptor during the period of the assay. To determine whether the Man-6-*P* receptor might be inactivated by the receptor-deficient lines, intact BW5147 and MOPC 315 cells were mixed in the ratios indicated in Table 1 and membranes were prepared; the BW5147 cells were prelabeled with [2-^{3}H]mannose so that the amount of the BW5147 membranes in the mixtures could be accurately determined. As shown in Table 1, the amount of specific (Man-6-*P*-inhibitable) ^{125}I-β-hexosaminidase binding to the membranes decreased as the ratio of BW5147 cells to MOPC 315 cells decreased. However, after correction for the quantity of BW5147 membrane present in each assay, the amounts of ^{125}I-β-hexosaminidase bound per μg of BW5147 membrane were very similar. These data indicate that MOPC 315 cells do not contain an inhibitor or inactivator of the Man-6-*P* receptor. Identical results were obtained with mixtures of BW5147 and $P388D_1$ cells.

Table 2 shows the Man-6-*P* receptor content of a number of cell lines. Receptor levels are expressed relative to the value observed with BW5147 membranes. Each of the human and hamster cell lines tested possesses receptor activity, although the amount ranged between 20% and 150% of the activity observed in BW5147 membranes. In contrast, four of six mouse cell lines contained either very low or undetectable receptor activity. The three lines with undetectable activity ($P388D_1$, J774, and L cells) secrete approximately 70% of their newly synthesized β-glucuronidase and β-hexosaminidase, whereas receptor-positive cells secrete much less enzyme. However, because the receptor-negative cells synthesize large amounts of these enzymes, the specific activity of the residual intracellular enzyme is actually higher than that found in some of the receptor-positive cells. For example, the specific activity of β-glucuronidase in $P388D_1$ cells is 7.6 units/mg compared to 0.63 unit/mg in BW5147 cells. Moreover, the receptor-deficient MOPC 315 cells secrete very little of their newly synthesized β-glucuronidase and β-hexosaminidase; the specific activity of β-glucuronidase in MOPC 315 cells is 5.4 units/mg.

Table 1. Man-6-*P* receptor activity in membranes prepared from mixtures of BW5147 and MOPC 315 cells

	Mixing ratio, BW5147 cells/MOPC 315 cells			
	1×10^8 / 0	7×10^7 / 2.4×10^7	3×10^7 / 5.6×10^7	0 / 8×10^7
Total membrane assayed, μg	40	40	40	40
^{125}I bound without Man-6-*P*,* cpm (*a*)	17,292	10,883	4,851	1,586
^{125}I bound with Man-6-*P*,* cpm (*b*)	972	856	939	1,072
Specific binding, cpm ($a - b$)	16,320	10,027	3,912	514
[^{3}H]Mannose/40 μg membrane, cpm	19,946	13,865	5,595	0
BW5147 membrane per assay, μg	40	28	11	0
Specific ^{125}I binding per μg BW5147 membrane, cpm	408	358	356	

Prior to mixing the cells, 6×10^7 BW5147 cells were labeled with 0.5 mCi of [2-^{3}H]mannose in 10 ml of glucose/bicarbonate-free minimal essential medium containing 20 mM Hepes, pH 7, and 10% dialyzed horse serum for 20 min. The labeling mixture was then diluted with 100 ml of minimal essential medium containing 10% fetal calf serum, 10 mM glucose, and 10 mM mannose and chased for 20 hr at 37°C. The ^{3}H-labeled BW5147 cells were added to 9×10^7 unlabeled BW5147 cells, then mixed with MOPC 315 cells in the indicated ratios. The cells were ruptured in the presence of 10 mM Man-6-*P* and the membranes were washed with saponin and Man-6-*P* before assaying.
* Average of duplicate determinations using an input of 7.6×10^5 cpm of ^{125}I-β-hexosaminidase.

Table 2. Levels of Man-6-*P* receptor and lysosomal enzyme secretion in various cell lines

Species	Cell line	Man-6-*P* receptor, %	Enzyme secreted, % β-Hex	Enzyme secreted, % β-Gluc
Human	U937 (monocyte)	43	14	19
	HL-60 (promyelocyte)	24	8	8
	HeLa	22	49	0
	Fibroblasts	151	19*	ND
Hamster	BHK	63	22	26
	CHO	147	13†	4†
Mouse	BW5147	100	0	8
	Swiss 3T3	17	ND	ND
	$P388D_1$ (macrophage)	<2	74	70
	J774.2 (macrophage)	<2	74	62
	L cells	<2	72	71
	MOPC 315	3	12	19

The Man-6-*P* receptor activities were determined with 40–50 μg of total membrane protein and are expressed as the percent of activity found with BW5147 membranes. The percent of newly synthesized β-hexosaminidase (β-Hex) and β-glucuronidase (β-Gluc) secreted was determined by growing cells for 24–48 hr in their standard medium containing 10% heat-inactivated fetal calf serum. Knowing the amount of each enzyme activity initially present, the increases both intra- and extracellularly were determined and the percent of newly synthesized enzyme secreted was calculated. ND, not determined.
* Value from ref. 7.
† Value from ref. 23.

Formation and Processing of Phosphorylated Oligosaccharides in the Man-6-*P* Receptor-Deficient Lines. To determine whether the Man-6-*P* receptor-deficient lines phosphorylate their lysosomal enzymes and metabolize the phosphomannosyl recognition marker as in receptor-positive lines, cells were labeled for 20 min with [2-^{3}H]mannose and chased for either 60 or 180 min in the presence of excess unlabeled mannose. The high-mannose oligosaccharides were then isolated from the glycoproteins and analyzed for the presence of phosphorylated oligosaccharides. Fig. 2 shows the QAE-Sephadex profiles of the high-mannose oligosaccharides isolated from CHO, mouse L, and MOPC 315 cells. In the case of the receptor-positive CHO cells, the major phosphorylated species after the 60-min chase contains a single phosphodiester, but smaller quantities of oligosaccharides containing one phosphomonoester, two phosphodiesters, and two phosphomonoesters are also present (Fig. 2*A*). After 180 min of chase, the number of phosphorylated oligosaccharides is greatly reduced (Fig. 2*B*); the amount of ^{3}H-labeled oligosaccharides eluting as phosphorylated species decreased from 8.1×10^4 cpm after 60 min of chase to 4.1×10^4 cpm after 180 min. This loss of phosphorylated oligosaccharides cannot be accounted for by enzyme secretion and is thought to occur as newly synthesized acid hydrolases reach lysosomes (8). A similar loss occurs in human fibroblasts and in

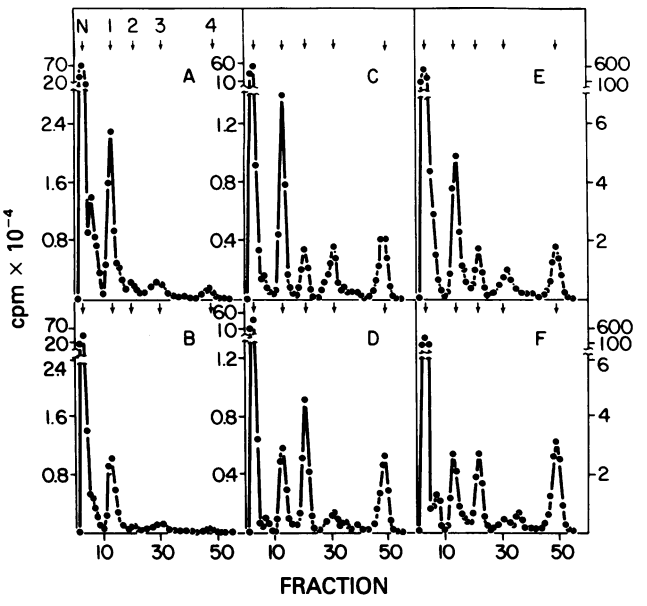

FIG. 2. Characterization of the high-mannose oligosaccharides isolated from CHO (*A* and *B*), L (*C* and *D*), or MOPC 315 (*E* and *F*) cells by QAE-Sephadex chromatography. The cells (5×10^7) were labeled with [2-^{3}H]mannose for 20 min and then chased for 1 hr (*A*, *C*, and *E*) or 3 hr (*B*, *D*, and *F*) in the presence of an excess of unlabeled mannose. The elution positions of neutral oligosaccharides (N) or high-mannose oligosaccharides containing one phosphodiester (1), one phosphomonoester (2), two phosphodiesters (3), or two phosphomonoesters (4) are indicated. The break in the ordinates represents a shift in two linear axes.

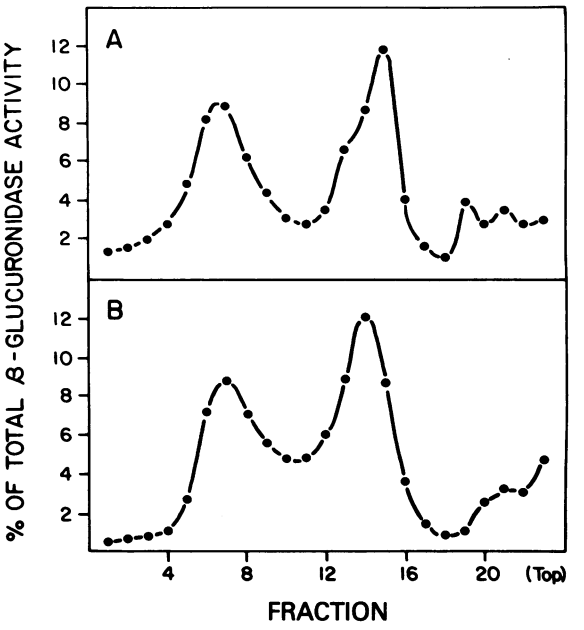

FIG. 3. Colloidal silica density gradient fractionation of lysates from MOPC 315 (*A*) or P388D$_1$ (*B*) cells.

BW5147 cells, where the $t_{1/2}$ of phosphorylated oligosaccharide loss is 1.4 hr (12).

The receptor-deficient MOPC 315 cells synthesize each of the phosphorylated oligosaccharide species (Fig. 2*E*). However, in contrast to CHO cells, the content of these oligosaccharides is stable during the 180-min chase period (Fig. 2*F*). The total quantity of [^{3}H]mannose-labeled oligosaccharides binding to QAE-Sephadex was 2.7×10^5 and 2.9×10^5 cpm after 60 and 180 min of chase, respectively. In addition, the predominant species recovered after the 180-min chase contained either one or two phosphomonoesters.

Similar results were obtained with receptor-negative mouse L cells (Fig. 2 *C* and *D*). In this cell line there was a decline in total cell-associated phosphorylated oligosaccharides during the 180-min chase period (from 1×10^5 cpm to 0.6×10^5 cpm). This was associated with a corresponding increase (0.3×10^5 cpm) in the recovery of phosphorylated oligosaccharides in the medium (data not shown). Thus, as with MOPC 315 cells, the total number of phosphorylated oligosaccharides remains constant and there is an accumulation of oligosaccharides that contain phosphomonoesters.

To investigate the possibility that intracellular hydrolases accumulate via a secretion–recapture mechanism in the receptor-negative lines, P388D$_1$ cells were labeled for 3 hr with [2-^{3}H]mannose. The medium, containing secretory products, was recovered and dialyzed extensively against fresh medium to remove free mannose. Greater than 30% of the ^{3}H-labeled high-mannose oligosaccharides recovered from the glycoproteins in the medium were phosphorylated, suggesting that lysosomal enzymes make up a large percentage of the glycoproteins secreted by P388D$_1$ cells. The dialyzed medium was reapplied to unlabeled P388D$_1$ cells for 3 hr; 0.2% of the radioactivity became cell associated (41 out of 18,075 cpm). Considering that P388D$_1$ cells contain 30% of their newly synthesized hydrolases intracellularly (Table 2), the uptake of 0.2% of the radioactivity over a 3-hr period is not sufficient to account for the sequestration of hydrolases in lysosomes by secretion–recapture.

Density Gradient Fractionation of Lysosomal Organelles. Lysates of P388D$_1$ and MOPC 315 cells were fractionated by colloidal silica density gradient centrifugation to examine the distribution of their lysosomal organelles. As shown in Fig. 3, the lysosomal hydrolase β-glucuronidase fractionates in a bimodal distribution for both P388D$_1$ and MOPC 315 cells. The small amount of activity at the top of the gradients most likely results from rupture of lysosomes during cell lysis. This same bimodal distribution of lysosomal hydrolase activity has been reported for human fibroblasts (17). The more dense fractions obtained from fibroblasts were shown by electron microscopy to contain primarily lysosomal organelles, whereas the less dense fractions contained a heterogenous mixture of subcellular organelles (17). Therefore, both P388D$_1$ and MOPC 315 cells possess dense organelles that contain acid hydrolase activity and probably correspond to lysosomes.

DISCUSSION

There is a considerable body of evidence which indicates that in some cell types the phosphomannosyl recognition marker of newly synthesized lysosomal enzymes is essential for targeting these proteins to lysosomes. Perhaps the strongest data in support of this conclusion come from studies of fibroblasts isolated from patients with I-cell disease (mucolipidosis II). These cells are unable to phosphorylate their newly synthesized lysosomal enzymes due to a lack of UDP-GlcNAc:lysosomal enzyme *N*-acetylglucosaminylphosphotransferase activity (24, 25). As a consequence, the enzymes are unable to bind to the Man-6-*P* receptor and are secreted into the extracellular milieu rather than being targeted to lysosomes (26). Whereas fibroblasts and several other cell types from these patients are deficient in lysosomal enzymes, other types of cells, such as hepatocytes, Kupffer cells, and leukocytes, have nearly normal levels of lysosomal enzymes (27, 28). Recent studies have demonstrated that all of these cell types are deficient in *N*-acetylglucosaminylphosphotransferase activity, indicating that there must be

mechanisms for localizing acid hydrolases to lysosomes that are independent of the phosphomannosyl recognition system. Our finding that several receptor-negative cell lines contain high levels of intracellular lysosomal enzymes provides further evidence for the existence of alternate lysosomal enzyme targeting mechanisms.

One explanation for the normal intracellular hydrolase activities in the Man-6-*P* receptor-deficient lines is that these cells secrete their newly synthesized lysosomal hydrolases and then subsequently internalize the secreted hydrolases via a cell surface receptor. Because [^{3}H]mannose-labeled lysosomal enzymes isolated from the medium of P388D$_1$ cells are not endocytosed upon readdition to unlabeled P388D$_1$ cells, secretion–recapture does not appear to be responsible for the intracellular hydrolase activity observed in these cells. Another explanation for the high intracellular lysosomal enzyme activity in the absence of the Man-6-*P* receptor is that the enzymes are not actually segregated into lysosomes but rather are packaged into vesicles that also contain nonlysosomal proteins that are passing through the Golgi complex. In this case, segregation of lysosomal enzymes from nonlysosomal secretory proteins would not be occurring. This seems very unlikely because MOPC 315 cells synthesize and secrete large quantities of IgA molecules but do not secrete the bulk of their newly synthesized lysosomal enzymes (Table 2). The presence in these cells of dense granules that contain lysosomal enzymes (Fig. 3) is further evidence for the actual segregation of the acid hydrolases in lysosomes.

Whereas three of the Man-6-*P* receptor-deficient lines possess no detectable receptor activity, the fourth, MOPC 315, consistently shows 3% of the activity found in BW5147 cells (Table 2). Although it is possible that this low level of receptor is sufficient for targeting of the acid hydrolases in MOPC 315 cells, two points suggest that this is not occurring. First, we have previously shown that the Man-6-*P* receptor in BW5147 cells is near maximal occupancy when only 10% of the newly synthesized lysosomal enzymes are bound (12); at 3% of the level found in BW5147 cells, the MOPC 315 receptor would have to recycle extremely rapidly to account for the delivery of all of the newly synthesized enzymes to lysosomes. Second, MOPC 315 cells resemble the receptor-negative cell lines in that they accumulate oligosaccharides possessing either one or two phosphomonoesters (Fig. 2). In contrast, the three Man-6-*P* receptor-positive lines that we have analyzed rapidly destroy the recognition signal. The fact that the receptor-negative cell lines accumulate oligosaccharides with phosphomonoesters may indicate that their newly synthesized lysosomal enzymes are packaged into vesicles that are deficient in acid phosphatase activity.

Although the subcellular distribution of acid hydrolases isolated from P388D$_1$ and MOPC 315 cells is similar to that observed in human fibroblasts (17) that utilize the Man-6-*P* receptor, it is tempting to speculate that the alternate targeting mechanism may result in the formation of a separate class of lysosomes. Evidence has previously been presented suggesting that lysosomal organelles are heterogenous (29, 30). The existence of separate mechanisms for the targeting of enzymes to each class of lysosome would enable cells to regulate the distribution of acid hydrolases between the different organelles. In addition the activity of each targeting pathway might vary in individual cells, depending upon their specific biological functions. For example, macrophages are known to discharge acid hydrolases upon the appropriate stimulus (31), making their lysosomes a type of secretory granule. The two macrophage lines we examined, P388D$_1$ and J774.2, do not possess the Man-6-*P* receptor, suggesting that the alternate targeting pathway may give rise to a secretory lysosome. Human fibroblasts, on the other hand, do not contain lysosomes for secretory purposes, and these cells appear to utilize only the Man-6-*P* receptor pathway to sequester their lysosomal enzymes; thus, the Man-6-*P* receptor pathway results in the formation of the fibroblast type of lysosome. It is possible that other cells possess both types of lysosomal organelles and that multiple pathways are operating simultaneously within these cells to deliver the acid hydrolases to their appropriate destinations.

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