Studies of the Biosynthesis of the Mannose 6-Phosphate Receptor in Receptor-positive and -deficient Cell Lines

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ABSTRACT Phosphomannosyl residues present on lysosomal enzymes are specifically recognized by the mannose 6-phosphate receptor protein. This interaction results in the selective targeting of lysosomal enzymes to lysosomes. While this pathway is operative in many cell types, we have found four cultured cell lines that are deficient in the ability to bind lysosomal enzymes containing phosphomannosyl residues to their intracellular or surface membranes (Gabel, C., D. Goldberg, and S. Kornfeld, 1983, *Proc. Natl. Acad. Sci. USA*, 80:775–779). These cells appear to segregate lysosomal enzymes by an alternate intracellular pathway. To determine the basis for the lack of mannose 6-phosphate receptor activity in these cell lines, we studied the biosynthesis of the receptor in receptor-positive (BW5147) and receptor-deficient ($P388D_1$ and MOPC 315) cells. The cells were labeled with [2-^{3}H]mannose or [^{35}S]methionine and the receptor was immunoprecipitated with an antireceptor antiserum. BW5147 cells synthesize a receptor protein whose size increases after translation/glycosylation. MOPC 315 cells produce an apparently normal receptor and degrade it rapidly. $P388D_1$ cells fail to synthesize any detectable receptor. The receptor from BW5147 and MOPC 315 cells is a glycoprotein with both high mannose and complex asparagine-linked oligosaccharides. The complex-type units become fully sialylated and remain so during long periods of chase.

It is now well established that the mannose 6-phosphate (Man-6-P)[1] receptor plays an important role in the targeting of acid hydrolases to lysosomes (1–5). This receptor binds phosphorylated high mannose-type oligosaccharides present on acid hydrolases and translocates these enzymes to lysosomes. The receptor has been purified from bovine liver (6) and rat chondrosarcoma (7), and appears to be a single protein of molecular weight 215,000. While Man-6-P receptor activity has been detected in numerous tissues and cultured cell lines (8–10), we have identified four mouse cell lines that are deficient in functional Man-6-P receptor (10). By using an iodinated lysosomal enzyme as a probe for receptor activity, we found that three of the lines have no demonstrable receptor, while the fourth line has a low level of activity. These cell lines are of interest for several reasons. First, they have high intracellular lysosomal enzyme activities contained within dense granules and they appear to use an alternate intracellular lysosomal enzyme targeting mechanism (10). In this regard, these cells are similar to some of the tissues and cell types in patients with I-cell disease, or Mucolipidosis II. The cells of these patients lack UDP-GlcNAc:lysosomal enzyme N-acetylglucosaminylphosphotransferase, the enzyme that phosphorylates mannose residues on newly synthesized lysosomal enzymes (11, 12). While this defect results in a failure of lysosomal enzyme targeting in cells such as fibroblasts, other cell types and tissues from these patients have normal intracellular levels of lysosomal enzymes (13, 14).

The receptor-negative cell lines are also of interest when considering mechanisms that might control the steady state level of receptor activity. The lack of receptor-binding activity in these cells could be due to a lack of receptor synthesis, the synthesis of an inactive receptor, an accelerated inactivation or breakdown of the receptor, or a combination of these factors.

In this report we characterize the synthesis of the Man-6-P receptor in a receptor-positive cell line and examine the basis for the lack of receptor activity in two of the receptor-deficient cell lines.

[1] *Abbreviations used in this paper*: Con A, concanavalin A; Man-6-P, mannose 6-phosphate; TBS, Tris-buffered saline; TIU, trypsin-inhibitory units; MEM, minimal essential medium.

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MATERIALS AND METHODS

Materials: [2-^{3}H]Mannose (14 Ci/mmol), [^{35}S]methionine (1,200 Ci/mmol) and En^{3}hance were from New England Nuclear (Boston, MA). Trasylol (aprotinin, 17 trypsin-inhibitory units (TIU)/ml), α-methylglucoside, and α-methylmannoside were from Sigma Chemical Co. (St. Louis, MO). Concanavalin A (Con A)-Sepharose and Protein A-Sepharose were from Pharmacia Fine Chemicals (Piscataway, NJ). The 3a70 scintillation mixture was from Research Products International Corp. (Mt. Prospect, IL). Pronase was from Calbiochem-Behring Corp. (San Diego, CA). XAR-5 film was from Kodak. Fetal calf serum was from KC Biologicals, Inc. (Kansas City, MO). Dialyzed horse serum was from Grand Island Biological Co. (Grand Island, NY).

Cell Culture: Murine BW5147 lymphoma cells, MOPC 315 plasmacytoma cells, and P388D$_1$ macrophages were all grown in suspension culture in α-minimal essential medium (MEM) supplemented with 10% fetal calf serum. All were rapidly growing at the time they were used for labeling experiments.

[2-^{3}H]Mannose Labeling for Immunoprecipitation and Oligosaccharide Isolation: Cells were collected by centrifugation and suspended in glucose/bicarbonate-free MEM containing 20 mM HEPES, pH 7, 10% dialyzed horse serum, and 0.1 mCi/ml of [2-^{3}H]mannose; the cells were labeled at a density of 1×10^7/ml. After a 20-min incubation at 37°C on a roller apparatus, the mixtures were adjusted to 10 mM in unlabeled mannose and glucose and diluted with a 10-fold excess of complete MEM containing 10% fetal calf serum to initiate the chase. After the indicated time at 37°C, cells were collected by centrifugation at 4°C, washed once with cold PBS, and the cell pellets frozen.

For immunoprecipitation of the Man-6-P receptor, the frozen cells ($1–2 \times 10^8$) were suspended in 0.5 ml of 25 mM HEPES, pH 7, 50 mM NaCl, 5 mM Na phosphate, 0.34 TIU/ml Trasylol, 10 mM EDTA, 1% Triton X-100, 0.1% deoxycholate (solubilization buffer), and were placed on ice. After 60 min, insoluble material was removed by centrifugation (15 min in an Eppendorf centrifuge) and the supernatant fluid recovered; the pellets were resuspended in 0.5 ml of solubilization buffer, sonicated by two 10-s bursts (100 W) on a Bronwill Biosonik IV (Rochester, NY) and incubated for an additional 30 min on ice. The extracts were again centrifuged for 15 min and the supernatant fluids combined. 25 μl of rabbit preimmune serum were added to each extract, the tubes were incubated for 2 h at 4°C, then 0.2 ml of a protein A-Sepharose suspension (1:1 in solubilization buffer) was added and the mixtures placed on ice for 20 min. The resin was removed by centrifugation, 0.05 ml of rabbit anti-bovine liver Man-6-P receptor serum (a gift of Dr. Ajit Varki of this laboratory) added, and the mixtures placed at 4°C overnight. The immune complexes were recovered by adding 0.2 ml of 1:1 protein A-Sepharose, incubating on ice for 20 min, and centrifuging; the supernatant fluids were retreated with 0.1 ml of 1:1 protein A-Sepharose and the Sepharose pellets combined with those recovered in the first treatment. The beads were washed five times with solubilization buffer and twice with 50 mM Tris, pH 6.8 (1 ml/wash). The beads were then suspended in 0.15 ml of 1% β-mercaptoethanol, 50 mM Tris, pH 6.8, 20% glycerol, 0.5% SDS, and boiled for 3 min. The Sepharose was removed by centrifugation and the samples subjected to SDS PAGE (15) using a 6% polyacrylamide slab gel. The gel was impregnated with En^{3}hance for autoradiography as described in the manufacturer's instructions.

Pronase Digestion of the Man-6-P Receptor: After autoradiography the appropriate regions of the dried polyacrylamide gel were excised, placed in 1 ml of 0.1 M Tris, pH 8, 0.1 M glucose 6-P, 20 mM $CaCl_2$, 10 mg/ml pronase, and incubated overnight as 56°C. The samples were then diluted with 2 ml of Tris-buffered saline (TBS), boiled for 10 min, clarified by centrifugation, and the supernatant fluid saved. The pellet was washed (with boiling) with another 2 ml, centrifuged, and the supernatant fluids combined. By this method, >90% of the radioactivity could be released from the gel. These digests were then used for Con A-Sepharose chromatography.

Con A-Sepharose Chromatography: Portions of each pronase digest were applied to a 1 ml pasteur pipette column of Con A-Sepharose equilibrated in TBS. The columns were washed sequentially with 4.5 ml of TBS, 9 ml of 10 mM α-methylglucoside in TBS, and 9 ml of 0.1 M α-methylmannoside in TBS warmed to 56°C (16, 17). Fractions of 1.5 ml were collected.

Further Structural Analysis of Man 6-P Receptor Oligosaccharides: The details of lentil lectin-Sepharose chromatography (which binds bi- and certain triantennary fucosylated oligosaccharides [17]), mixed glycosidase digestion (to confirm the presence of complex-type oligosaccharides and assess the existence of terminal sialic acid), mannose:fucose ratio determination (to assess degree of fucosylation), and methylation analysis (to assess degree of substitution of each mannose and substituent linkages) are described elsewhere (17).

Labeling with [^{35}S]Methionine: This procedure was identical to that described by Sahagian and Neufeld (31), except that the labeling medium was methionine-free minimal essential medium supplemented with 0.25 mCi/ml [^{35}S]methionine. The antiserum used was kindly provided by Drs. Sahagian and Neufeld.

Binding of Man-6-P Receptor to a Lysosomal Enzyme-Affinity Column: 2×10^8 BW5147 cells were labeled with [2-^{3}H]mannose as described above and were not frozen, but instead were suspended in 6 ml of 50 mM Tris, 5 mM PO_4, 0.34 TIU/ml Trasylol, 10 mM Man-6-P, pH 7.5. The cells were disrupted by sonication as before, and 0.3 ml of 10% Saponin was added. Membranes were sedimented at 80,000 *g* for 30 min, the supernatant fluid was removed, and the pellet was resuspended in 6 ml of 25 mM HEPES, pH 7.0, 0.1 M NaCl, 0.17 TIU/ml Trasylol, 0.5% saponin, 5 mM PO_4, 10 mM Man-6-P. The membranes were incubated at room temperature for 30 min, resedimented, and the Man-6-P wash repeated. The membranes were then washed twice with 5 ml of the same buffer without Man-6-P. The final membrane pellet was resuspended in 4 ml of 25 mM HEPES, pH 6.8, 10 mM EDTA, 0.34 TIU/ml Trasylol, 50 mM NaCl, 1% Triton X-100, 0.1% Na deoxycholate. This mixture was incubated overnight at 0°C, centrifuged at 80,000 *g* for 30 min, and the supernatant fluid recovered. All centrifugations were done in the cold.

The solubilized preparation was passed over a column of *Dictyostelium discoideum* differentiation secretions coupled to Affigel 10 and equilibrated in 0.1 M citrate-phosphate buffer, pH 6, containing 75 mM NaCl, 0.05% Triton X-100, 0.01% NaN_3, 0.5 mg/ml bovine serum albumin. These secretions are rich in phosphorylated lysosomal enzymes (18) and this column has been shown to bind the Man-6-P receptor specifically (19, 20). After loading, the column was washed extensively with equilibration buffer and then was eluted with phosphate-buffered saline containing 10 mM Man-6-P, 1% Triton X-100, 0.5% Na deoxycholate. The starting material, run-through, and Man-6-P eluate were all brought to 1% Triton X-100, 0.5% deoxycholate, 10 mM Man-6-P, pH 7, and were immunoprecipitated as described above.

RESULTS

Characterization of the Rabbit Anti-bovine Liver Man-6-P Receptor Serum

Bovine liver Man-6-P receptor was purified as previously described using an affinity column of *Dictyostelium discoideum* lysosomal enzymes attached to Affigel-10 (19), and the purified protein was used to immunize rabbits. To verify that the rabbit anti-bovine Man-6-P receptor serum cross-reacts with the Man-6-P receptor present in mouse cells, we pulse-labeled receptor-positive BW5147 cells for 20 min with [^{3}H]mannose, chased for 2 h, lysed by sonication, and a membrane fraction prepared. The membranes were washed with saponin and Man-6-P to remove endogenously bound lysosomal enzymes from the membrane-associated Man-6-P receptor. The washed membranes were then extracted with Triton X-100, the solubilized material applied to the Man-6-P receptor affinity column, and after extensive washing the column was eluted with 5 mM Man-6-P. Both the affinity column run-through and the Man-6-P eluate were treated with the rabbit anti-bovine receptor serum and equal portions of the resulting immunoprecipitates were analyzed by SDS gel electrophoresis; the autoradiogram of the gel is shown in Fig. 1. Lane *1* in Fig. 1 shows an immunoprecipitate of the Triton X-100 extract prior to the affinity column; a single major ^{3}H-labeled protein is present and migrates in the vicinity of an iodinated Man-6-P receptor standard purified from adult mouse liver. Fig. 1's lanes *2* and *3* show the immunoprecipitates derived from the affinity column run-through and Man-6-P eluate, respectively. Note that the immunoprecipitable material binds to the column (i.e., is absent from the run-through fraction and is recovered in the Man-6-P eluate). These data indicate that the rabbit anti-bovine liver receptor serum cross-reacts with a single major [^{3}H]mannose-labeled protein from BW5147 cells that binds to the lysosomal en-

zyme affinity column and is eluted by Man-6-P. By virtue of its properties in common with the bovine liver Man-6-P receptor (6, 7), the ^{3}H-labeled component is presumed to be the Man-6-P receptor. It should be noted that the BW5147 cell receptor shifts to a slightly lower apparent molecular weight following the affinity chromatography step (Fig. 1). The basis for this conversion is unknown, but may be caused by a slime mold glycosidase or protease that is present on the affinity column.

Newly Synthesized Man-6-P Receptor Undergoes Several Shifts in Apparent Molecular Weight

To analyze the kinetics of biosynthesis of the Man-6-P receptor, BW5147 cells were labeled for 20 min with [2-^{3}H]mannose and chased for various periods of time in the presence of excess unlabeled mannose. The cells were lysed by sonication in Triton X-100 and the labeled receptor was precipitated from the Triton X-100 extracts with the rabbit antiserum. The immunoprecipitates were then analyzed by SDS gel electrophoresis. Fig. 2 shows an autoradiogram of the gel. After a 10-min chase, two ^{3}H-labeled high molecular weight species are immunoprecipitated from BW5147 cells; one form migrates slightly faster than the ^{125}I mouse liver Man-6-P receptor standard (apparent molecular weight 215,000) and the other form migrates with the standard. The immunoprecipitate from the cells which had been chased for 2 h contains the same two species observed after the 10-min chase, and a third form that migrates slightly more slowly. A single species of Man-6-P receptor is recovered from BW5147 cells following 24 h of chase; this form migrates faster than the largest species observed following the 2-h chase.

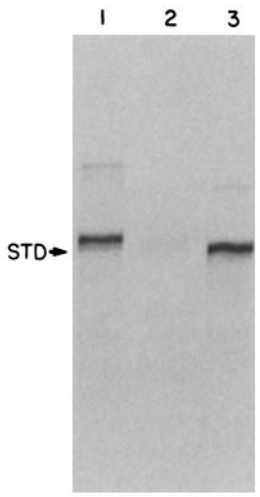

FIGURE 1 Lysosomal enzyme-binding capability of the immunoprecipitable receptor protein. BW5147 cells were labeled with [2-^{3}H]mannose for 20 min, chased for 2 h and lysed as described in Materials and Methods. Membranes were prepared, washed with Man-6-P to free occupied receptor, solubilized, and passed over a column of *D. discoideum* lysosomal enzymes coupled to AffiGel 10. After extensive washing the column was eluted with Man-6-P. Run-through, Man-6-P eluate, and unfractionated samples were immunoprecipitated. Lane *1*, total extract before affinity chromatography; lane *2*, column run-through; lane *3*, Man-6-P eluate. *STD*, [^{125}I]mouse liver Man-6-P receptor, prepared as described previously (19).

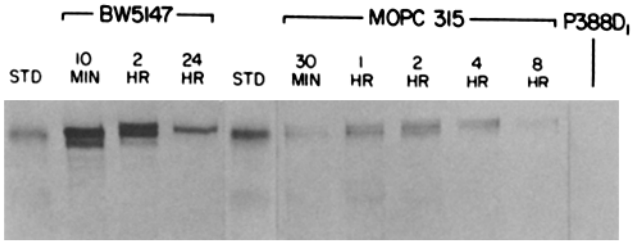

FIGURE 2 Pulse-chase kinetics of the biosynthesis of the Man-6-P receptor in BW5147, MOPC 315, and P388D$_1$ cells. Cells were labeled for 20 min with [2-^{3}H]mannose and chased for the times indicated. The Man-6-P receptor protein was solubilized, immunoprecipitated, and analyzed on a 6% SDS polyacrylamide gel. The first five lanes (BW5147) and last six lanes are from separate but identical experiments. Similar results were obtained in another experiment. The fluorograph of the first five lanes was exposed for 8 h, and that of the next six lanes for 40 h.

Immunoprecipitation from Man-6-P Receptor-deficient Cell Lines

Pulse-chase experiments similar to the one described above were performed using receptor-deficient MOPC 315 cells and receptor-negative P388D$_1$ cells to determine whether these cell lines synthesize a protein that cross-reacts with the receptor antiserum. The MOPC 315 cells have 3% receptor activity relative to the level found in BW5147 cells while the P388D$_1$ cells have <2% the activity of BW5147 cells (undetectable) (10). As shown in Fig. 2, MOPC 315 cells synthesize several [^{3}H]mannose-labeled species that are recognized by the antiserum. After a 30-min chase, a small quantity of ^{3}H-labeled receptor is recovered and this increases during the next 30 min of chase. The increase in the total amount of radioactivity precipitated by the antiserum indicates that the chase is not totally efficient in MOPC 315 cells; this most likely reflects a large internal pool of [^{3}H]Man-6-P and/or GDP-mannose. The two forms of the Man-6-P receptor immunoprecipitated from the MOPC 315 cell extracts during the first 60 min of chase are similar in size to the two species present after 10 min of chase in BW5147 cells. The immunoprecipitate obtained from MOPC 315 cells after 2 h of chase contains a higher molecular weight form of the receptor not seen at the earlier times and this becomes the predominant species with longer times of chase (4 and 8 h).

In contrast to MOPC 315 cells, P388D$_1$ cells do not synthesize any radioactive proteins that are precipitated by the anti-Man-6-P receptor serum (Fig. 2), even though the cells incorporate a similar amount of [^{3}H]mannose into total cellular glycoproteins during the 20-min pulse. When the autoradiography was carried out for a 125-fold longer period of time than shown in the figure, it was still not possible to detect any Man-6-P receptor. On the basis of this observation, we estimate that P388D$_1$ cells synthesize receptor at <0.2% of the rate of BW5147 cells. In a separate experiment the P388D$_1$ cells were labeled for 20 min with [2-^{3}H]mannose and chased for 2, 4, 8 and 16 h. No immunoprecipitable Man-6-P receptor was detected at any time point. To exclude the possibility that the receptor was being synthesized and then degraded when the cells were lysed, ^{125}I-labeled purified murine liver Man-6-P receptor was incubated with crude extracts of P388D$_1$ cells at 4°C and 22°C for 4 h. The receptor remained intact as judged by its subsequent migration on SDS gels (not shown).

Immunoprecipitation of [^{35}S]Methionine-labeled Receptor

The various cell lines were next labeled with [^{35}S]methionine for 30 min and chased for 1, 2, 4, and 6 h before harvesting and immunoprecipitating the receptor. As in the case of the [^{3}H]mannose-labeling experiments, receptor synthesis was detected in both the BW5147 and the MOPC 315 cells, but the receptor turned over much more rapidly in the latter cell type (Fig. 3). Again, receptor synthesis could not be

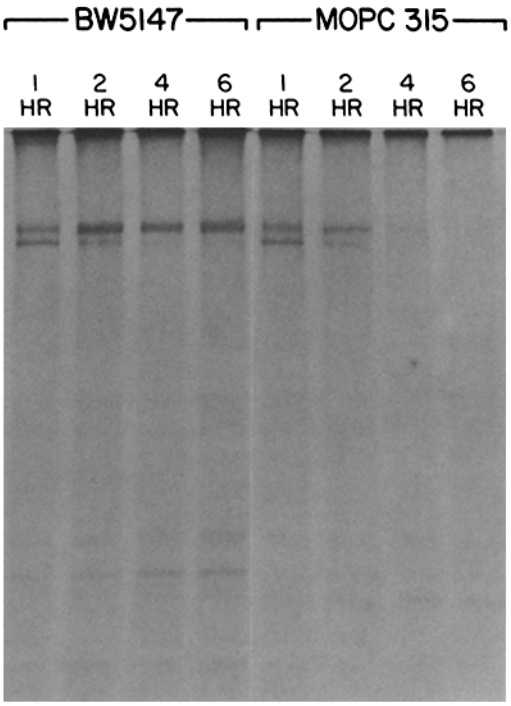

FIGURE 3 Incorporation of [^{35}S]methionine into the Man-6-P receptor of BW5147 and MOPC 315 cells. Cells were labeled for 30 min with 0.25 mCi/ml [^{35}S]methionine and chased for the times indicated. The Man 6-P receptor protein was solubilized, immunoprecipitated, and analyzed on a 6% SDS polyacrylamide gel.

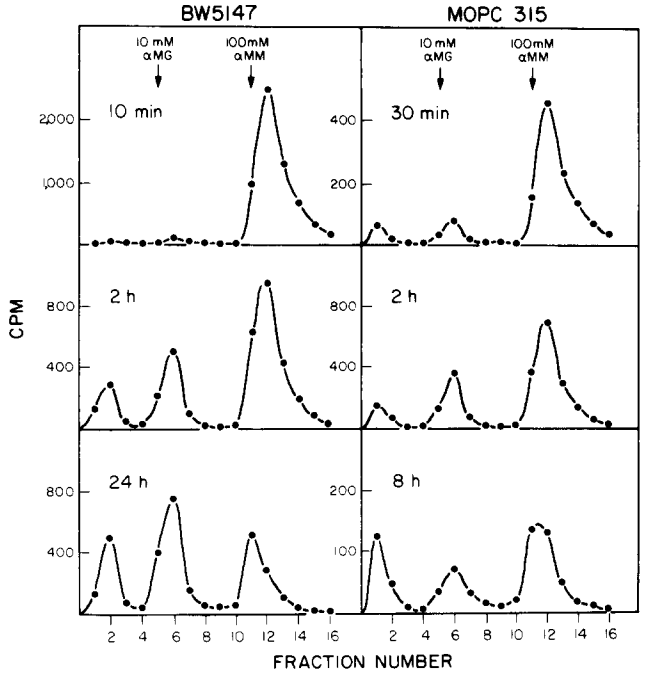

FIGURE 4 Con A-Sepharose chromatography of Man-6-P receptor glycopeptides isolated from BW5147 and MOPC 315 cells. After the pulse-chase analysis shown in Fig. 2, the Man-6-P receptor was eluted from the gels, digested with pronase, and aliquots fractionated on Con A-Sepharose. The columns were washed with TBS and eluted with 10 mM α-methylglucoside and 100 mM α-methylmannoside, at the indicated points. 100% of each MOPC 315 pronase digest, 20% of the BW5147 10-min and 2-h digests, and 30% of the BW5147 24-h digest were applied to the Con A-Sepharose columns.

detected in the P388D$_1$ cells (not shown). These findings exclude the possibility that the results obtained with [^{3}H]-mannose labeling were due to alterations in glycosylation or oligosaccharide processing in the receptor-deficient cell lines.

In these experiments a different anti-Man-6-P receptor antiserum was used since it gave immunoprecipitates that were less contaminated with extraneous proteins. However, similar results were obtained with the other antiserum. One interesting difference between these antisera is that the one used in the [^{35}S]methionine experiment shown in Fig. 3 does not precipitate the initial, fast-migrating receptor band (compare Fig. 3 with Fig. 2). This finding is consistent with the observation of Sahagian and Neufeld (31) that this particular antiserum will not precipitate the Man-6-P receptor of Chinese hamster ovary cells until 50 min after synthesis.

Structure of the Asn-linked Oligosaccharides

To analyze the nature of the oligosaccharides present on the receptor at the various chase times, we excised the radioactive bands en bloc from the dried gels of the [^{3}H]mannose experiment and treated with pronase to generate soluble glycopeptides. The glycopeptides were then fractionated on Con A-Sepharose. Fig. 4 shows the Con A-Sepharose elution profiles for the receptor glycopeptides from BW5147 cells and MOPC 315 cells. Under these elution conditions, tri- and tetraantennary complex-type glycopeptides pass through the column (peak I), biantennary complex-type glycopeptides bind and are eluted with 10 mM α-methylglucose (peak II), and high mannose-type units bind and are eluted with 100 mM α-methylmannose (peak III) (16, 17). As shown in the figure, the receptor oligosaccharides are initially of the high mannose-type and are processed to the complex-type with increasing times of the chase. However, even after long chase times some of the high mannose-type oligosaccharides remain. The Con A-Sepharose profile obtained after an intermediate chase (9 h, not shown) had a pattern virtually identical to the one depicted here for a 24-h chase suggesting that a stable pattern had been reached. It appears, therefore, that the mature receptor retains some high mannose-type structures. The size of the BW5147 high mannose oligosaccharides was determined by digesting the α-methylmannoside-eluted material with endo-β-*N*-acetylglucosaminidase H to release the oligosaccharides from the underlying peptides. The released oligosaccharides were then analyzed by HPLC as shown in Fig. 5. After the 20-min pulse and 10-min chase, the predominant oligosaccharide contains nine mannose residues. There are smaller quantities of oligosaccharides containing eight mannoses or nine mannoses plus one glucose residue (Fig. 5*A*). With longer times of chase, there is a decrease in the total quantity of high mannose oligosaccharides (Fig. 4) and those that persist show signs of mannose processing. For example, after 2 h of chase the major oligosaccharide species still contains nine mannose residues but there is an increase in those possessing seven and eight mannose residues (Fig. 5*B*). Moreover, oligosaccharides possessing eight mannose residues predominate after a 9-h chase (Fig. 5*C*).

The partial structures of the complex-type oligosaccharide were determined as follows: first, strong acid hydrolysis followed by paper chromatography of peaks I and II from Con A-Sepharose yielded a mannose:fucose ratio of 3:1.2. Second, 83% of the radioactivity present in peak II from Con A-Sepharose bound to lentil lectin-Sepharose and was eluted with 0.5 M α-methylmannoside. Methylation of this material

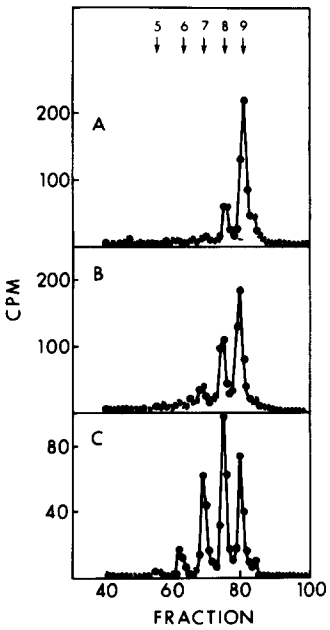

FIGURE 5 Size of the high mannose oligosaccharides isolated from the BW5147 cell Man-6-P receptor. The 0.1 M α-methylmannoside eluates from the Con A-Sepharose columns (Fig. 4) were desalted, concentrated by rotary evaporation under reduced pressure, and digested with 1 mU of Endo H. The digests were applied to Amberlite MB3 minicolumns in H_2O and an aliquot of released, neutral oligosaccharides was taken to dryness, dissolved in 35% aqueous acetonitrile, and applied to an AX5 micropak high performance liquid chromatography column (Varian, Sunnyvale, CA). The oligosaccharides were eluted using a 60-ml linear gradient from 35% to 65% aqueous acetonitrile (21); 0.6-ml fractions were collected and diluted with 4 ml of scintillation fluor for radioactivity determinations. The oligosaccharides were isolated from the BW5147 cell Man-6-P receptor following a 20-min labeling with [^{3}H]mannose and chase times of 10 min, 2 h, and 9 h. The arrows indicate the elution position of the following high mannose oligosaccharide units: $Man_5GlcNAc$ (5), $Man_6GlcNAc$ (6), $Man_7GlcNAc$ (7), $Man_8GlcNAc$ (8), and $Man_9GlcNAc$ (9).

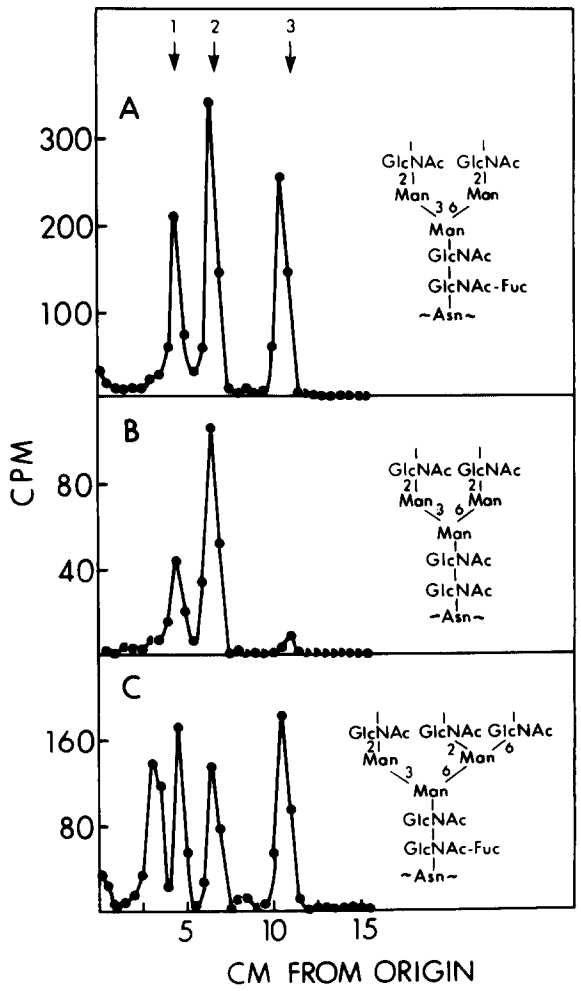

FIGURE 6 Methylation analysis of the [^{3}H]mannose-labeled complex type oligosaccharide units isolated from the BW5147 cell Man-6-P receptor. The Con A-Sepharose run-through oligosaccharides from cells labeled for 30 min and chased for 2 and 9 h, were pooled (peak I) and fractionated using lentil lectin-Sepharose as described elsewhere (17). Similarly, the 10 mM α-methylmannoside eluates were pooled (peak II) and fractioned using lentil lectin-Sepharose. 62% of the radioactivity from Con A peak I and 83% of that from peak II bound to lentil lectin-Sepharose and was eluted with 0.5 M α-methylmannoside. The resulting four fractions were desalted on Sephadex G-25 and methylated as described elsewhere (17). The thin-layer chromatograms of the methylated monosaccharides obtained after acid hydrolysis are shown for the following: *A*, Con A peak II/lentil lectin bound; *B*, Con A peak II/not bound by lentil lectin; *C*, Con A peak I/lentil lectin bound. The deduced minimal structures for the oligosaccharide units are shown in the inset to each panel. The arrows in *A* indicate the migration of the following methylated monosaccharides: 2,4-dimethylmannose (1), 3,4,6-trimethylmannose (2), and 2,3,4-trimethylfucose (3).

yielded 2,4-dimethylmannose, 3,4,6-trimethylmannose, and 2,3,4-trimethylfucose in a ratio of 1:1.7:1.3 (Fig. 6*A*). These properties indicate that this fraction contains a biantennary oligosaccharide possessing a core fucose residue (17) as shown in the *inset* to Fig. 6*A*. Methylation of the Con A peak II material that did not bind to lentil lectin-Sepharose yielded 2,4-dimethylmannose, 3,4,6-trimethylmannose, and 2,3,4-trimethylfucose in a 1:2.3:0.2 ratio (Fig. 6*B*); these properties are consistent with a nonfucosylated, biantennary oligosaccharide as shown in the inset of Fig. 6*B*. Third, 62% of the radioactivity present in the Con A-Sepharose peak I material bound to lentil lectin-Sepharose and was eluted with 0.5 M α-methylmannoside. Methylation of this material (Fig. 6*C*) yielded 3,4-dimethylmannose, 2,4-dimethylmannose, 3,4,6-trimethylmannose, and 2,3,4-trimethylfucose in a 1.15:1:0.96:1.34 ratio, which is consistent with a triantennary structure as shown in the *inset* to Fig. 6*C*. The Con A-Sepharose peak I material that did not bind to lentil lectin-Sepharose gave a complex methylation pattern suggestive of a mixture of tri- and tetraantennary oligosaccharides (not shown).

To determine whether the complex-type oligosaccharides contain terminal sialic acid residues, aliquots of peaks I and II from the Con A-Sepharose columns were digested with a mixture of α-mannosidase, β-galactosidase, and β-*N*-acetylglucosaminidase before and after treatment with neuraminidase or mild acid; the extent of [^{3}H]mannose release was then assessed by paper chromatography. As shown in Table I, mixed glycosidase digestion of the BW5147 Man-6-P receptor complex-type glycopeptide fractions released free mannose only after pretreatment with neuraminidase or mild acid. This indicates that the complex-type asparagine-linked units are fully sialylated. Moreover, the glycopeptides recovered from the receptor after 24 h of chase remain sialylated as evidenced by the low release of [^{3}H]mannose in the absence of desialylation. Mixed glycosidase digestion of the Man-6-P receptor

TABLE I

Mixed Glycosidase Digestion of Man-6-P Receptor Glycopeptides

Source of receptor	Chase time	Con A fraction	% ^{3}H-Mannose Released		
			−Neur-aminidase	+Neur-aminidase	+Mild Acid
	h				
BW5147	2 + 9*	Peak I	0	31	58
		Peak II	10	42	62
MOPC 315	2 + 21*	Peak II	0	63	ND
BW5147	24	Peak I	0	ND	42
		Peak II	19	ND	75

The complex-type glycopeptides isolated from the Man-6-P receptor following a 20-min labeling with [2-^{3}H]mannose and various chase times were desialylated either by treatment with *Vibrio cholerae* neuraminidase (0.01 IU for 5 h at 37°C in 50 mM Na acetate, pH 5.5, 150 mM NaCl, 4 mM $CaCl_2$) or by mild acid hydrolysis (2 N HOAc at 100°C for 60 min). The acetic acid was removed by rotary evaporation under reduced pressure following acid hydrolysis. The glycopeptides (±desialylation) were then treated with 0.02 U of jack bean β-*N*-acetylglucosaminidase, 0.014 units of jack bean β-galactosidase, and 0.235 units of jack bean α-mannosidase in 0.025 ml of 40 mM NaAc, pH 4.6, at 37°C for 18 h. To assess the amount of [^{3}H]mannose released by the mixed glycosidase digestion, the reaction mixtures were spotted directly onto Whatman #1 paper and chromatographed for 16 h in ethyl acetate/pyridine/acetic acid/water (5/5/1/3). The chromatograms were cut into 1 cm strips and 0.4 ml of H_2O and 4 ml of scintillation fluid were added to each for determination of radioactivity. Mild acid hydrolysis alone did not release any radioactivity from the glycopeptides. *ND*, not determined

* The receptor glycopeptides analyzed here were from an experiment similar to that depicted in Fig. 4; 2 + 9 and 2 + 21 refer to analyses of combined fractions from equal portions of two different chase times.

glycopeptides isolated from MOPC 315 cells indicated that these oligosaccharides also contain terminal sialic acid residues (Table I).

DISCUSSION

The Man-6-P receptor is an important component of a pathway used in targeting acid hydrolases to lysosomes. Using Chinese hamster ovary cells, it has been shown that proteins possessing the Man-6-P recognition signal bind to cell surface Man-6-P receptors and are clustered in coated pits before their appearance in lysosomal granules (22). Mutant Chinese hamster ovary cells, selected for resistance to a ricin-phosphomannose conjugate, are deficient in cell surface Man-6-P receptor activity and secrete larger quantities of newly synthesized acid hydrolases than their wild-type counterparts. These data suggest that the reduced receptor levels prevent the efficient delivery of acid hydrolases to lysosomes (23, 24). Kinetic analysis of the binding of newly synthesized acid hydrolases to the BW5147 cell Man-6-P receptor suggests that intracellular receptor binding precedes entry of the hydrolases into lysosomes (4). Moreover, the Man-6-P receptor is present in a large number of cells and tissues from different species (6–10) indicating that it is an integral part of a general pathway for achieving lysosomal enzyme segregation.

Despite its apparent involvement in the targeting pathway, there are cells that lack (or contain barely detectable) Man-6-P receptor activity and yet possess high intralysosomal levels of various acid hydrolases (10). The data presented here provide an explanation for the decreased receptor activity in two of the receptor-deficient cell lines. The receptor-negative $P388D_1$ cells do not synthesize any protein that is precipitable by the Man-6-P receptor antisera. In contrast, receptor-deficient MOPC 315 cells synthesize a receptor that turns over very rapidly. The mechanism of this rapid turnover is currently unknown.

The Man-6-P receptor synthesized by both BW5147 and MOPC 315 cells is a glycoprotein that undergoes a series of posttranslational modifications as assessed by SDS gel electrophoresis (Fig. 2). After a 20-min pulse with [^{3}H]mannose and 10-min chase, two forms of the Man-6-P receptor are recovered from the BW5147 cells. Since the receptor contains only high mannose-type asparagine-linked oligosaccharides at this time, the difference in apparent molecular weight observed between the two forms can not be accounted for by processing of the oligosaccharides to complex-type structures. With further time, an increase in the apparent molecular weight of the Man-6-P receptor occurs concomitant with the conversion of the high mannose oligosaccharides to complex-type units that are highly sialylated. Since sialylation of oligosaccharide units on glycoproteins can result in an apparent increase in the molecular weight observed by SDS gel electrophoresis, it is possible that this second increment in apparent molecular weight may result from oligosaccharide processing. The mature form of the receptor isolated after 24 h of chase migrates slightly faster on the SDS gels than the largest form observed after 2 h of chase (Fig. 2). Since the complex-type oligosaccharides recovered from the receptor at this time are still fully sialylated, loss of sialic acid residues from the asparagine-linked units cannot account for this shift. It is possible, however, that the decrease in apparent molecular weight reflects an alteration in O-linked oligosaccharide chains or a proteolytic reaction.

The presence of both fucose and sialic acid residues on the Man-6-P receptor oligosaccharides indicates that the newly synthesized receptor passes through the *trans* face of the Golgi apparatus during its maturation, similar to the pathway taken by other secretory and membrane glycoproteins (25–27). However, unlike most other glycoproteins that contain sialylated oligosaccharides, the receptor remains almost entirely intracellular. In fact, we have not been able to detect any receptor activity on the surface of BW5147 cells (4), and even in fibroblasts where surface receptor is easily demonstrable, >80% of the receptor is intracellular (28).

Several investigators have presented evidence for the recycling of both the cell-surface and intracellular Man-6-P receptor (3, 4). It has also been shown that low pH accelerates the dissociation of the Man-6-P receptor-ligand complex (3, 6). These observations have led to the proposal that following binding of ligand to the receptor, the complex is translocated to an acidic granule where the low pH causes dissociation and completes the delivery process. The receptor can then recycle and bind another ligand (3). According to this model, the receptor is transiently exposed to a low pH, during which time the various acid glycosidases that are carried as ligands could have the opportunity to degrade the receptor oligosaccharides. Yet analysis of the oligosaccharides indicates that they remain intact (e.g., fully sialylated) for up to 24 h of chase. There are several possible explanations for this curious observation. One possibility is that the Man-6-P receptor does not actually enter an acidified granule or, if it does, the oligosaccharides are somehow protected from the action of the glycosidases. A more intriguing possibility is that the receptor oligosaccharides are actually partially degraded but upon recycling to the Golgi where the ligands are picked up, the oligosaccharides are reglycosylated. Consequently, at steady state there would be no apparent change in the oligo-

saccharide structures. Since reglycosylation of glycoproteins and glycopeptides has been demonstrated in other systems (29, 30), this explanation can fully account for the findings with the Man-6-P receptor.

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REFERENCES

1. Hickman, S., and E. F. Neufeld. 1972. A hypothesis for I-cell disease: defective hydrolases that do not enter lysosomes. *Biochem. Biophys. Res. Commun.* 49:992–999.
2. Kaplan, A., D. T. Achord, and W. S. Sly. 1977. Phosphohexosyl components of a lysosomal enzyme are recognized by pinocytosis receptors on human fibroblasts. *Proc. Natl. Acad. Sci. USA.* 74:2026–2030.
3. Gonzalez-Noriega, A., J. H. Grubb, V. Talkad, and W. S. Sly. 1980. Chloroquine inhibits lysosomal enzyme pinocytosis and enhances lysosomal enzyme secretion by impairing receptor recycling. *J. Cell Biol.* 85:839–852.
4. Gabel, C. A., D. E. Goldberg, and S. Kornfeld. 1982. Lysosomal enzyme oligosaccharide phosphorylation in mouse lymphoma cells:specificity and kinetics of binding to the mannose 6-phosphate receptor in vivo. *J. Cell Biol.* 95:536–542.
5. Sly, W. S., and H. D. Fischer. 1982. The phosphomannosyl recognition system for intracellular and intercellular transport of lysosomal enzymes. *J. Cell Biochem.* 18:67–85.
6. Sahagian, G. G., J. Distler, and G. W. Jourdian. 1981. Characterization of a membrane-associated receptor from bovine liver that binds phosphomannosyl residues of bovine testicular β-galactosidase. *Proc. Natl. Acad. Sci. USA.* 78:4289–4293.
7. Steiner, A., and L. H. Rome. 1982. Assay and purification of a solubilized membrane receptor that binds the lysosomal enzyme α-L-iduronidase. *Arch. Biochem. Biophys.* 214:681–687.
8. Fischer, H. D., A. Gonzalez-Noriega, W. S. Sly, and D. J. Morre. 1980. Phosphomannosyl-enzyme receptors in rat liver. *J. Biol. Chem.* 255:9608–9615.
9. Hasilik, A., B. Voss, and K. von Figura. 1981. Transport and processing of lysosomal enzymes by smooth muscle cells and endothelial cells. *Exp. Cell Res.* 133:23–30.
10. Gabel, C. A., D. E. Goldberg, and S. Kornfeld. 1983. Identification and characterization of cells deficient in the mannose 6-phosphate receptor:evidence for an alternate pathway for lysosomal enzyme targeting. *Proc. Natl. Acad. Sci. USA.* 80:775–779.
11. Hasilik, A., A. Waheed, and K. von Figura. 1981. Enzymatic phosphorylation of lysosomal enzymes in the presence of UDP-N-acetyl-glucosamine. Absence of the activity in I-cell fibroblasts. *Biochem. Biophys. Res. Commun.* 98:761–767.
12. Reitman, M. L., A. Varki, and S. Kornfeld. 1981. Fibroblasts from patients with I-cell disease and pseudo-Hurler polydystrophy are deficient in UDP-N-acetylglucosamine:glycoprotein N-acetylglucosaminylphosphotransferase activity. *J. Clin. Invest.* 67:1574–1579.
13. Owada, M., and E. F. Neufeld. 1982. Is there a mechanism for introducing acid hydrolases into liver lysosomes that is independent of mannose 6-phosphate recognition? Evidence from I-cell disease. *Biochem. Biophys. Res. Commun.* 105:814–820.
14. Waheed, A., R. Pohlmann, A. Hasilik, K. von Figura, A. van Elsen, and J. G. Leroy. 1982. Deficiency of UDP-N-acetylglucosamine: lysosomal enzyme N-acetylglucosamine 1-phosphotransferase in organs of I-cell patients. *Biochem. Biophys. Res. Commun.* 105:1052–1058.
15. Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
16. Narasimhan, S., J. R. Wilson, E. Martin, and H. Schachter. 1979. A structural basis for four distinct elution profiles on concanavalin A-Sepharose affinity chromatography of glycopeptides. *Can. J. Biochem.* 57:83–96.
17. Kornfeld, K., M. L. Reitman, and R. Kornfeld. 1981. The carbohydrate-binding specificity of pea and lentil lectins. *J. Biol. Chem.* 256:6633–6640.
18. Freeze, H. H., A. L. Miller, and A. Kaplan. 1980. Acid hydrolases from *D. discoideum* contain phosphomannosyl recognition markers. *J. Biol. Chem.* 255:11081–11084.
19. Varki, A., and S. Kornfeld. 1983. The spectrum of anionic oligosaccharides released by endo H from glycoproteins:structural studies and interactions with the phosphomannosyl receptor. *J. Biol. Chem.* 258:2808–2818.
20. Fischer, H. D., K. E. Creek, and W. S. Sly. 1982. Binding of phosphorylated oligosaccharides to immobilized phosphomannosyl receptors. *J. Biol. Chem.* 257:9938–9943.
21. Mellis, S., and J. Baenziger. 1981. Separation of neutral oligosaccharides by high-performance liquid chromatography. *Anal. Biochem.* 114:276–280.
22. Willingham, M. C., I. H. Pastan, G. G. Sahagian, G. W. Jourdian, and E. F. Neufeld. 1981. Morphologic study of the internalization of a lysosomal enzyme by the mannose 6-phosphate receptor in cultured Chinese hamster ovary cells. *Proc. Natl. Acad. Sci. USA.* 78:6967–6971.
23. Robbins, A. R., R. Myerowitz, R. J. Youle, G. J. Murray, and D. M. Neville, Jr. 1981. The mannose 6-phosphate receptor of Chinese hamster ovary cells: isolation of mutants with altered receptors. *J. Biol. Chem.* 256:10618–10622.
24. Robbins, A. R., and R. Myerowitz. 1981. The mannose 6-phosphate receptor of Chinese hamster ovary cells:compartmentalization of acid hydrolases in mutants with altered receptors. *J. Biol. Chem.* 256:10623–10627.
25. Roth, J., and E. G. Berger. 1982. Immunocytochemical localization of galactosyltransferase in HeLa cells:codistribution with thiamine pyrophosphatase in trans-Golgi dictyosomae. *J. Cell Biol.* 92:223–229.
26. Dunphy, W. G., E. Fries, L. J. Urbani, and J. E. Rothman. 1981. Early and late functions associated with the Golgi apparatus reside in distinct compartments. *Proc. Natl. Acad. Sci. USA.* 78:7453–7457.
27. Goldberg, D. E., and S. Kornfeld. 1983. Evidence for extensive subcellular organization of asparagine-linked oligosaccharide processing and lysosomal enzyme phosphorylation. *J. Biol. Chem.* 258:3159–3165.
28. Fischer, H. D., A. Gonzalez-Noriega, and W. S. Sly. 1980. β-Glucuronidase binding to human fibroblast membrane receptors. *J. Biol. Chem.* 255:5069–5074.
29. Regoeczi, E., P. A. Chindemi, M. T. Debanne, and P. A. Charlwood. 1982. Partial resialylation of human asialotransferrin type 3 in the rat. *Proc. Natl. Acad. Sci. USA.* 79:2226–2230.
30. Kreisel, W., B. A. Volk, R. Buchsel, and W. Reutter. 1980. Different half-lives of the carbohydrate and protein moieties of a 110,000-dalton glycoprotein isolated from plasma membranes of rat liver. *Proc. Natl. Acad. Sci. USA.* 77:1828–1831.
31. Sahagian, G. G., and E. F. Neufeld. 1983. Biosynthesis and turnover of the mannose 6-phosphate receptor in cultured Chinese hamster ovary cells. *J. Biol. Chem.* 258:7121–7128.