Mannose 6-Phosphate Receptor-mediated Endocytosis of Acid Hydrolases: 
Internalization of β-Glucuronidase Is Accompanied by a 
Limited Dephosphorylation

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Abstract. Endocytosis of acid hydrolases via the cell surface mannose 6-phosphate (Man 6-P) receptor results in the delivery of the enzymes to lysosomes. To examine the fate of the ligand-associated phosphorylated high mannose oligosaccharides, we have analyzed the asparagine-linked oligosaccharides attached to β-glucuronidase after uptake and processing by Man 6-P receptor-positive mouse L cells. β-Glucuronidase, double-labeled with [2-3H]mannose and [35S]methionine, was isolated from the growth medium of mouse P388D1 cells. 80% of the [3H]mannose associated with the secreted enzyme was recovered as high mannose-type oligosaccharides, and 24–37% of these units were phosphorylated. Three species of phosphorylated oligosaccharides were identified; high mannose-type units containing either one or two phosphomonoesters, and hybrid-type units containing one phosphomonoester and one sialic acid residue. After endocytosis by the L cells, the β-glucuronidase molecules migrated faster on an SDS gel, suggesting that the enzymes had been processed within lysosomes. Examination of the cell-associated β-glucuronidase molecules indicated that: (a) the percentage of phosphorylated oligosaccharides remained comparable to the input form of the enzyme, even after a 24-h chase period, (b) the presence of a single species of phosphorylated oligosaccharide that contained one phosphomonoester, and (c) the positioning of the phosphate within the intracellular monophosphorylated species was comparable to the positioning of the phosphate within the two phosphomonoester species originally secreted by the P388D1 cells. Therefore, the internalized β-glucuronidase molecules undergo a limited dephosphorylation; oligosaccharides containing two phosphomonoesters are converted to monophosphorylated species, but the one phosphomonoester forms are conserved. A comparison of the phosphorylated oligosaccharides recovered from ligands internalized by the L cells at 37°C and 20°C indicated that: (a) molecules internalized at 20°C retain a higher percentage of phosphorylated structures; and (b) at both temperatures the predominant phosphorylated oligosaccharide contains a single phosphomonoester group. The results indicate that the Man 6-P recognition marker persists after endocytosis and delivery to lysosomes and support the possibility that the limited dephosphorylation of the oligosaccharides may occur en route to these organelles.

The mannose 6-phosphate (Man 6-P) receptor is an important component in the delivery of newly synthesized acid hydrolases to lysosomes (32, 37). Lysosomal enzyme biogenesis begins in the rough endoplasmic reticulum, where the enzymes attain asparagine-linked oligosaccharides during their translation (5, 27, 34). Upon transfer to the Golgi apparatus, the enzymes are phosphorylated by UDP-N-acetylglucosamine/lysosomal enzyme N-acetylglucosaminylphosphotransferase to generate a phosphodiester linkage between the C-1 position of N-acetylglucosamine and the C-6 position of mannose residues within the oligosaccharide (21, 33, 38). In a second step, the N-acetylglucosamine residues are removed by a specific Golgi-associated phosphoglycosidase (40, 42) to reveal the Man 6-P recognition marker. The Man 6-P receptor then binds to the phosphorylated high mannose oligosaccharides and translocates with the attached ligand to lysosomes (32, 37). The low pH of the vesicle is thought to cause dissociation of the receptor–ligand complex, thus allowing the unoccupied receptor to recycle to the Golgi apparatus and bind another ligand (18).

While the 215-kD Man 6-P receptor is a common element in the targeting of newly synthesized acid hydrolases to lysosomes (7), it is not a universal requirement. For example, some cells lack (or are deficient in) the receptor yet they actively sequester acid hydrolases within lysosomes (11, 16); therefore, an alternate delivery mechanism must exist within

1. Abbreviations used in this paper: Endo H, endo-β-N-acetylglucosaminidase H; HPLC, high-performance liquid chromatography; Man 6-P, mannose 6-phosphate; MEM, a-minimum essential medium; Rec+, receptor positive; TBS, 10 mM Tris, pH 8, 150 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 0.02% NaN3,

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these cells. Interestingly, Hoflack and Kornfeld (22, 23) have recently found that both the 215-kD Man 6-P receptor-deficient and receptor-positive cells contain a 46-kD protein which, in the presence of cations, will also bind to phosphorylated high mannose oligosaccharides. Both its cation dependence and its immunological properties indicate that the 46-kD binding protein is unrelated to the 215-kD Man 6-P receptor (23). The existence of multiple Man 6-P binding proteins raises the possibility that each protein may be involved in the formation of unique classes of lysosomes.

We have previously observed that cell lines that lack the 215-kD Man 6-P receptor are inefficient in the degradation of the Man 6-P recognition marker (II, 12). This is in contrast to the situation in many Man 6-P receptor-positive cells where the kinetics of dephosphorylation parallel entry of the newly synthesized enzymes into lysosomes, suggesting that the recognition marker is destroyed intralysosomally by a resident phosphatase (10, II). Longevity of the Man 6-P recognition marker has also recently been observed in a Man 6-P receptor-positive (Rec +) line of mouse L cells. Unlike the cells previously analyzed that contain the 215-kD receptor (10, II), the Rec + L cells accumulate acid hydrolases at steady state that retain the Man 6-P recognition marker; surprisingly, the phosphorylated molecules are associated with lysosomes (9).

To learn more about the processing of the Man 6-P recognition marker and of the pathways involved in lysosomal enzyme targeting, we have analyzed the fate of phosphorylated high mannose oligosaccharides after endocytosis of β-glucuronidase into Man 6-P Rec + mouse L cells and human fibroblasts. Structural analysis of the oligosaccharides recovered from β-glucuronidase before and after endocytosis indicated that: (a) the percentage of the high mannose oligosaccharides bearing the Man 6-P recognition marker declined maximally by only 33 %, (b) oligosaccharides originally possessing two phosphomonoesters or one phosphomonoester and one sialic acid residue were converted upon endocytosis to monophosphorylated species, and (c) the location of the remaining phosphomonoester group within the high mannose oligosaccharide was not specific to either the α-1,3 or α-1,6 branch of the oligosaccharide unit. The results indicate that receptor-mediated internalization is accompanied by a limited dephosphorylation of the β-glucuronidase oligosaccharides, and support the hypothesis that dephosphorylation of the Man 6-P recognition marker may occur before the enzyme reaches lysosomes.

Materials and Methods

Maintenance of Cells and Metabolic Labeling

All cells were grown as monolayers and were maintained in α-minimal essential medium (MEM) containing 10 % fetal bovine serum. Normal human fibroblasts were kindly provided by Dr. Larry Witte, Columbia University. The Man 6-P receptor-positive line of mouse L cells was previously described (9).

For production of radiolabeled β-glucuronidase, mouse P388D1 cells were grown in two T-150 flasks. The growth medium was discarded and each flask received 25 ml of complete MEM; 10 % fetal bovine serum containing 3-4 ml of the dialyzed P388D1 secretions. After an overnight incubation at 37°C, the medium was recovered (post-uptake supernatant), and the cells were either harvested immediately or 80 ml of fresh MEM, containing 10 % fetal bovine serum, was added and the dishes were replaced at 37°C for an additional overnight incubation.

To harvest, the cells were washed twice while attached to the dishes with 10 mM phosphate, pH 7, 150 mM NaCl, then dislodged with a rubber policeman. The cells were collected by centrifugation and quick frozen in a dry ice-ethanol bath. The frozen cells were suspended in 1.2 ml of 25 mM Hapes, pH 7, 0.1 M NaCl, 5 mM phosphate, 0.15 trypsin inhibitory units/ml of Aprotinin (Sigma Chemical Co., St. Louis, MO), 1 % Triton X-100, 0.2 % deoxycholate, 5 mM Man 6-P (solubilization buffer). The suspension was incubated on ice for 30 min, clarified in an Eppendorf centrifuge for 5 min, and the resulting supernatant and pellet were recovered. The pellet was resuspended in 1.2 ml of the solubilization buffer by a brief burst of a probe sonicator, incubated for an additional 20 min on ice, then combined with the original supernatant and centrifuged at 10,000 g for 30 min at 4°C. The resulting supernatant (cell-associated fraction) was recovered and pre-cleared by addition of 0.05 % of a 10 % fixed Staphylococcus aureus suspension (Pansorbin; Calbiochem-Behring Corp., La Jolla, CA) incubated on ice for 30 min, then clarified by centrifugation. Each supernatant received the appropriate amount of goat anti-rat preputial β-glucuronidase serum (a generous gift of Dr. Roger Gans) and was incubated overnight at 4°C. The immune complexes were recovered by adsorption to Pansorbin, and the bacteria were washed five times (by repeated centrifugations) with 25 mM Hapes, pH 7, 0.1 M NaCl, 1 % Triton X-80, 0.2 % deoxycholate, 5 mM glucose 6-phosphate and once with 50 mM Tris, pH 6.8. The washed bacterial pellet was suspended in 0.12 ml of disaggregation buffer (28), boiled for 3 min, and the bacteria were removed by centrifugation. The supernatant was loaded onto a 10 % polyacrylamide gel (28), and after electrophoresis the gel was stained, soaked in an enhancer (Amplify; Amersham Corp.), and dried; fluorography was performed at ~70°C. In some experiments, the immunoprecipitated β-glucuronidase was solubilized with 50 mM Tris, pH 6.8, 1 % SDS, 15 mM dithiothreitol rather than the disaggregation buffer and digested with 0.5 μl of endo-β-N-acetylgalactosaminidase H (Endo H; Miles-Yeda, Rehovot, Israel) as previously described (35) before application to the gel.

β-Glucuronidase was recovered from the input P388D1 secretions after adjusting the solutions to 25 mM in Hapes, pH 7, 1 % Triton X-80, and 5 mM Man 6-P. The solutions were preclarified, and β-glucuronidase was immunoprecipitated as described above for the cell-associated fractions. To isolate β-glucuronidase from the post-uptake supernatant, the solution was adjusted to 20 mM in Hapes, pH 7, and solid ammonium sulfate was added to a final concentration of 70 % saturation. The precipitate was allowed to form on ice for 30 min, then was collected by centrifugation and redissolved in a minimum volume of HBS. After dialysis versus HBS, the solution was adjusted to 1 % in Triton X-80 and 5 mM in Man 6-P, and β-glucuronidase was recovered as described above for the input fraction.

Isolation and Characterization of Phosphorylated Oligosaccharides

The regions of the dried gel containing radiolabeled β-glucuronidase were excised and the radiolabel activity solubilized by adding 1.5 ml of 0.1 M Tris, pH 8, 20 mM CaCl2, 0.2 M β-glycerophosphate containing 30 mg/ml of protamine (preincubated at 55°C for 20 min). After an overnight incubation at 56°C, the digests were boiled, clarified by centrifugation, and the supernatants were desalted using a Sephadex G-25 column. The excluded glycopep-
tides were dried by rotary evaporation under reduced pressure, dissolved in 2 ml of 10 mM Tris, pH 8, 150 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 0.02% NaN₃ (TBS), and applied to a 1.5 ml column of Con A-Sepharose equilibrated in TBS. The column was eluted sequentially with 6 ml of TBS (peak I), 8 ml of 10 mM α-methylglucoside in TBS (peak II), and 10 ml of 100 mM α-methylmannoside in TBS at 56°C (peak III).

The glycopeptides eluted with α-methylmannoside were desalted on a Sephadex G-25 column and digested overnight at 37°C in 0.05 ml of citrate/phosphate buffer, pH 5.6, with 1.3 mU of Endo H. The digests were dried by rotary evaporation under reduced pressure, dissolved in 2 ml of 10 mM Tris, pH 8, 150 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, and 2 ml of 10 mM α-methylglucoside in TBS (peak I), and 10 ml of 100 mM α-methylmannoside in TBS at 56°C (peak III).

The column was eluted using a 200-ml linear gradient of ammonium acetate (peak I), 8 ml of 10 mM α-methylglucoside in TBS (peak II), and 10 ml of 100 mM α-methylmannoside in TBS at 56°C (peak III).

Alkaline phosphatase digestion of the phosphorylated oligosaccharides was performed as previously described (39). For QAE-Sephadex analysis, the alkaline phosphatase digests were diluted with 1.5 ml of 2 mM Tris base and applied to pasteur pipet columns of QAE-Sephadex equilibrated in 2 mM Tris base; bound oligosaccharides were eluted by the stepwise addition of higher concentrations of NaCl (39). When the dephosphorylated oligosaccharides were to be analyzed by high-performance liquid chromatography (HPLC), the alkaline phosphatase digests were diluted with 1 ml of H₂O and applied to 2-ml columns of Amberlite MB3 mixed bed ion-exchange resin; the columns were eluted with 10 ml of H₂O. HPLC analysis was performed as described (29).

Acetolysis of the phosphorylated high mannose oligosaccharides was carried out as described (39). The negatively charged fragments were recovered by applying the acetolysis mix to QAE-Sephadex and eluting the bound fragments with 0.1 M NaCl (39). The charged fragments were deionized by Dowex AG 50W chromatography, treated with alkaline phosphatase, and the dephosphorylated components were sized by descending paper chromatography using ethyl acetate/pyridine/acetic acid/H₂O (5:5:1:3) as solvent. The chromatogram was cut into 1-cm sections and the radioactivity associated with each was determined by liquid scintillation counting. The standards noted in the chromatogram were prepared by subjecting a mixture of neutral [³H]mannose-labeled high mannose oligosaccharides to the acetolysis procedure; the migration position of the fragments was deduced by comparison to published reports (39).

Results

Receptor-mediated Internalization of β-Glucuronidase

Mouse P388D₁ macrophages secrete high levels of β-glucuronidase (17, 24) as well as large quantities of phosphorylated oligosaccharides (41). We reasoned, therefore, that the culture supernatant isolated from P388D₁ cells should be highly enriched in phosphorylated β-glucuronidase and that the secreted molecules should be excellent ligands for the Man 6-P receptor. P388D₁ cells were grown for 3 d in the presence of [2-³H]mannose and [³²P]methionine, and the secreted double-labeled β-glucuronidase molecules were recovered by ammonium sulfate fractionation of the medium. After extensive dialysis, the labeled P388D₁ secretions were incubated with Rec⁺ mouse L cells in the presence and absence of 5 mM Man 6-P, and the cell-associated β-glucuronidase molecules were recovered by immunoprecipitation and analyzed by SDS gel electrophoresis. As shown in Fig. 1 A, in the absence of Man 6-P radiolabeled β-glucuronidase was recovered from the cells (lane 1), whereas in the presence of the hapten sugar no labeled molecules were im-

Figure 1. SDS gel electrophoresis of immunoprecipitated β-glucuronidase. Rec⁺ L cells were incubated with double-labeled P388D₁ secretions, then harvested and solubilized with Triton X-100. β-Glucuronidase was recovered from the detergent extracts by immunoprecipitation, and the precipitates were analyzed by SDS gel electrophoresis and fluorography. (A) L cells (confluent monolayers in p-10 dishes) were incubated for 22 h with the labeled P388D₁ secretions (containing 2 × 10⁻³ U of β-glucuronidase) in the absence (lane 1) or presence (lane 2) of 5 mM Man 6-P. (B) L cells (confluent monolayers in T-150 flasks) were incubated for 24 h with the labeled P388D₁ secretions (containing 7.6 × 10⁻³ U of β-glucuronidase) and β-glucuronidase was subsequently immunoprecipitated from the post-uptake supernatant (lane 1), the original P388D₁ secretions (lane 2), or the detergent extract of the cells after the 24-h uptake (lane 3) or 24-h uptake/7-h chase (lane 4). The fluorograph of the gels is shown; the arrow marks the migration position of rat preputial β-glucuronidase.
The regions of the dried gel shown in Fig. 1 B were excised, and the amount of radioactivity recovered in the various forms of β-glucuronidase was determined after solubilizing the proteins by pronase digestion. As shown in Table I, 65% of the [3H]mannose and 76% of the [35S]methionine-labeled radioactivity added as β-glucuronidase became associated with the cells during the 24-h duration of the uptake experiment. The ratio of $\frac{\text{[3H]}}{\text{[35S]}}$ was comparable (4.2) for the input and post-uptake forms of β-glucuronidase but was slightly lower (3.6) for the cell-associated form, suggesting that mannose residues may be lost upon internalization. Taken together, the results indicate that a large percentage of the input β-glucuronidase molecules are internalized via the surface Man 6-P receptor. The long incubation periods used in these studies were required for the cells to accumulate sufficient radioactivity for the subsequent oligosaccharide structural analyses.

**Comparison of the β-Glucuronidase Oligosaccharides**

Rec + L cells were incubated with the double-labeled P388D1 secretions for 25 h, and they were then divided into two populations: half of the cells were harvested immediately and the other half were chased for an additional 24 h in the absence of the ligand before harvesting. The β-glucuronidase molecules recovered by immunoprecipitation from the cell extracts were separated by SDS gel electrophoresis, and the high molecular weight glycopeptides generated by pronase digestion were fractionated by Con A-Sepharose chromatography.

**Table I. Quantitation of Immunoprecipitated β-Glucuronidase**

<table>
<thead>
<tr>
<th>Source of β-glucuronidase</th>
<th>cpm Recovered</th>
<th>% of Input</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$^3$H</td>
<td>$^{35}$S</td>
</tr>
<tr>
<td>Input P388D1 secretions</td>
<td>11,400</td>
<td>2,700</td>
</tr>
<tr>
<td>Post-uptake supernatant</td>
<td>3,740</td>
<td>880</td>
</tr>
<tr>
<td>Rec + L cell-associated</td>
<td>9,900</td>
<td>2,750</td>
</tr>
</tbody>
</table>

Rec + L cells were incubated with the double-labeled P388D1 secretions for 24 h at 37°C. The post-uptake supernatant was recovered, and the cells were solubilized with Triton X-100. β-Glucuronidase was isolated from each of the indicated fractions by immunoprecipitation, and the precipitates were subjected to SDS gel electrophoresis. After fluorography, the regions of the gel containing β-glucuronidase were excised and the radioactivity was solubilized by pronase digestion. Aliquots of the pronase digest were taken for liquid scintillation counting to determine the amount of radioactivity associated with each form. The β-glucuronidase immunoprecipitated from the input P388D1 secretions was derived from 3 ml of the preparation. For the uptake experiment, however, 4 ml of the secretions were applied to the L cells. Therefore, the values in parentheses represent the calculated cpm (assuming 100% recovery) that would be associated with β-glucuronidase from 4 ml of the original P388D1 secretions.
raphy. 80% of the [3H]mannose-labeled radioactivity associated with β-glucuronidase isolated from the original P388D1 secretions bound tightly to Con A-Sepharose and was eluted with α-methylmannoside (peak III, Table II); glycopeptides in this fraction contain high mannose and hybrid-type asparagine-linked oligosaccharides, whereas the glycopeptides that do not bind or that bind and elute with α-methylglucoside contain complex-type units (3). Similarly, 83 and 79% of the [3H]-labeled radioactivity associated with β-glucuronidase after the 25-h uptake, and the subsequent 24-h chase, respectively, eluted with α-methylmannoside. Thus, there was no change in the proportion of complex- to high mannose-type oligosaccharides associated with β-glucuronidase after endocytosis. As expected, the residual [35S]methionine associated with the desalted glycopeptides was recovered in peak I from Con A-Sepharose; the extensive pronase digestion is intended to degrade the polypeptide to its constituent amino acids which (in the absence of an attached oligosaccharide) should not bind to the lectin.

The peak III glycopeptides that eluted with α-methylmannoside were digested with Endo H, and the released oligosaccharides were applied to a column of QAE-Sephadex; negatively charged units bind to the resin and can be differentially eluted using a linear gradient of ammonium acetate at pH 5.3 (41). Fig. 3 shows the elution profiles for the β-glucuronidase oligosaccharides isolated from the input P388D1 secretions (A), the Rec+ L cells after a 25-h uptake (B), and the Rec+ L cells after a 25-h uptake/24-h chase (C). Radioactivity associated with the input enzyme eluted as expected for negatively charged oligosaccharides containing one phosphomonoester, one phosphomonoester and one sialic acid residue, and two phosphomonoesters. Previous studies examining total P388D1 cell secretions observed the same three phosphorylated species (41). Overall, 24% of the [3H]mannose-labeled radioactivity eluted as the phosphorylated species, with the two phosphomonoester form predominating (11.3%). Similarly, 21 and 26% of the radioactivity associated with β-glucuronidase recovered from the cells after endocytosis eluted as phosphorylated oligosaccharides. In contrast to the input enzyme, however, the L cell–associated enzyme contained primarily negatively charged oligosaccharides associated with the β-glucuronidase after endocytosis.

### Table II. Con A-Sepharose Fractionation of the Pronase Digests of β-Glucuronidase

<table>
<thead>
<tr>
<th>Source of β-glucuronidase</th>
<th>Con A-Sepharose peak</th>
<th>Total cpm recovered</th>
<th>[% of ( ^{3}H ) cpm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Input P388D1 secretions</td>
<td>I</td>
<td>752</td>
<td>110 (12)</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>550</td>
<td>0 (9)</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>5,082</td>
<td>80 (83)</td>
</tr>
<tr>
<td>L cell–associated 25-h uptake</td>
<td>I</td>
<td>816</td>
<td>120 (8)</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>850</td>
<td>0 (9)</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>8,272</td>
<td>83 (83)</td>
</tr>
<tr>
<td>L cell–associated 25-h uptake/24-h chase</td>
<td>I</td>
<td>1,106</td>
<td>200 (13)</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>720</td>
<td>0 (8)</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>6,886</td>
<td>79 (79)</td>
</tr>
</tbody>
</table>

The glycopeptides generated by pronase digestion of immunoprecipitated β-glucuronidase were desalted on Sephadex G-25 (the majority of the [35S]methionine radioactivity was lost during this step) then fractionated on Con A-Sepharose. Under the conditions used, tri- and/or tetraantennary complex-type glycopeptides do not bind to Con A-Sepharose and are recovered by washing the column with TBS (peak I), biantennary complex-type glycopeptides bind to the column and are eluted with 10 mM α-methylglucoside in TBS (peak II), and glycopeptides containing high mannose-type oligosaccharides are eluted with 0.1 M α-methylmannoside in TBS (peak III) (3).
from the original P388D, secretions contained the same three species of phosphorylated oligosaccharides noted above. In this preparation, 33% of the [3H]mannose-labeled radioactivity eluted as negatively charged oligosaccharides. The β-glucuronidase internalized by the Rec+L cells also contained 33% of its radioactivity in negatively charged species, with one phosphomonoester species again predominating (Fig. 4C). 76% of the input [35S]methionine associated with β-glucuronidase was internalized by the cells in this experiment (Table I). If the cells had selectively sequestered only enzyme molecules containing oligosaccharides with one phosphomonoester, then the enzyme remaining in the medium would be depleted of the one phosphomonoester containing units. As shown in Fig. 4B, however, the post-uptake supernatant β-glucuronidase molecules contained the same three species of phosphorylated oligosaccharides, and their relative amounts were comparable, as those originally added to the cells. As such, the intracellular accumulation of oligosaccharides containing one phosphomonoester does not occur by a selective uptake but, rather, by a conversion of the internalized oligosaccharides possessing two phosphomonoesters or one phosphomonoester/one sialic acid residue to species that elute as the one phosphomonoester forms.

**Generality of the Processing**

To determine if cells other than the Rec+L cells process the phosphorylated oligosaccharides on internalized acid hydrolases in a comparable fashion, the double-labeled P388D, secretions were applied to human fibroblasts. These cells...
### Table III. Summary of the Endocytosis-induced Dephosphorylation of β-Glucuronidase

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Internalized by</th>
<th>Conditions</th>
<th>% Phosphorylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Rec⁺ L cells</td>
<td>24-h uptake/7-h chase</td>
<td>Input 33</td>
</tr>
<tr>
<td>B</td>
<td>Rec⁺ L cells</td>
<td>23-h uptake</td>
<td>Input 37</td>
</tr>
<tr>
<td>C</td>
<td>Rec⁺ L cells</td>
<td>20-h uptake/22-h chase</td>
<td>Input 31</td>
</tr>
<tr>
<td>D</td>
<td>Rec⁺ L cells</td>
<td>25-h uptake/24-h chase</td>
<td>Input 24</td>
</tr>
<tr>
<td></td>
<td>Normal human fibroblasts</td>
<td>25-h uptake/24-h chase</td>
<td>Post-uptake 33</td>
</tr>
<tr>
<td></td>
<td>Normal human fibroblasts</td>
<td>25-h uptake/24-h chase</td>
<td>Post-uptake 27</td>
</tr>
<tr>
<td></td>
<td>Normal human fibroblasts</td>
<td>25-h uptake/24-h chase</td>
<td>Post-uptake 21</td>
</tr>
<tr>
<td></td>
<td>Normal human fibroblasts</td>
<td>25-h uptake/24-h chase</td>
<td>Post-uptake 16</td>
</tr>
<tr>
<td></td>
<td>Normal human fibroblasts</td>
<td>25-h uptake/24-h chase</td>
<td>Post-uptake 18</td>
</tr>
</tbody>
</table>

Four separate preparations (A–D) of P388D<sub>2</sub> secretions were isolated and used for the indicated uptake experiment. The percentage of the total [³H]mannose associated with high mannose-type oligosaccharides recovered from β-glucuronidase as phosphorylated species before (Input) and after (Post-uptake) endocytosis is indicated.

possess the Man 6-P receptor at their cell surface (6, 25) and ligands internalized via the cell surface receptor are known to reach lysosomes (30). Fibroblasts were grown for 24 h in the presence of the labeled secretions and chased for an additional 24 h in fresh medium lacking the ligand. As with Rec⁺ L cells, the β-glucuronidase molecules recovered from the fibroblasts migrated slightly faster than the molecules secreted by the P388D<sub>2</sub> cells (data not shown). Moreover, as shown in Fig. 5, the internalized molecules remained phosphorylated as 18% of the radioactivity recovered from the fibroblast-associated β-glucuronidase eluted as phosphorylated species; oligosaccharides containing one phosphomonoester predominated, representing 91% of the negatively charged species. It should be noted that the β-glucuronidase applied to the fibroblasts was the same as used in Fig. 3; 24% of the [³H]mannose-labeled radioactivity associated with the enzyme was incorporated into phosphorylated species (Fig. 3 A).

A comparison of the extent of phosphorylation of the oligosaccharides recovered from β-glucuronidase before and after uptake, is shown in Table III, along with a summary of several different experiments. The percentage of the radioactivity recovered as phosphorylated units from the cell-associated enzyme was always less than or equal to the input value. Unexpectedly, however, the overall percentage never decreased by more than 33% (e.g., 21% after uptake vs. 31% input). In many cases, such as experiments A and D, there was essentially no change in the percentage of phosphorylated molecules after endocytosis. Taken together, the data indicate that endocytosis via the Man 6-P receptor into both Rec⁺ L cells and human fibroblasts is accompanied by a limited dephosphorylation, such that the absolute number of phosphorylated units remains comparable but units containing two phosphates are degraded to the monophosphorylated species.

### Structural Analysis of the Phosphomonoester-Containing Oligosaccharides

The oligosaccharides suspected of containing one phosphomonoester isolated from the internalized β-glucuronidase molecules were treated with alkaline phosphatase to verify the presence of the phosphate monoester. As shown in Fig. 6 A, the radioactivity associated with the untreated oligosaccharides bound to QAE-Sephadex and eluted with 70 mM NaCl; this concentration of NaCl has been shown previously to elute oligosaccharides containing one phosphomonoester (39). After digestion with alkaline phosphatase, however, the radioactivity ran through the QAE-Sephardex column, Fig. 6 B, indicating that the oligosaccharides initially bound via a phosphomonoester group.

The size of the one phosphomonoester-containing oligosaccharides was assessed by HPLC analysis after dephosphorylation with alkaline phosphatase. As shown in Fig. 7, the molecules isolated either before (A) or after (B) internalization eluted in the same position as standard high mannose-type oligosaccharides containing six, seven, or eight mannose residues; in both populations the Man-GlcNAc species predominated. HPLC analysis of the neutral oligosaccharides isolated from β-glucuronidase indicated that these units contained primarily nine mannose residues and that no change occurred after endocytosis (data not shown). As such, there is no significant loss of mannose residues from

![Figure 6. Alkaline phosphatase sensitivity of the negatively charged oligosaccharides recovered after endocytosis.](image-url)
either the neutral or phosphorylated oligosaccharides upon endocytosis.

Finally, to determine whether the phosphates within the one phosphomonoester-containing units are localized to a specific branch of the oligosaccharides, the position of the phosphate groups was determined. Acetolysis (26) was used to selectively hydrolyze the high mannose units at α-1,6-linkages, and the resulting negatively charged fragments were recovered by QAE-Sephadex chromatography. The isolated fragments were then dephosphorylated by alkaline phosphatase digestion and sized by paper chromatography. Acetolysis of the two phosphomonoester-containing oligosaccharides isolated from total P388D1 cell secretions (A) and from β-glucuronidase internalized by the Rec+L cells (B). The elution position of the following high mannose oligosaccharides are indicated in A: ManαGlcNAc (5), ManβGlcNAc (6), Man6GlcNAc (7), Man7GlcNAc (8), and Man8GlcNAc (9).

75% of the molecules contain the phosphate on the α-1,6-branch of the core β-linked mannose residue and 25% on the α-1,3-branch, as shown in the inset to Fig. 8B; the exact location within the α-1,6-branch is not known. Importantly, the comparable positioning of the phosphate moiety between the α-1,3- and α-1,6-branch of the two phosphomonoester species before uptake with the distribution of the phosphate within the one phosphomonoester oligosaccharide post-uptake, suggests that phosphate loss occurs randomly upon endocytosis.

**Ligands Internalized at 20°C Are Partially Dephosphorylated**

The stability of the one phosphomonoester species within the L cells coupled with the random loss of a single phosphate from diphosphorylated units suggested that a partial dephosphorylation of β-glucuronidase may occur as the enzyme is transported to lysosomes. To investigate this possibility, the Rec+L cells were incubated with the double-labeled P388D1 secretions at 20°C; at temperatures near 20°C, cell surface receptors mediate endocytosis but the ligands fail to reach lysosomes (4, 19, 31). After an overnight incubation, the cell-associated phosphorylated oligosaccharides were isolated

![Figure 7](https://example.com/figure7.png)  
**Figure 7.** HPLC analysis of the monophosphorylated high mannose-type oligosaccharides. The one phosphomonoester containing oligosaccharides was digested with alkaline phosphatase, and the dephosphorylated molecules were sized by HPLC. The chromatograms show the elution of [3H]mannose-labeled oligosaccharides isolated from total P388D1 cell secretions (A) and from β-glucuronidase internalized by the Rec+L cells (B). The elution position of the following high mannose oligosaccharides are indicated in A: ManαGlcNAc (5), ManβGlcNAc (6), Man6GlcNAc (7), Man7GlcNAc (8), and Man8GlcNAc (9).

![Figure 8](https://example.com/figure8.png)  
**Figure 8** Paper chromatography of the phosphorylated acetolysis fragments. After degradation of the phosphorylated oligosaccharides by acetolysis, the negatively charged fragments were isolated by QAE-Sephadex chromatography and dephosphorylated by alkaline phosphatase digestion. The [3H]mannose-labeled fragments were then fractionated by descending paper chromatography. (A) Fragments generated from the two phosphomonoester-containing oligosaccharides secreted by P388D1 cells; (B) fragments generated from the one phosphomonoester containing oligosaccharides recovered from the Rec+L cells after endocytosis of P388D1 secretions. The migration position of ManαGlcNAc (1), ManβGlcNAc (2), Man7GlcNAc (3), mannobiose (4), and mannose (5) are indicated in A. The inset in B shows the proposed distribution of the phosphate moiety within the monophosphorylated structures recovered from the L cells. Asterisks, mannose residues that may be phosphorylated (39); M and G, mannose and N-acetylglucosaminitol residues, respectively.
Table IV. Comparison of the Phosphorylated Oligosaccharides Recovered from L Cells after Endocytosis of Ligands at 20° and 37°C

<table>
<thead>
<tr>
<th>Source of high mannose-type oligosaccharides</th>
<th>% of the Total cpm recovered from QAE-Sephadex as</th>
<th>Overall % phosphorylation</th>
<th>Ratio of 2:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Post-uptake supernatant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>37°C</td>
<td>53</td>
<td>2.9</td>
<td>10</td>
</tr>
<tr>
<td>20°C</td>
<td>48</td>
<td>2.4</td>
<td>11.2</td>
</tr>
<tr>
<td>Rec+ L cell-associated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>37°C</td>
<td>79</td>
<td>7.2</td>
<td>9.4</td>
</tr>
<tr>
<td>20°C</td>
<td>54</td>
<td>6.4</td>
<td>29</td>
</tr>
</tbody>
</table>

Rec+ L cells were incubated with double-labeled P388D1, secretions containing 0.16 mU of β-glucuronidase activity in 10 ml of α-MEM, 10% FBS per 10-cm dish) at either 37° or 20°C. The 20°C cultures were incubated in a Gas Pak System chamber (BBL Microbiology Systems, Cockeysville, MD) which was flushed with 5% CO₂ in air before closing. After a 21.5-h incubation, 1 ml of 55 mM Man 6-P in α-MEM, 10% fetal bovine serum was added to each dish, and the cells were incubated for an additional 60 min; the Man 6-P was added to remove ligands bound to the cell surface. At the end of the incubation, the dishes were placed on ice, the post-uptake supernatants were recovered, and the cells were scraped into PBS containing 2 mg/ml BSA and collected by centrifugation. The cells were washed twice with the PBS, BSA solution by repeated centrifugation. The post-uptake supernatants were adjusted to 0.1 M Tris, pH 8, 20 mM CaCl₂, and 200 mM β-glycerophosphate, and pronase was added to achieve a final concentration of 2.3 mg/ml. The cell pellets were resuspended in 2 ml of 0.1 M Tris, pH 8, 20 mM CaCl₂, 200 mM β-glycerophosphate containing 5 mg/ml pronase. The pronase digestions were incubated overnight at 36°C, boiled for 3 min, and clarified by centrifugation. The supernatants were dried by rotary evaporation, dissolved in 1 ml of H₂O, and desalted on a Sephadex G-25 column. The excluded glycopeptides were dried by rotary evaporation and fractionated on Con A-Sepharose. The high mannose-type units were desalted on Sephadex G-25, digested with Endo H, and the digests were applied directly to QAE-Sephadex columns. The columns were eluted with a gradient of ammonium acetate (4), and aliquots of each fraction were analyzed by liquid scintillation counting. The values in the table represent the total amount of radioactivity eluting from the columns in the position expected for the indicated species. The ratio of diphosphorylated to monophosphorylated oligosaccharides (ratio of 2:1) was determined by dividing the radioactivity recovered as the 2 PM species by the sum of the 1 PM and the 1 PM/1 SA species. The [³H]mannose-labeled radioactivity eluted from the columns as neutral oligosaccharides (N) or units containing 1 sialic acid residue (1 SA), 1 phosphomonoester (1 PM), 1 phosphomonoester and 1 sialic acid residue (1 PM/1 SA), and 2 phosphomonoesters (2 PM).

and compared to the corresponding structures recovered from cells incubated at 37°C and from the post-uptake supernatant. For these experiments the bulk phosphorylated oligosaccharide content (the P388D1, secretions contain many phosphorylated acid hydrolases) was examined rather than those recovered from β-glucuronidase individually; the 20°C cultures accumulated <40% of the [³H]-labeled radioactivity internalized by those at 37°C.

As shown in Table IV, the post-uptake supernatant fractions contained the same three species of phosphorylated oligosaccharides as recovered from β-glucuronidase; overall, 39-45% of the radioactivity associated with the high mannose-type oligosaccharides eluted from QAE-Sephadex as phosphorylated units. The major phosphorylated oligosaccharide recovered from the L cells after a 37°C incubation contained a single phosphomonoester group, as was the case with the internalized β-glucuronidase molecules (Fig. 3). However, in contrast to β-glucuronidase, which possessed a similar percentage of phosphorylated oligosaccharides before and after endocytosis, the percentage of phosphorylated high mannose-type oligosaccharides associated with the bulk ligand pool decreased from near 40 to 11% upon association with the L cells.

The high mannose-type oligosaccharides recovered from L cells incubated at 20°C contained similar phosphorylated oligosaccharides (Table IV). Importantly, however, the distribution of radioactivity within the phosphorylated species is altered relative to the post-uptake supernatant fraction. The ratio of radioactivity recovered as diphosphorylated to monophosphorylated units is 0.58-0.72 for the post-uptake supernatant oligosaccharides but 0.15 and 0.17 for those associated with the L cells at 20° and 37°C, respectively. The predominance of the one phosphomonoester species within the cells indicates that oligosaccharides with two phosphomonoesters are converted to the monophosphorylated species at 20°C. The lower overall percentage of phosphorylation of the oligosaccharides internalized at 37°C as compared with those internalized at 20°C indicates that some ligands are completely dephosphorylated within the cell at a site distal to the 20°C temperature block. The dissimilar processing of the phosphorylated oligosaccharides suggests that the ligands internalized at 20°C do not achieve the same destination as those internalized at 37°C.

Discussion

The 215-kD Man 6-P receptor is unusual in that it operates in the transport of both intra- and extracellular ligands. The intracellular Man 6-P receptor is localized within the Golgi apparatus (1, 14) where it functions to transport newly synthesized acid hydrolases to lysosomes (32, 37). The function of the cell surface receptor is not known, but morphological and biochemical studies (30, 43) have established that the surface Man 6-P receptors also deliver their ligands to lysosomal vesicles. In human fibroblasts, the cell surface molecules represent only 20% of the total cell-associated Man 6-P receptor activity (6), and these appear to be in equilibrium with intracellular forms (13). To date, there is no evidence that the 46-kD Man 6-P binding protein described by Hoflack and Kornfeld (22, 23) is present at the cell surface. Therefore, internalization of exogenous ligands containing the Man 6-P recognition marker presumably occurs only via the 215-kD Man 6-P receptor.

β-Glucuronidase secreted by P388D1 cells was found to be an excellent ligand for the surface receptor. Structural analysis of the secreted molecules indicated that 24-37% of the Endo H-releasable oligosaccharides contain the Man 6-P recognition marker. The phosphorylated oligosaccharides were composed of three distinct species; high mannose type units possessing either one or two phosphomonoesters and hybrid-type structures containing one phosphomonoester and one sialic acid residue. Application of the double-labeled P388D1 cell secretions to either Rec+ mouse L cells or human fibroblasts resulted in the association of a high-
per centage of the input β-glucuronidase molecules with the cells. Three lines of evidence suggest that the cell-associated molecules were actually internalized. First, Man 6-P inhibited the association of β-glucuronidase as would be expected for molecules internalized via the surface Man 6-P receptor (25). Second, the cell-associated forms of β-glucuronidase showed an altered mobility on SDS gel electrophoresis, suggesting that they had undergone a proteolytic maturation; the proteolysis most likely occurs in lysosomes (8). Finally, as discussed below, the cell-associated molecules possessed an altered complement of phosphorylated oligosaccharides in comparison to those present on the originally secreted or non-internalized enzymes.

β-Glucuronidase recovered from the cells after endocytosis remained phosphorylated as evidenced by the persistence of the negatively charged oligosaccharides. In some experiments (see Table III), uptake was accompanied by a 30% decrease in the number of phosphorylated structures while in others the percentage of negatively charged units was comparable before and after uptake; the significance of this variability is unclear. While the overall percentage remained comparable, there was always a dramatic difference in the composition of the negatively charged oligosaccharides. The cell-associated molecules contained primarily a single species of negatively charged oligosaccharide possessing one phosphomonoester; these oligosaccharides accounted for ~90% of the negatively charged structures. In contrast, the one phosphomonoester species accounted for only 35% of the negatively charged oligosaccharides associated with the original P388D1-secreted β-glucuronidase.

The intracellular accumulation of oligosaccharides containing a single phosphomonoester did not arise by a selective uptake of β-glucuronidase molecules containing these structures. This is expected, as the Man 6-P receptor binds oligosaccharides containing either one or two phosphates (10); in fact, the best ligands are oligosaccharides containing two phosphomonoesters (2, 41). Moreover, the high percentage of phosphorylation associated with the internalized molecules did not reflect a selective loss of nonphosphorylated structures, as both the Con A-Sepharose profiles and the size content of negatively charged units must have resulted from: (a) conservation of those oligosaccharides originally containing a single phosphomonoester, and (b) conversion of oligosaccharides with two phosphomonoesters or one phosphomonoester and one sialic acid residue to species containing a single phosphomonoester. Glaser and co-workers (15) previously noted that “high uptake” forms of β-glucuronidase became less negative (as judged by isoelectric focusing gels) after endocytosis; on this basis, it was concluded that the recognition marker is destroyed upon internalization. The phosphorylated acid hydrolases do not reach lysosomes. Previous studies have also indicated that ligands internalized by cells near 20°C fail to reach lysosomes (4, 19, 31). For example, asialoorosomucoid is internalized at 16°C by the hepatocyte asialoglycoprotein receptor, but the cell-associated molecules reside in endosomal structures rather than achieving their normal destination within lysosomes (31). The limited dephosphorylation of the acid hydrolases at the prelysosomal location may reflect an unusual specificity of the processing phosphatase. Alternatively, while passing through the various intracellular compartments during its transport to lysosomes, β-glucuronidase may remain associated with a binding protein that protects a single phosphate moiety on the oligosaccharides from the phosphatase. Candidates for such a protein include the 215-kD Man 6-P receptor and the 46-kD cation-dependent Man 6-P binding protein. It is hoped that future localization and characterization of the processing phosphatase may lead to a better understanding of the intracellular pathways traversed by receptor-bound acid hydrolases and of the function of the various Man 6-P binding proteins.

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References

ture selectively inhibits fusion between pinocytotic vesicles and lysosomes during heterophagy of ~251-asialofetuin by the perfused rat liver.


