Identification of Mannose 6-Phosphate Receptors in Rabbit Alveolar Macrophages*

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Mannose 6-phosphate is an important recognition site involved in transport of newly synthesized lysosomal enzymes from the endoplasmic reticulum to lysosomes. The current study is the first demonstration of functional mannose phosphate receptors in macrophages. The receptor appears to be similar in many respects to that expressed in fibroblasts. Binding at 4°C of a mannose-6-P-containing ligand, α-mannosidase from H. holstii, was specific and saturable (K_D = 1.6 nM). In the presence of permeabilizing agents (saponin and digitonin), macrophage mannose-6-P receptors gave a distribution of 15–20% on the surface and 80–85% inside. Uptake studies gave a K_app value of 4.9 nM. Mannose-6-P, H. holstii phosphomannan, and fructose 1-phosphate were effective inhibitors of α-mannosidase uptake. Inhibitors of mannose uptake, such as β-glucuronidase, mannose-bovine serum albumin, fucose-bovine serum albumin, or mannann had no effect on α-mannosidase uptake. Likewise, an inhibitor (fucoidin) of the macrophage receptor which recognizes negatively charged proteins did not inhibit α-mannosidase uptake. Uptake was linear over 90 min and inhibited by chloroquine, suggesting that surface receptors recycle.

These data demonstrate that macrophages contain receptors which specifically recognize mannose-6-P units and are distinct from the well characterized mannose receptors. The finding that the mannose-6-P receptors play a role at the surface, together with the fact that most of the receptors are intracellular (similar to the mannose receptor) suggests that both carbohydrate receptors play a regulatory role at the surface and intracellularly in transport of lysosomal enzymes.

Mannose 6-phosphate is an important recognition site in the intracellular transport of lysosomal enzymes in fibroblasts (1). Several workers have demonstrated that mannose-6-P rafts on high mannose chains of newly synthesized hydrolases are recognized by specific receptors which act to direct the proteins from Golgi membranes to the lysosome.

Recently it has been shown that the continuous macrophage-like cell line P388D1, synthesizes phosphate-containing β-glucuronidase analogous to the system in fibroblasts (2). In addition, Jessup and Dean (3) reported that P388D1 cells spontaneously secrete large quantities of β-hexosaminidase containing the mannose-6-P group. These workers suggested that these macrophages share a common pathway with fibroblasts for packaging of lysosomal enzymes. However, functional mannose-6-P receptors in macrophages have not been reported, and Gabel et al. have shown that P388D1 cells lack these receptors (4).

We have studied the receptor-mediated uptake and binding of mannose-containing ligands by primary macrophages (5, 6). This receptor appears to bind ligands at the cell surface, followed by internalization of the receptor-ligand complex. The ligand is delivered to the lysosome for subsequent degradation, and kinetic data suggest that the receptor is recycled back to the cell surface for another round of uptake (7). In the present study, we demonstrate that primary macrophages contain high affinity cell surface receptors which specifically recognize mannose-6-P units, and that rabbit alveolar macrophages express both mannose and mannose phosphate receptors.

**EXPERIMENTAL PROCEDURES**

*Materials*

Tissue culture medium, Hanks' balanced salt solution, and fetal and newborn calf serum were obtained from Grand Island Biological Co. Silicone oil (DCS50) was purchased from Accufilm (Elizabethtown, KY). Mineral oil was from Taylor Chemical Co. (St. Louis, MO). HEPES, TES, chloroquine, yeast mannann, mannose-6-P, fucoidin, mannose-1-P, fructose-1-P, BSA, potassium iodide, and sodium metabisulfite were purchased from Sigma. Chloramphenicol was obtained from Eastman-Kodak (Rochester, NY), and Na2SO4 from Amersham (Arlington Heights, IL). The high molecular weight phosphomannan from H. holstii (Y-5448) was a gift from Dr. Morey Slodki of the Northern Regional Research Center, USDA, Peoria, IL.

*Ligands*

Mannose-BSA, fucose-BSA, and galactose-BSA were prepared by the method of Lee et al. (8). α-N-Mannosidase was purified from the growth medium of H. holstii as described by Freeze et al. (9). β-Glucuronidase was labeled as previously described (10). α-Mannosidase was labeled similarly, except that the buffer used during the iodination was 0.1 M sodium phosphate, pH 6.5.

*Cells*

Alveolar macrophages were obtained from rabbits by pulmonary lavage as described previously (6). Cells were collected in saline,

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1. The abbreviations used are: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; MEM, minimal essential medium; HBSS, Hanks' balanced salt solution; TES, 2-(4-2-hydroxyethyl)-1-tris(hydroxymethyl)methyl] aminoethanesulfonic acid; LDL, low density lipoprotein.
pelled, and resuspended in Hanks' BSA assay medium. Cells were used for suspension assays in this medium or pelleted and suspended in RPMI containing 10% newborn or fetal calf serum and plated at 5 x 10^6 cells/well in 24-well Linbro plates. Nonadherent cells were removed by washing, and Hanks' BSA assay medium was added for subsequent uptake studies.

**Uptake and Binding Assays**

**Suspension Cells**—Uptake and binding assays using alveolar macrophages in suspension were run as previously described (5) in Hanks'/BSA assay medium. Uptake is defined as the cell-associated radioactivity plus degraded material in the medium after incubation at 37 °C. Binding is defined as the cell-associated radioactivity after incubation at 4 °C. In all cases, specific binding or uptake is that which can be eliminated by a large excess of a competitive ligand (yeast mannan (1-2 mg/ml) or mannose-6-P (10 mM)).

**Plated Cells**—Alveolar macrophages were allowed to adhere. Assay buffer (400 μl) containing ligand with or without inhibitors was added and uptake allowed to proceed for the times indicated. The medium containing excess ligand as well as degraded material was removed for subsequent trichloroacetic acid precipitation. The cell layer was washed twice with Hanks' and the cells solubilized for counting in 0.25 ml of 0.1% Triton X-100 in water.

**Binding Assays in the Presence of Saponin or Digitonin**—Cells were preincubated in suspension at 4 °C for 15 min in the presence or absence of 0.5% saponin in Hanks'/BSA assay medium, containing 1 mM phenylmethylsulfonyl fluoride and 5 μM of leupeptin. Ligand was then added with or without mannose-6-P at 4 °C, and binding allowed to proceed for 2 h. The cells were then spun through oil as previously described (5). Experiments with digitonin were performed as described by Weigel and Oka (11). Briefly, binding of α-mannosidase into cells in suspension was carried out at 4 °C for 120 min in the presence or absence of 0.05% digitonin. Digitonin was added as a stock solution (14 mg/ml) in absolute ethanol. Control (no digitonin) cells received an equal volume of absolute ethanol.

**RESULTS**

**Binding and Uptake of α-Mannosidase by Rabbit Alveolar Macrophages**—It has previously been shown that freshly isolated rabbit alveolar macrophages express a high affinity mannose receptor on their surfaces (6). To examine the presence of a mannose-6-P receptor on macrophages which also express a mannose-specific receptor, it was necessary to use a ligand which would exhibit a high affinity for the mannose-6-P receptor with relatively lower affinity for the mannose receptor. Freeze et al. (9, 12) have shown that α-mannosidase isolated from *D. discoideum* contains the phosphomannosyl recognition marker and is a ligand for the mannose-6-P receptor on fibroblasts. From their studies, they found that most, if not all, of the enzyme molecules are taken up by a mannose-6-P-inhibitable process. Additionally, the results of uptake studies suggested that slime mold α-mannosidase interacted with the same mannose-6-P receptor on fibroblasts as did mammalian “high uptake” enzymes.

The dependence of uptake on ligand concentration was examined with macrophages incubated with ligand for 10 min at 37 °C (Fig. 1, left). The concentration of ligand required to produce half-maximal uptake (Kuptake) was estimated graphically as 4.9 x 10^-6 M. This is in excellent agreement with reported values for uptake of this enzyme (9) and mammalian enzymes (13, 14) by fibroblasts. The concentration dependence of binding at 60 min and 4 °C is shown in Fig. 1 (right). A Scatchard plot of the data gave a Kd value of 1.6 x 10^-6 M, with a single class of binding sites.

**Specificity of Uptake of α-Mannosidase by Macrophages**—

The specificity of uptake of α-mannosidase was tested using various sugars, sugar phosphates, mannans, neoglycoproteins, and lysosomal hydrolases as inhibitors. The results are shown in Table I. Mannose-6-P and fructose-1-P show almost equal inhibitory potency, with 50% inhibition at concentrations of 0.5-1.0 mM. Mannose-1-P showed no inhibition at 1 mM, and only 33% inhibition at 40 mM. Sugars and neoglycoproteins which have been shown to be effective inhibitors of uptake by the mannose receptor (6) did not inhibit α-mannosidase uptake. Galactose-BSA also did not inhibit. Unlabeled α-mannosidase inhibited 50% of the uptake of labeled enzyme at a concentration of 4 nM, in close agreement with the Kuptake of 4.9 nM calculated from Fig. 1.β-Glucuronidase, a ligand for the mannose receptor, showed no inhibition at 100 μg/ml. The large molecular weight phosphomannan of *H. holstii*, an effective inhibitor of uptake by the fibroblast mannose phosphate receptor (15, 16), gave 50% inhibition of uptake of α-mannosidase at 0.15 μg/ml.

**TABLE I**

**Effect of inhibitors on uptake of α-mannosidase by macrophages**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simple sugars</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mannose-6-P</td>
<td>0.5 mM</td>
<td>50</td>
</tr>
<tr>
<td>Fructose-1-P</td>
<td>1 mM</td>
<td>57</td>
</tr>
<tr>
<td>Mannose-1-P</td>
<td>40 mM</td>
<td>33</td>
</tr>
<tr>
<td>Mannose</td>
<td>50 mM</td>
<td>0</td>
</tr>
<tr>
<td>Fucose</td>
<td>50 mM</td>
<td>0</td>
</tr>
<tr>
<td>Lysosomal hydrolases</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Mannosidase (<em>D. discoideum</em>)</td>
<td>4 nM</td>
<td>50</td>
</tr>
<tr>
<td>β-Glucuronidase (rat preputial)</td>
<td>100 μg/ml</td>
<td>0</td>
</tr>
<tr>
<td>Neoglycoproteins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mannose-BSA</td>
<td>10 μg/ml</td>
<td>0</td>
</tr>
<tr>
<td>Fucose-BSA</td>
<td>10 μg/ml</td>
<td>0</td>
</tr>
<tr>
<td>Galactose-BSA</td>
<td>10 μg/ml</td>
<td>0</td>
</tr>
<tr>
<td>Phosphomannan (<em>H. holstii</em>)</td>
<td>0.15 μg/ml</td>
<td>50</td>
</tr>
<tr>
<td>Fucoidin</td>
<td>1 mg/ml</td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 1. Uptake (left) and binding (right) of 125I-α-mannosidase by rabbit alveolar macrophages. Left, cells (5 x 10^6) in 0.1 ml were incubated for 10 min at 37 °C with increasing concentrations of ligand. Non-specific uptake was determined by adding 10 mM mannose-6-P to companion assays. Inset, a double reciprocal plot of the uptake data; Kuptake = 4.9 nM. Right, binding was determined by incubating cells with ligand at 4 °C for 60 min. Inset, Scatchard plot of the data; K = 1.6 nM.

1. H. H. Freeze, personal communication.
fucoidin did not inhibit α-mannosidase uptake at a concentration of 1 mg/ml.

**Uptake of α-Mannosidase in the Absence of Calcium**—It has been shown that uptake mediated by the macrophage mannos receptor is absolutely dependent on the presence of Ca²⁺ in the assay medium (6), whereas mannose-6-P specific uptake in fibroblasts does not require Ca²⁺ (18). To further verify that α-mannosidase is recognized specifically by a mannose phosphate receptor and not the mannose receptor, uptake of β-glucuronidase (a mannose receptor ligand) and α-mannosidase by rabbit macrophages in the presence and absence of Ca²⁺ was compared. As shown in Table II, there was little uptake of β-glucuronidase in the absence of Ca²⁺, whereas the mannose-6-P-inhibitable uptake of α-mannosidase was essentially the same with or without Ca²⁺.

**Time Course of Uptake of α-Mannosidase by Rabbit Macrophages and Inhibition by Chloroquine**—The uptake of α-mannosidase with increasing time is shown in Fig. 2. Total specific uptake proceeded linearly up to 90 min. During this period the cell-associated ligand decreased as ligand degradation products began to appear in the medium. Uptake by both the mannose phosphate receptor in human fibroblasts (19) and the mannose receptor in rat macrophages (20) was also linear with time, suggesting either a reutilization of receptors or replenishment of the cell surface complement from a large intracellular pool. From studies on inhibition of uptake by chloroquine and other amines in both systems (19, 20) it would appear that receptors on the surface deliver ligand to the interior of the cell, and then return to the surface. In the present study, chloroquine inhibited uptake of α-mannosidase by rabbit macrophages (Fig. 3). At 90 min, uptake in the presence of 1 mM chloroquine was only 6% of the control level.

**Intracellular and Cell Surface Mannose Phosphate Receptors**—Both saponin (21) and digitonin (11) have been shown to permeabilize cells, allowing soluble ligands to bind to intracellular membranes. Rabbit macrophages were treated with either saponin or digitonin and the total ligand bound was measured. Data from an experiment using cells in the presence or absence of 0.055% digitonin are shown in Fig. 4. Similar results were obtained with cells treated with 0.5% saponin. Binding in the absence of digitonin represents the cell surface content of receptors; saturation is attained at approximately 100 ng/assay or 1 μg/ml. Saturation in permeabilized cells occurs at approximately 8 μg/ml. At 800 ng of ligand added, cells with digitonin bound approximately 9 times more ligand, suggesting that 80–90% of the receptors are localized on intracellular membranes.

**Table II**

<table>
<thead>
<tr>
<th>LIGAND  ADDED (ng/assay)</th>
<th>-EDTA/+Ca²⁺</th>
<th>+EDTA/-Ca²⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Glucuronidase</td>
<td>19.9</td>
<td>1.1</td>
</tr>
<tr>
<td>α-Mannosidase</td>
<td>3.0</td>
<td>4.1</td>
</tr>
</tbody>
</table>

**Fig. 2.** Time course of uptake of ¹²⁵I-labeled α-mannosidase by rabbit alveolar macrophages. Cells were collected by lavage, washed with HBSS, then resuspended in MEM containing 10% newborn calf serum and seeded into 24 well Linbro plates at 5 X 10⁴ cells/well. To each well of adherent cells was added 0.4 ml of Hanks'/BSA assay medium containing 160 ng of ¹²⁵I-labeled α-mannosidase with or without 10 mM mannose-6-P. At varying times the reaction was terminated by removing the medium, washing the cells twice with HBSS, then solubilizing the cells in 0.1% Triton X-100 in water. Degraded ligand in the spent medium was quantitated by adding trichloroacetic acid to a final concentration of 10%. O—O, total uptake (cellular + degraded); □—□, cell-associated uptake; O—O, degraded ligand.

**Fig. 3.** Effect of chloroquine on uptake of α-mannosidase by rabbit alveolar macrophages. The time course of uptake of α-mannosidase was measured as described in Fig. 2, using 5 X 10⁴ macrophages/well in 24-well plates. Hanks'/BSA assay medium (0.4 ml) containing 160 ng of ¹²⁵I-labeled α-mannosidase with and without 1 mM chloroquine was added, and total specific uptake measured from 15 to 90 min. Total uptake was corrected for nonspecific uptake in the presence of 10 mM mannose-6-P.

**Fig. 4.** Binding of α-mannosidase to rabbit alveolar macrophages in the presence and absence of 0.055% digitonin. Cells (5 X 10⁴) were incubated with ligand at 4 °C for 2 h as described in Fig. 1 over a concentration range of 0.50 to 8 μg/ml. To one set of tubes, digitonin (14 mg/ml in ethanol) was added to a final concentration of 0.055%. Control tubes received an equivalent concentration of ethanol. The reaction was terminated by spinning the cells through oil (9) and counting the pellets.
DISCUSSION

Mannose-6-P has been shown to be a specific recognition marker responsible for intracellular transport of newly synthesized lysosomal enzymes from the endoplasmic reticulum to lysosomes. It has been proposed that enzymes bearing mannose-6-P are transported by receptors in the endoplasmic reticulum, through the Golgi, and on to lysosomes. The enzymes are released from receptors in the lysosomal acidic environment, followed by removal of the recognition marker (1). Many of the acid hydrolases that reach lysosomes appear to be delivered via this mechanism. Much of the information concerning the mechanism of enzyme transport has come from studies on fibroblasts. However, mannose phosphate receptors have been found in a variety of whole tissue homogenates (21), and it is assumed that the mannose-6-P recognition signal is a general mechanism for lysosomal enzyme targeting (22).

Little information is available as to the importance of the mannose-6-P receptor in macrophages. Several reports have appeared demonstrating secretion of phosphorylated hydrolases by macrophage cell lines (2, 3, 23) and the presence of mannose-6-P receptors in human monocyte-like transformed cells (22).

The present report is the first demonstration of functional mannose-6-P receptors in freshly isolated macrophages. The receptor is similar in kinetic parameters to the fibroblast receptor (14), and the inhibitory potency of fructose-1-P and mannose-6-P agrees with previously reported data (9, 13, 24).

Since macrophages are known to contain a variety of receptors on their surface (25), it was imperative to show unequivocally that the α-mannosidase was being recognized by a specific mannose-6-P receptor. Two receptors which could potentially contribute to the recognition of α-mannosidase by rabbit macrophages are the well characterized mannose-specific receptor in alveolar macrophages (5, 6) and the "scavenger receptor" described by Goldstein et al. (17). Since lysosomal enzymes contain a variety of carbohydrate structures, including terminal mannose as well as mannose-6-P, recognition of the slime mold α-mannosidase could involve both the mannose and the mannose-6-P receptors. Unlike mannose-6-P-containing ligands isolated from mammalian sources, the D. discoideum α-mannosidase contains a high proportion of phosphorylated mannose residues. Uptake by rabbit cells was inhibited only by phosphate-containing ligands known to block uptake into fibroblasts (mannose-6-P, fructose-1-P, and the phosphorylated mannan from H. holsti). None of the compounds which block uptake via the macrophage mannose receptor inhibited α-mannosidase uptake (mannose, fucose, mannose-BSA, fucose-BSA, and rat preputial β-glucuronidase). In addition, uptake through the mannose receptor is dependent on the presence of Ca\(^{2+}\) (6). When uptake of α-mannosidase was measured in the absence of Ca\(^{2+}\) and in the presence of 10 mM EDTA, mannose-6-P-inhibitable uptake was essentially the same as in Ca\(^{2+}\)-containing media (Table II). Diment and Dean have reported that fibroblast β-glucuronidase is taken up by peritoneal macrophages, through the mannose receptor, although the same ligand is recognized by the fibroblast mannose phosphate receptor (26). They concluded that mouse peritoneal macrophages probably do not contain mannose phosphate receptors. Using the slime mold α-mannosidase, mannose phosphate specific uptake has been detected in mouse peritoneal macrophages,\(^2\) although considerably less than measured in rabbit alveolar macrophages. It is possible that the 

\(^2\) V. Shepherd and P. D. Stahl, unpublished results.

The primary function of the mannose phosphate receptor appears to be the delivery of newly synthesized acid hydrolases to lysosomes. This is consistent with the findings of Fischer et al. (21) that greater than 80% of the enzyme population is found on intracellular membranes in fibroblasts. Using saponin or digitonin to permeabilize cells, a similar distribution of receptors in rabbit alveolar macrophages was found. Approximately 90% of the total binding sites are inside the cell (Fig. 4). The 10% of receptors found on fibroblast surfaces may function to recapture enzyme that contains the mannose-6-P recognition marker, and thus act as a pinocytosis receptor. Previous pinocytic studies in fibroblasts have shown that surface receptors bind and internalize mannose-6-P containing ligands, followed by delivery to lysosomes. The rate of uptake is unchanged up to 3 h, and receptors are reutilized every 5 min (1). Uptake of α-mannosidase by rabbit alveolar macrophages is linear for 90 min (Fig. 2), and continued uptake is inhibited by the lysosomotropic drug chloroquine (Fig. 3). As in fibroblasts, the receptor on the surface may be serving a pinocytic role, perhaps recapturing enzyme which has escaped the intracellular packaging mechanism. The mannose receptor likewise plays a pinocytic role in macrophages, and appears to recognize lysosomal enzymes that have escaped the intracellular packaging mechanism. Although an intracellular function has not been found for the mannose receptor, this receptor also has a large percentage (80%) of the total receptors inside (7). It is interesting to speculate that in the macrophage, which secretes biologically active compounds and ingests soluble and particulate ligands, both mannose and mannose-6-P receptors may play a regulatory role at the surface as well as in transport and delivery within the cell.

REFERENCES

Identification of mannose 6-phosphate receptors in rabbit alveolar macrophages.

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