Mannose 6-Phosphate Receptors on the Plasma Membrane on Rat Retinal Pigment Epithelial Cells

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The retinal pigment epithelium (RPE) phagocytizes the tips of photoreceptor outer segments (OS) during normal eye function. It is not known what ligand on OS is recognized by the RPE for removal from the interphotoreceptor matrix. It is possible that a sugar residue on a cell surface glycoconjugate of either the OS or RPE mediates the phagocytic interaction. Pinocytic experiments with a soluble mannose 6-phosphate ligand (125I-labelled mannosidase) showed that similar quantities of ligand were bound by RPE explants from Long Evans rat retinas and from Royal College of Surgeons (RCS/p') rat retinas known to be defective in the phagocytosis of OS. The addition of mannose 6-phosphate reduced the total counts of bound alpha-mannosidase by 23% in both normal and dystrophic RPE explants. Mannose 6-phosphate receptors were visualized on normal and dystrophic RPE plasma membranes by immunocytochemical techniques. Further, phagocytosis was studied using phospho-mannan-coated beads as phagocytic particles. Dystrophic RPE phagocytized phosphomannan-coated beads by a mannose 6-phosphate specific mechanism as shown by a significant reduction in the number of these coated beads taken up in the presence of the competing sugar. In contrast, normal RPE showed no uptake of phosphomannan-coated beads. Our findings indicate that a mannose 6-phosphate receptor is on the apical plasma membrane of rat RPE. This receptor may not be involved in normal OS phagocytic recognition, but may function in the trafficking of lysosomal enzymes by RPE cells. Invest Ophthalmol Vis Sci 29:291–297, 1988
phagocytic uptake of particulate mannose 6-phosphate.

Materials and Methods

Animals

Rats used in this study were 10-13 day postnatal Long Evans rats (normal retina) and Royal College of Surgeons (RCS/p⁺) rats (dystrophic retina model). Eight to 12 animals were used for each experimental condition. The rats were maintained on a 12 hr light:12 hr dark cycle with light intensities not exceeding 10 foot-candles (ft-cd) in the cages. The treatment of experimental animals in this study was in compliance with the ARVO Resolution on the Use of Animals in Research.

Eye Dissection

Explants were prepared as previously described. Briefly, eyes were enucleated during the end of the light cycle, soaked in RPMI 1640 with Hepes at 4°C during the dark cycle. At the beginning of the light cycle, the anterior eye was cut at the ora serrata. The cornea and lens were discarded. The eye was quartered and the neural retina allowed to detach.

Bead Coating

Bead coating followed the procedures of Seyfried-Williams and McLaughlin and Seyfried-Williams et al. Briefly, polybead-carboxylated monodispersed microspheres (1.0 μm diameter, 4.55 × 10¹⁰ beads/ml) (Polysciences, Warrington, PA) were reacted with N-ethyl-5-phenyl-isoxozolium 3' sulfonate (Wodward's reagent) (Sigma, St. Louis, MO) at 22°C for 30 min, then rinsed with PBS. The activated beads were coupled with the large molecular weight fragment of phosphomannan prepared by acid hydrolysis of native Hansenula holstii phosphomannan.

Pinocytic Experiments

Alpha-mannosidase from Dictyostelium discoideum was iodinated by the chloramine T method as described by Shepherd et al. Five to seven explants were added per well of a 24 well tissue culture dish. ¹²⁵I-labelled alpha-mannosidase was added in a total volume of 400 μl of Hank's balanced salt solution containing 1% BSA in the presence or absence of 10 mM mannose 6-phosphate. Explants were incubated at 37°C for 2 hr, washed, and bound ¹²⁵I-alpha-mannosidase was counted. Specific activity of the ligand for experiments with dystrophic RPE explants was 15,000 cpm/ng; for normal explants, 18,000 cpm/ng. Assays were done in triplicate, and results presented are representative of three separate experiments.

Immunocytochemistry

RPE explants from normal and dystrophic rats were prepared as described above. The explants were fixed for 4 hr in 2% paraformaldehyde, 0.075 M lysine, 0.01 M periodate in 0.037 M phosphate buffer. The tissue was rinsed and nonspecific binding blocked by incubation in PBS containing 5% normal goat serum (Miles, Naperville, IL). Either rabbit antihuman liver mannose 6-phosphate receptor or normal rabbit serum was added to the explants at concentrations of 1:10 in PBS/5% normal goat serum/1% BSA and incubated at 4°C overnight. The tissue was washed in Tris-HCl (pH 8.2)/normal goat serum/BSA, and goat anti-rabbit IgG conjugated to 15 nm gold (Janssen, Piscataway, NJ) was added overnight at 4°C. The tissue was rinsed, fixed for 1 hr in 2% glutaraldehyde, 2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.3). The tissue was washed three times in 0.1 M cacodylate buffer, osmicated for 1 hr, uranyl acetate en bloc stained for 1 hr, dehydrated in ethanol, and embedded in Epon/Araldite.

Phagocytic Experiments

Explants, consisting of sclera, choroid, and RPE, were incubated for 1 hr in RPMI 1640 medium containing glutamine (Gibco, Grand Island, NY) at 37°C in a 5% CO₂ atmosphere. Fresh media was added and phosphomannan-coated beads (50 μl) in the same media were added four times at 30 min intervals at a concentration of 4.55 × 10¹⁰ beads/ml. Specificity of uptake was demonstrated by the addition of 100 mM mannose 6-phosphate (Sigma) together with phosphomannan beads. Uncoated beads and mannose 6-phosphate (100 mM) were added to the medium of RCS/p⁺ explant cultures as a positive control for phagocytosis. The explants were incubated with the beads for 5 hr and were then washed and fixed for electron microscopy in 2% glutaraldehyde, 2% paraformaldehyde, 0.5% acrolein in a 0.1 M cacodylate buffer (pH 7.3). The tissue was further processed for electron microscopy as described in the methods for immunocytochemistry.

Statistical Analysis

The number of beads phagocytized by normal and dystrophic RPE explants was analyzed by electron microscopy. Blocks were randomly selected and sectioned so that beads were counted in an average of 24 cell profiles per animal. The number of cell profiles which contained beads in their cytoplasm was quantitated and expressed as a percent of the total cell profiles counted. Morphological criteria for inclusion of RPE explants in the quantitative analysis were: RPE cells were present as monolayers lying on...
Bruch's membrane, cell junctions between RPE lateral plasma membranes were intact, and beads, but few OS, were seen near the microvilli. The Mann-Whitney U Test was used to compare uptake in the two strains of rats for each bead coating.

**Results**

**Pinocytic Experiments**

Dystrophic RPE explants bound a total of 14.8 ng of ligand added. The addition of 10 mM mannose 6-phosphate blocked binding by approximately 23%, resulting in 3.4 ng recognized by a mannose 6-phosphate specific process. Similarly, normal rat RPE explants bound almost identical amounts of ligand: total binding was 14.2 ng with 3.2 ng specific for mannose 6-phosphate recognition. Background levels (nonspecific binding) were consistently high in all experiments most likely due to the use of tissue explants.

**Immunocytochemistry**

Using en bloc immunogold labeling of RPE cells in explants, anti-mannose 6-phosphate receptor reactivity (arrowheads) on the apical plasma membrane. No label is present on the outer segment tips (OS) (×25,143). Fig. 2. Label (arrow) is present in a coated pit region of the apical plasma membrane in dystrophic RPE. PG, pigment granule (×100,000). Fig. 3. Normal RPE has anti-mannose 6-phosphate receptor-15 nm gold labelling (arrow) in a coated pit (×100,000).
Phagocytic Experiments

Transmission electron microscopy demonstrated that phosphomannan-coated beads were avidly phagocytized by dystrophic RPE explants (Fig. 4). Numerous beads were observed in the cytoplasm and between microvillous membranes. Normal RPE did not phagocytize the phosphomannan-coated beads as beads were observed in close proximity with microvilli, but were not observed in the cytoplasm (Fig. 5). Specificity of uptake of phosphomannan-coated beads by dystrophic RPE was shown by a significant reduction in the numbers of beads phagocytized with the addition of 100 mM mannose 6-phosphate in the explant culture medium (Fig. 6). In the presence of the competing sugar, beads are only seen in an extracellular location. The addition of 100 mM mannose 6-phosphate did not change the ability of the RPE cells to ingest beads with an irrelevant coating (uncoated) as uncoated beads were taken up in similar numbers by dystrophic RPE in the presence (Fig. 7) or absence (not shown) of the competing sugar. The results of bead uptake are summarized in Table 1. Beads were incubated with the RPE explants for 4 hr in RPMI 1640 containing glutamine at 37°C in a 5% CO₂ atmosphere. Beads were counted in three or four animals with an average of 21 cell profiles scored per animal. Each experiment was performed two times. Dystrophic RPE phagocytized 6.9 (SEM 2.4) phosphomannan-coated beads/cell profile \((P < 0.01)\) as compared to normal RPE which did not phagocytize any phosphomannan-coated beads. The dystrophic RPE cell profiles which contained beads represented 48.6% of the total number of cell profiles counted. Accordingly, 51.4% of the dystrophic RPE cell profiles counted contained no beads.

Discussion

The phosphomannosyl receptor has been well characterized on many cell types such as macrophages, fibroblasts, CHO cells, hepatocytes, lymphocytes, and natural killer cells and has been shown to function primarily in the translocation of newly synthesized lysosomal enzymes from Golgi to lysosomes. Approximately 80%
of the receptor is inside the cell, with 20% located on the plasma membrane. This plasma membrane component, at least in fibroblasts, may be a normal consequence of the pathway of membrane recycling, and may represent movement of intracellular receptor transiently to the outside.

Several recent reports have suggested the existence of phosphomannosyl receptors on RPE. Wilcox reported that the addition of 20 mM mannose 6-phosphate to the medium of cultured human RPE enhanced release of several lysosomal enzymes. Kean et al have demonstrated the binding of mannose 6-phosphate-BSA to cultured chick embryo RPE cells and Shirakawa and Kean have shown that phosphomannan and mannose 6-phosphate inhibit the binding of rhodopsin-liposomes to RPE cells from cultured chick embryos.

Our study presents, for the first time, direct evidence that RPE cells express a phosphomannan receptor on their surface. Normal and dystrophic rat RPE cells bind similar levels of soluble ligand, suggesting that pinocytic mannose 6-phosphate receptors are present in both animals. These pinocytosis data are confirmed by the immunocytochemical results which show the presence of mannose 6-phosphate receptors on dystrophic and normal RPE. The receptor is localized on the apical surface of the RPE cells in regions resembling coated pits which is similar to the localization of surface mannose 6-phosphate receptors on CHO fibroblasts and hepatocytes.

The pinocytic mannose 6-phosphate receptor on the surface of RPE cells may have a function similar to the mannose 6-phosphate receptor reported on the surface of fibroblasts. In these cells, 80-90% of the receptor is thought to function within the Golgi system by compartmentalizing lysosomal enzymes (bearing mannose 6-phosphate sugars), through the endoplasmic reticulum, peripheral Golgi elements (GERL) and into lysosomes. In some way, a small

Table 1. Number of phosphomannan-coated beads phagocytized by dystrophic* and normal† RPE explants

<table>
<thead>
<tr>
<th>RPE</th>
<th>Competing sugar</th>
<th>Beads/cell profile</th>
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<tbody>
<tr>
<td>Dystrophic</td>
<td>None</td>
<td>6.9 (2.4)</td>
</tr>
<tr>
<td>Dystrophic</td>
<td>Mannose 6-phosphate</td>
<td>0</td>
</tr>
<tr>
<td>Normal</td>
<td>None</td>
<td>0</td>
</tr>
</tbody>
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* RCS/pc rat retinas.
† Long-Evans rat retinas.
Beads were incubated with RPE explants for 4 hr in RPMI 1640 + glutamine medium at 37°C in a 5% CO2 atmosphere. Beads were counted in three or four animals with an average of 21 cell profiles scored per animal from two separate experiments. All values are expressed as the mean ± SE. The number of cell profiles containing beads is expressed as a percentage as compared to the total number of cell profiles counted; dystrophic RPE, 48.7% (8.7).
percentage of receptor reaches the plasma membrane, clusters in coated pit regions, and is internalized through the coated pit-receptosome-Golgi pathway.25

Alternatively the receptor on RPE cells may more closely resemble the receptor on macrophages, which may have additional roles. It has been shown that macrophage cell lines and freshly isolated macrophages secrete phosphorylated enzymes.36 In cultures where high levels of enzymes are present extracellularly, phosphomannan receptor levels are low. When extracellular enzymes are low, receptor levels are high. Thus the macrophage and the RPE phosphomannan receptor may be involved in packaging newly synthesized enzymes within lysosomes as well as “recapturing” secreted phosphorylated enzymes for delivery to lysosomes.

The role of mannose phosphate receptors in cell-particle recognition has been well documented by Rosen and his coworkers who have shown that the mannose phosphate receptor on lymphocytes specifically recognizes a mannose 6-phosphate determinant on endothelial cells of post-capillary venules in the course of lymphocyte recirculation through lymphoid organs.28 Lymphocytes, but not thymocytes, also bind specifically to beads which had been conjugated to phosphomannan.29

Our pinocytosis data suggest that normal and dystrophic RPE both express cell surface mannose phosphate receptors. However, when RPE explants are exposed to phosphomannan coated beads, only dystrophic RPE phagocytize the beads in a mannose phosphate dependent manner. There are several possible explanations. It has been shown that dystrophic RPE is not deficient in phagocytosis, but in the specific recognition of OS and subsequent internalization. The phagocytic mechanism is intact as evidenced by successful ingestion of carbon particles,12 latex beads17 and beads coated with Con A, *Ulex europaeus* agglutinin, *Lens culinaris* agglutinin,8 succinyalted wheat germ agglutinin and N-acetyl glucosamine.9 Although both normal and dystrophic RPE express pinocytic mannose phosphate receptors, these may only recognize and internalize soluble ligands. A different receptor or recognition event may be involved in phosphomannan bead uptake, a recognition that is unrelated to (1) the pinocytic receptor and (2) the OS-recognition defect in dystrophic RPE.

The presence of mannose 6-phosphate receptors on RPE explants has been demonstrated in this study, but no connection of that receptor to the recognition of OS in vivo has been established. We are currently pursuing the study of other carbohydrate-dependent cell-particle and cell-protein recognition systems by the RPE.

Key words: mannose 6-phosphate receptor, phagocytosis, pinocytosis, retinal pigment epithelium, RCS/p⁺ rats

Acknowledgments

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References

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