Transmembrane Orientation of the Mannose 6-Phosphate Receptor in Isolated Clathrin-coated Vesicles*

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The mannose 6-phosphate (Man-6-P) receptor is an integral membrane glycoprotein which mediates intracellular transport and receptor-mediated endocytosis of lysosomal proteins. Clathrin-coated vesicles, which have been shown to be significantly involved in these processes, have also been shown to be a major subcellular site of the receptor. In order to define the orientation of the Man-6-P receptor within the coated vesicle membrane, highly purified preparations of coated vesicles were prepared from bovine brain employing D2O/sucrose gradient centrifugation and Sephacryl S-1000 column chromatography. Using [35S]methionine-labeled lysosomal enzymes secreted by Chinese hamster ovary cells as receptor ligand, significant binding activity was detected only upon permeabilization of the coated vesicle membranes with detergent. Prior treatment of intact vesicles with proteinase K resulted in similar binding activity upon permeabilization. However, examination of the receptor by sodium deoxycholate, Man-6-P, p-hydroxymercuribenzoate, and carboxypeptidase Y revealed that proteinase K treatment of intact vesicles reduced the size of the receptor by 12,000 daltons. A similar decrease in size was obtained when the vesicles were treated with carboxypeptidase Y. These results suggest that the Man-6-P receptor is a transmembrane protein with its lysosomal enzyme binding site oriented toward the lumen of the coated vesicle and its C-terminal end exposed to the exterior or cytoplasmic portion of the vesicle membrane.

Both lysosomal enzymes and secretory proteins share a common site of synthesis on ribosomes of the rough endoplasmic reticulum (1–3). The sorting of these two classes of proteins depends on construction of Man-6-P residues on asparagine-linked oligosaccharides of the enzymes, a process which occurs post-translationally after the proteins are translated. This modification serves to transport the enzymes to the Golgi apparatus (4–7). This modification serves as a recognition marker which signals binding of the enzymes to the Man-6-P receptor and subsequent transport to lysosomes (for reviews, see Refs. 8–11).

Studies with human fibroblasts have shown that lysosomal enzymes are transported to lysosomes through a predominantly intracellular route (12–15). Morphological studies (16–18) suggest that this process involves binding of lysosomal enzymes to Man-6-P receptors in the Golgi apparatus and subsequent transport to lysosomes via Golgi-derived clathrin-coated vesicles. Significant portions of most lysosomal enzymes escape direct lysosomal delivery and are secreted (19). In many cell types these secreted enzymes are recaptured by Man-6-P receptors on the cell surface and are delivered to lysosomes by the process of receptor-mediated endocytosis involving cell surface clathrin-coated pits and vesicles (20). The involvement of coated pits and vesicles in endocytosis and intracellular transport of lysosomal enzymes has been substantiated by the demonstration of Man-6-P receptors and lysosomal enzyme "precursors" in purified preparations of coated vesicles (21, 22).

The Man-6-P receptor has been purified to near homogeneity from bovine liver, a number of other mammalian tissues, and established cell lines (23–35). Biosynthetic studies in Chinese hamster ovary cells have shown that the receptor is composed of a single polypeptide of Mr = 215,000 that is extensively cross-linked with disulfide bonds and contains phosphoserine residues (26). In the present study, antibodies against purified bovine Man-6-P receptor were used to demonstrate the transmembrane nature and orientation of the Man-6-P receptor in isolated rat liver and bovine brain coated vesicles.

EXPERIMENTAL PROCEDURES

Materials

Ultrapure sucrose, phenylmethylsulfonyl fluoride, and Proteinase K were purchased from Bethesda Research Laboratories; [35S]methionine (carrier free) and [3H]-l-Protein A (30 μCi/μg) from Amersham; Protein A-Sepharose and Sephacryl S-1000 from Pharmacia; sodium deoxycholate, Man-6-P, p-hydroxymercuribenzoate, and carboxypeptidase Y from Sigma; and deuterium oxide (D2O) from Aldrich. All other chemicals were reagent grade or better. Chinese hamster ovary cells (strain WTB) were provided by Dr. April Robbins, National Institutes of Health. Bovine brains were obtained from a local slaughterhouse and were kept at 4 °C until processed within 2 h of slaughter. The antisera used in this study was raised against purified bovine Man-6-P receptor as previously described (24, 26). This antibody specifically precipitates Man-6-P receptor from cells and tissues of several mammalian species including cow, man, rat, mouse, and Chinese hamster and does not inhibit binding of lysosomal enzymes to the receptor (24).1

Methods

Isolation of Coated Vesicles—Coated vesicles were isolated from fresh bovine brain according to the method of Nandi et al. (27). Enrichment of the coated vesicles from the original homogenate consisted of a series of low speed (10,000 × g) and high speed (100,000 × g) centrifugations performed at 4 °C. Single-step gradient centrifugation (at 18 °C) through an 8% sucrose/D2O solution maintained

1 G. G. Sahagian, unpublished results.
at pH 6.5 with similar buffer salts as included in the homogenization buffer (Buffer A: 0.1 M MES, pH 6.5, 1 mM EGTA, 0.5 mM MgCl₂, and 0.02% w/v NaN₃) removed greater than 85% of the remaining contaminating smooth membrane material. Final purification of the clathrin-coated vesicles was achieved by Sephacryl S-1000 gel filtration column chromatography (28). In a typical purification, 40 mg of coated vesicle was resuspended in 85-cm column of Sephacryl S-1000 pre-equilibrated with Buffer A. Smooth membrane contaminating material, consisting of sheets and vesicles, was eluted in the void volume peak. A second well defined peak in the included volume consisted almost entirely of clathrin-coated vesicles as determined by thin section electron microscopy (29, 30). Fractions containing the coated vesicles were pooled and pelleted by centrifugation at 100,000 x g for 60 min. The supernatant was gently aspirated and the clear pellet of coated vesicles was allowed to resuspend in 10 ml of Buffer A overnight at 4 °C. Final protein concentrations were approximately 2 mg/ml.

Coated vesicles were isolated from rat liver in a similar manner with the exception that the single-step gradient centrifugation was done using a 17% sucrose/D₂O solution as described previously (29).

Preparation of [³⁵S]-labeled Lysosomal Enzymes—Lysosomal enzymes secreted by amniotic treated Chinese hamster ovary cells were prepared as follows and were used as ligand for quantitative mannose-6-phosphate receptor binding activity. Two T150 flasks, each containing 2 x 10⁷ cells, were biosynthetically labeled for 16 h at 35 °C with 2 ml of [³⁵S]methionine in the presence of 10 mM NH₄Cl as previously described (28). Lysosomal enzymes were isolated from the medium by affinity chromatography using a column of immobilized mannose-6-phosphate receptor.

The affinity column was prepared by packing 5 ml of Protein-A-Sepharose into a 1.6-cm diameter column and sequentially passing through it the following solutions at 4 °C: 25 ml of Buffer B (10 mM NaH₂PO₄, 0.15 M NaCl, pH 7.0), 3 ml of rabbit anti-bovine Man-6-P receptor serum, 10 ml of Buffer B, 500 ml of a detergent extract of bovine liver acetone powder (24), 100 ml of Buffer B containing 1% Triton X-100, and 25 ml of Buffer B. The medium from the abused cells was made 0.1 M in Tris-HCl, pH 7.5, and applied to the affinity column at 4 °C. The column was washed with 50 ml of Buffer B and the bound radioactivity (approximately 3 x 10⁶ cpm) was eluted with Buffer B containing 5 mM Man-6-P. When stored in this buffer at 4 °C, the labeled ligand retained its ability to bind to the receptor for at least two weeks.

Binding of [³⁵S]-labeled Lysosomal Enzymes to Coated Vesicles—Just prior to use, the labeled enzyme preparation was dialyzed against Buffer B to remove Man-6-P. Coated vesicles (0.1-0.2 mg of protein in 100 µl of Buffer A) were incubated with 10,000 cpm of labeled enzyme for 1 h on ice; when indicated, sodium deoxycholate or Man-6-P was added to the vesicles 5 min prior to addition of the ligand. Aliquots (100 µl) of the incubation mixtures were layered over 0.4 M sucrose (100 µl) and centrifuged at 4 °C in a Beckman airfuge at 20 p.s.i. for 30 min to pellet the vesicles. The pellets were gently rinsed with 100 µl of Buffer A and counted.

Proteolytic Treatment of Coated Vesicles—Treatment of coated vesicles with Proteinase K or carboxypeptidase Y was carried out in Buffer A for 60 min at room temperature. Reactions were terminated by the addition of phenylmethylsulfonyl fluoride (100 mM in absolute ethanol) to a final concentration of 2 mM and allowed to incubate for an additional 15 min.

Electrophoresis and Immunoblotting—Electrophoresis of proteins was performed in sodium dodecyl sulfate-polyacrylamide gels as described by Laemmli (31) with the exception that the ratio of bisacrylamide to total acrylamide was reduced to 1:95. Samples containing 25-50 µg of coated vesicle protein in 25-µl volume were solubilized by addition of an equal volume of buffer containing 200 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 50 mM glycerol, 10 mM dithiothreitol, and bromphenol blue. Electrophoresis was carried out after heating the samples at 95 °C for 2 min. Those samples used for immunoblotting were solubilized and electrophoresed in the absence of dithiothreitol. For detection of total coated vesicle protein, gels were stained for 20 min with 0.1% (w/v) Coomassie Brilliant Blue R in 50% trichloroacetic acid and destained with 7% acetic acid. Man-6-P receptor was sensitive to proteolysis and was therefore excised by the addition of phenylmethylsulfonyl fluoride (100 µM in absolute ethanol) to a final concentration of 2 mM and allowed to incubate for an additional 15 min. Proteinase K was used in these and subsequent experiments because of its ability to completely degrade proteins down to their corresponding amino acids. Deoxycholate at 0.1% was used to permeabilize the coated vesicle membrane. This concentration produced a maximal permeabilization of the vesicles as determined by the release of endogenous β-hexosaminidase activity (Table III). Under identical conditions, approximately 50% of the Man-6-P receptor was solubilized as shown by immunoblotting of detergent-treated vesicles before and after centrifugation.

Transmembrane Orientation of the Man-6-P Receptor—Immunoblotting of Proteinase K-treated coated vesicles with anti-Man-6-P receptor antibody revealed that a portion of the receptor was sensitive to proteinase K and was therefore exposed to the outer surface of the vesicle membrane (Fig. 1). Under nonreducing conditions, the receptor in both intact and deoxycholate-permeabilized vesicles migrated as a single major band with an apparent M, of 202,000. This molecular weight is identical to that previously determined for nonreduced receptor isolated from bovine liver. Samples were electrophoresed without prior exposure to reducing agents.

Table I

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<th>Pretreatment</th>
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<tr>
<td>Man-6-P</td>
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<td>Deoxycholate + Man-6-P</td>
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Table II

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<td>Deoxycholate</td>
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<td>Proteinase K + Deoxycholate</td>
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Results

Orientation of Lysosomal Enzyme Binding Sites in Isolated Coated Vesicles—The data shown in Tables I and II suggest that the lysosomal enzyme binding site of the Man-6-P receptor is located on the inner surface of the coated vesicle membrane. Significant Man-6-P inhibitable binding of [³⁵S]-labeled lysosomal enzymes to the vesicles was detected only upon permeabilization of the coated vesicles with detergent (Table I). This binding was unaffected by prior treatment of intact vesicles with Proteinase K under conditions which resulted in proteolysis of clathrin and several other proteins located on the outer surface of the vesicle membranes (Table II). Proteinase K was used in these and subsequent experiments because of its ability to completely degrade proteins down to their corresponding amino acids. Deoxycholate at 0.1% was used to permeabilize the coated vesicle membrane. This concentration produced a maximal permeabilization of the vesicles as determined by the release of endogenous β-hexosaminidase activity (Table III). Under identical conditions, approximately 50% of the Man-6-P receptor was solubilized as shown by immunoblotting of detergent-treated vesicles before and after centrifugation.

The abbreviations used are: MES, 4-morpholineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N''-tetraacetic acid.
The effects of increasing concentrations of Proteinase K on proteolysis of the Man-6-P receptor and the major coated vesicle proteins are shown in Fig. 2. Degradation of the 12,000-dalton proteinase-sensitive portion of the receptor was complete after treatment with 3 µg/ml of Proteinase K. At higher concentrations of the protease, no further degradation of the receptor was observed. With the exception of the heavy chain of clathrin (M, = 180,000), most detectable coated vesicle proteins were degraded at similarly low concentrations of the enzyme. Clathrin was also degraded by treatment with Proteinase K; however, higher concentrations (100 µg/ml) were required. The resistance of the receptor to further proteolysis under conditions that promote degradation of both clathrin and other external proteins suggests that the remaining 190,000-dalton portion of the receptor is located either within or on the inner surface of the vesicle membrane.

Complete degradation of the receptor by Proteinase K upon exposure of the vesicles to deoxycholate (Fig. 1) suggests that the proteinase-resistant portion of the receptor is, in fact, protected from proteolysis rather than intrinsically resistant to the protease. The results noted in Fig. 3 (left panel) reveal that the loss of this protection parallels the increase in permeability of the coated vesicles (Table III), suggesting that such protection is afforded by the vesicle membrane. The additional immunoreactive bands of M, 96,000, 70,000, and 52,000 that appear at intermediate deoxycholate concentrations (Fig. 3, left panel) presumably arise from limited proteolysis of the receptor as the proteinase gains access to the intravesicular space.

Treatment of coated vesicles with Proteinase K at sufficiently high concentrations of the enzyme results in efficient degradation of both clathrin and other external proteins. However, examination of the effect of deoxycholate on degradation of coated vesicle proteins at low Proteinase K concentration (Fig. 3, right panel) reveals that the rate of degradation of clathrin is stimulated by addition of detergent. Deoxycholate does not destroy the integrity of the clathrin coat since clathrin and other major coated vesicle proteins can still be pelleted after treatment of the vesicles with the detergent (data not shown). It is conceivable that the increased degradation of clathrin in the presence of detergent is a result of membrane solubilization and therefore an increase in accessibility of clathrin to proteolysis. Such an explanation is plausible in light of the reported interaction

because of a diminished reactivity of the antibody for reduced receptor (26). In contrast to intact coated vesicles, the receptor of Proteinase K-treated vesicles migrated with an apparent M, of 190,000, i.e. 12,000 daltons lower than the unproteolyzed receptor. Clathrin and other major coated vesicle proteins were at least partially degraded by this treatment. Centrifugation studies revealed that the Man-6-P receptor remained associated with the vesicles after treatment with Proteinase K. Treatment of deoxycholate-permeabilized vesicles with Proteinase K resulted in loss of detectable receptor and in degradation of the major coated vesicle proteins. Similar results were obtained for coated vesicles isolated from rat liver (data not shown).
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Table 1: Effect of deoxycholate concentration on the degradation of coated vesicle proteins by proteinase K.

<table>
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<th>DOC (%)</th>
<th>0</th>
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<tr>
<td>PK (10 μg/ml)</td>
<td>+</td>
<td>+</td>
<td>+</td>
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Fig. 3. Effect of deoxycholate concentration on the degradation of coated vesicle proteins by proteinase K. Vesicles were permeabilized with the indicated concentration of deoxycholate (DOC), treated with Proteinase K (PK) at 10 μg/ml and subjected to electrophoresis in a slab gel with a total concentration of 7% (left panel) or 9% (right panel).

Fig. 4. Effect of carboxypeptidase Y treatment on coated vesicle proteins. Intact vesicles or vesicles permeabilized with 0.1% deoxycholate (DOC) were treated with carboxypeptidase Y (CPY) at the indicated concentration and/or 100 μg/ml of Proteinase K (PK) and subjected to electrophoresis in a 7% (left panel) or 12.5% (right panel) acrylamide slab gel.

Fig. 5. Effect of inhibitors on the degradation of coated vesicle proteins by carboxypeptidase Y. Carboxypeptidase Y (CPY) at 3 mg/ml in Buffer A was preincubated for 30 min at room temperature with 2 mM phenylmethylsulfonyl fluoride (PMSF) or 10 mM p-hydroxymercuribenzoate (PHMB). Coated vesicles were then incubated with the treated enzyme in the usual manner and subjected to electrophoresis in a 7% (left panel) or 12.5% (right panel) acrylamide slab gel.

The unique resistance of clathrin to proteolysis as well as its increased sensitivity in the presence of detergent has been described previously for several other proteases (34, 35).

External Orientation of the C Terminus of the Mannose 6-Phosphate Receptor—Treatment of coated vesicles with the exopeptidase carboxypeptidase Y resulted in the same 12,000-dalton reduction in the size of the receptor as produced by Proteinase K (Fig. 4). Likewise, simultaneous treatment with both enzymes resulted in a similar change, suggesting that each enzyme acts upon an identical structural domain of the receptor. Treatment of the coated vesicles with carboxypeptidase Y in the presence of 0.1% deoxycholate, which was shown to permeabilize the vesicle membranes and partially solubilize the receptor, resulted in further degradation of the receptor. The appearance of discrete bands might possibly be explained by slow proteolysis in regions of the receptor less sensitive to the enzyme, such as sites of glycosylation or regions of extensive secondary or tertiary structure. In order to rule out the possibility that proteolysis of the receptor was the result of contaminating proteases in the carboxypeptidase Y preparation, the effects of inhibitors of the carboxypeptidase were examined. p-Hydroxymercuribenzoate and phenylmethylsulfonyl fluoride, each having different modes of action, were shown to inhibit proteolysis of the receptor (Fig. 5).

DISCUSSION

The role of clathrin-coated vesicles in intracellular trafficking of macromolecules is now well established (36, 37). It has been shown that coated vesicles originating from the cell surface serve as the primary vehicle of transport for a variety of ligands during the process of receptor-mediated endocytosis (38). Subsequent to coated vesicle formation and dissociation of the clathrin coat, the newly formed endosomes undergo a number of fusion events with prelysosomal and lysosomal organelles that result ultimately in delivery of the endocytosed ligands to lysosomes. Prior to these events, however, endosomes, and perhaps coated vesicles as well, undergo acidification soon after formation. This intravesicular pH change results in release of lysosomal enzymes as well as other ligands from their receptors and permits recycling of unoccupied receptors back to the plasma membrane for reutilization (39).

Morphological studies by Geuze et al. (40) on the endocytosis of asialoglycoproteins suggest that segregation of the receptors occurs in characteristic tubulovesicular structures...
referred to as the compartment for the uncoupling of receptor and ligand (CURL). Receptor recycling is thought to initially involve accumulation of unoccupied receptors in the tubular domains of the CURL membrane. These tubular structures appear to then pinch off and recycle back to and fuse with the plasma membrane. The concentration of ligand-receptor complexes in clathrin-coated pits and the selective internalization of these complexes into coated vesicles have suggested a possible transmembrane interaction of receptors with either clathrin or some clathrin-associated protein(s). Likewise, interaction of receptors with membrane or cytoplasmic factors may also be involved in concentrating receptors in the CURL membrane for recycling. Interactions responsible for the internalization and recycling of Man-6-P receptors during endocytosis may also be responsible for similar processes that occur during intracellular lysosomal enzyme transport (discussed in Ref. 41).

The present study demonstrates that the Man-6-P receptor associated with clathrin-coated vesicles is a transmembrane protein. In confirmation of the results of Campbell et al. (21), the binding site of the receptor was found to be located at the inner face of the vesicle where it was not only inaccessible to ligand binding but also protected from proteolysis. However, a 12,000-molecular weight portion of the receptor polypeptide containing the C-terminal end was shown to be susceptible to proteolysis by both Proteinase K and carboxypeptidase Y. Since no further reduction in size was noted upon treatment with proteases that produce extensive degradation of clathrin and other external coated vesicle proteins (Figs. 2 and 4), proteolysis by both Proteinase K and carboxypeptidase Y may also be involved in concentrating receptors in the CURL membrane and cytoplasmic factors responsible for receptor internalization and recycling.

After this study was submitted for publication, von Figura et al. (46) reported that the Man-6-P receptor in membranes isolated from fibroblasts and HepG2 cells has a similar transmembrane orientation.

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REFERENCES