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Assembly of the mannose-6-phosphate receptor into reconstituted clathrin coats

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In ionic conditions in which clathrin coats are stable, the mannose-6-phosphate receptor associates with the 100-kd/50-kd coat complexes purified from bullock brain coated vesicles. These aggregates exist as striking spherical structures of 300 – 1000 Å diameter. When clathrin is included in the assembly mixture, cages are formed which apparently encapsulate these aggregates, giving, in the absence of lipid, structures resembling full coated vesicles.

Key words: mannose-6-phosphate receptor/coated vesicles/clathrin

Introduction

Coated pits and vesicles are thought to mediate the transfer of selected membrane receptors and their ligands from one cell compartment to another on both endocytic and secretory pathways (Goldstein *et al.*, 1979; Pearse and Bretscher, 1981). As a coated pit forms, certain receptors are concentrated in the coated region of membrane, whereas other membrane proteins are excluded (Bretscher *et al.*, 1980). This process is essential to the efficient transfer of the selected receptors and their ligands to the appropriate intracellular compartment. If a receptor is deficient in its ability to localize in coated regions of membrane, such as the JD mutant form of the low density lipoprotein (LDL) receptor, the efficiency of its uptake is drastically reduced (Anderson *et al.*, 1976, 1977). Uptake by the cell of iron bound to transferrin is dependent on the recycling of the transferrin receptor, from the plasma membrane *via* coated vesicles, through the endosome system and back (Karin and Mintz, 1981; Bleil and Bretscher, 1982). In normal fibroblasts, a group of lysosomal enzymes are labelled with mannose-6-phosphate (Sly and Fischer, 1982) causing them to bind to the mannose-6-phosphate receptor, which is concentrated in *cis* Golgi cisternae (Brown and Farquhar, 1984a). This receptor enters coated pits, which are believed to bud off as coated vesicles, presumably concentrating the lysosomal enzymes and facilitating their ultimate delivery to lysosomes. In I-cell fibroblasts (in mucolipidosis II disease) whose lysosomal enzymes lack the mannose-6-phosphate recognition markers (Neufeld and McKusik, 1983), the mannose-6-phosphate receptors are almost exclusively trapped in the *cis* Golgi region and accumulate in numerous coated pits at this presumptive sorting site (Brown and Farquhar, 1984b).

Thus it is important to understand the nature of the interaction between particular receptors and the coat proteins. To this end, I have attempted to reconstitute purified receptors with coat proteins. As the structure of coated vesicles is apparently preserved during extraction with 1% Triton X-100 (Pearse, 1982), one may think of it as being held together largely by protein-protein interactions even in the absence of the ordered lipid membrane of the vesicle. Therefore, the possibility exists of trying to reconstitute the structure in the absence of lipids. The success of this approach, with the incorporation of the mannose-6-phosphate receptor into coats, is described here.

Results

Co-assembly of mannose-6-phosphate receptors and 100-kd/50-kd coat complexes

Striking spherical aggregates of variable diameter (300 – 1000 Å are formed when the mannose-6-phosphate receptor and the 100-kd/50-kd coat complexes are together dialyzed into reconstitution buffer A. These spherical particles are concentrated in the pellet by centrifugation for 1 h at 100 000 *g*. An electron micrograph of a sample from such a pellet is shown in Figure 1.

In contrast, when the assembly mixture contains mannose-6-phosphate receptor in the absence of coat proteins, essentially no material pellets in the standard centrifugation step and the receptor remains soluble in the supernatant.

The pellets containing spherical aggregates assembled from mixtures of both receptor and coat proteins were further analyzed by agarose gel electrophoresis. A typical gel is shown in Figure 2. Free mannose-6-phosphate receptor migrates at about half the rate of bovine serum albumin (BSA). Free 100-kd/50-kd complexes remain close to the origin. However, a substantial proportion of the pelleted material from an assembly mixture containing both receptor and coat proteins migrates as a sharp band, intermediate in mobility between the free components. This pellet also appears to contain some free 100-kd/50-kd complex which probably accounts, with BSA, for the background of small particles seen in the electron micrograph of a sample (Figure 1).

To determine the composition of the intermediate band seen in the assembled mixture on agarose gel electrophoresis, an equivalent lane was analyzed in a second dimension by SDS-polyacrylamide gel electrophoresis. In this experiment, a higher

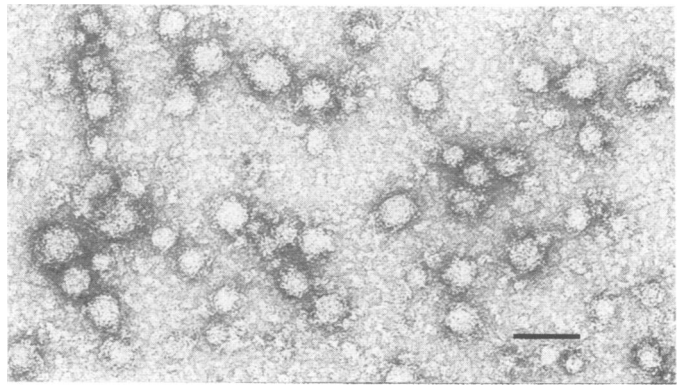

Fig. 1. Particles pelleted from an assembly mixture containing 60 μg/ml 100-kd/50-kd complexes, ~50 μg/ml mannose-6-phosphate receptor and 1 mg/ml BSA. The bar line represents 1000 Å.

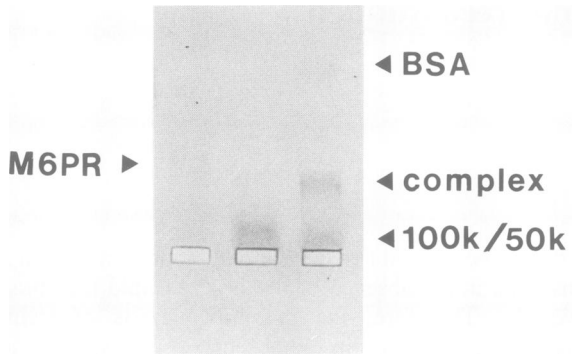

Fig. 2. Analysis of samples by agarose gel electrophoresis (from left to right: (**1**) mannose-6-phosphate receptor (the position to which the receptor migrates is indicated by the label); (**2**) 100-kd/50-kd complex; (**3**) particles pelleted from an assembly mixture containing 60 μg/ml 100-kd/50-kd complexes, ~50 μg/ml mannose-6-phosphate receptor and 1 mg/ml BSA (Yield ~20–30 μg). (The position to which marker BSA migrates is indicated by the label.)

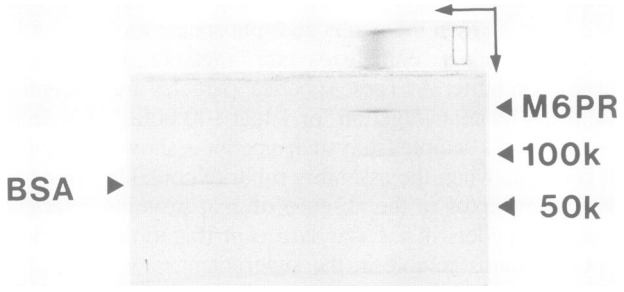

Fig. 3. Analyis of particles pelleted from an assembly mixture containing 60 μg/ml 100-kd/50-kd complexes, ~100 μg/ml mannose-6-phosphate receptor and 1 mg/ml BSA. Electrophoresis in the first dimension was from right to left on an agarose gel and electrophoresis in the second dimension was from top to bottom on a 7.5% SDS-polyacrylamide gel. The 50-kd polypeptide stained weakly on the second dimension gel, however, it co-migrated with the 100-kd polypeptides on the first dimension as expected, as these proteins occur as 100-kd/50-kd complexes (Pearse and Robinson, 1984).

ratio of receptor to 100-kd/50-kd protein was used in the initial assembly mixture, resulting in a greater portion of the pelleted material migrating as a single apparent complex. The analysis in the second dimension (Figure 3) shows that essentially all the receptor migrates in this region combined with a substantial portion of the 100-kd/50-kd complex. It looks as if the different polypeptides of the heterogeneous 100-kd polypeptide family associated with the receptor appear in the same ratio as in the free 100-kd/50-kd complexes. This suggests that under these conditions, the mannose-6-phosphate receptor does not selectively bind distinct members of the 100-kd polypeptide family. However, the 100-kd proteins may interact with each other and thus get carried along together with the receptor in the aggregate. Clearly, other proteins in the assembly mixture, the chief of which is BSA, do not interact with the receptor.

The precise ratio of 100-kd protein to receptor in the complex has not yet been determined but it varies depending on the ratio of these proteins in the initial assembly mixtures. However, in the electrophoretic analysis shown (Figure 3) there appears to be slightly more than one receptor molecule (mol. wt. 215 000) to one 100-kd molecule migrating together on the gel.

Thus it seems likely that the large spherical particles seen in the electron microscope (Figure 1) are composed of both receptor and 100-kd/50-kd complex as these proteins co-migrate as a single band on electrophoresis in agarose gel.

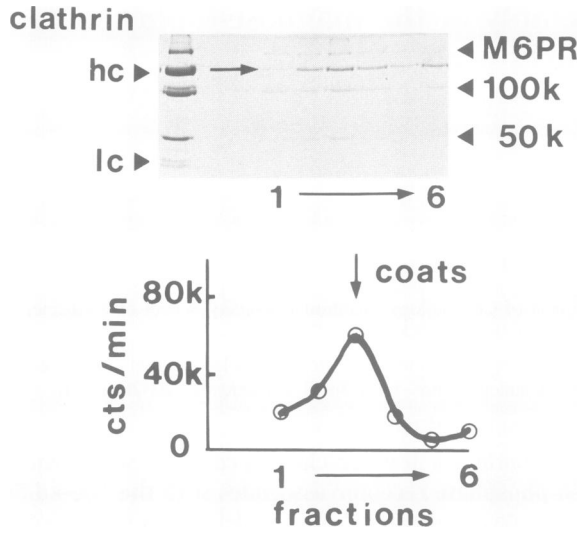

Fig. 4. (**upper**) Analysis of particles pelleted from an assembly mixture containing 50 μg/ml clathrin, 50 μg/ml 100-kd/50-kd complexes and ~50 μg/ml mannose-6-phosphate receptor by SDS-polyacrylamide gel electrophoresis before (left hand lane) and after separation on a 10–90% ^2H_2O gradient (**lanes 1–6**). (**lower**) Sedimentation profile of ^{125}I-labelled mannose-6-phosphate receptor in the gradient fractions (1–**6**) analyzed above by SDS-polyacrylamide gel electrophoresis.

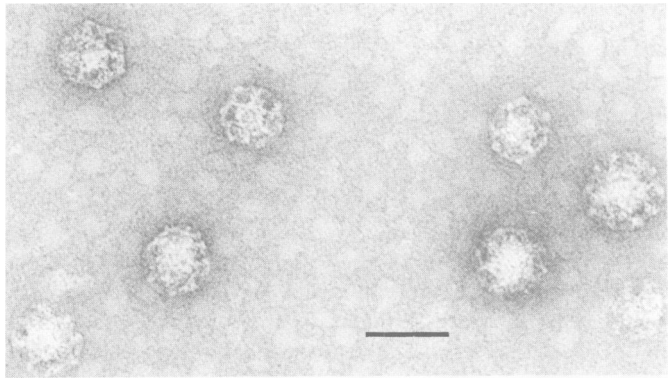

Fig. 5. Sample of coats containing mannose-6-phosphate receptor from fraction 2 of the 10–90% ^2H_2O gradient separation shown in Figure 4. The bar represents 1000 Å. Although fraction 3 contained more coats than fraction 2, this micrograph was chosen as it shows views of small coats analogous to those obtained for samples of empty coats (Pearse and Robinson, 1984).

Addition of clathrin to the assembly mixture

Addition of clathrin alone to receptor in an assembly experiment does not precipitate an appreciable amount of receptor compared with its addition in combination with the 100-kd/50-kd polypeptides. When all the coat proteins are present, coats are formed during the overnight dialysis (against buffer A) which apparently bring down the receptor on centrifugation. The composition of the pellet obtained is shown in the upper portion of Figure 4. The coats contained in this material were separated by centrifugation on the 10–90% ^2H_2O gradient in assembly buffer, as shown also in Figure 4. The receptor, in this experiment labelled with ^{125}I, co-sediments with the coats [Figure 4 (lower portion)]. An electron micrograph of a sample of the purified coats from fraction 2 is shown in Figure 5. In contrast to coats made in the absence of receptor (Pearse and Robinson, 1984), most of the coats shown are remarkable for their dense central region. These presumably contain receptor and are rem-

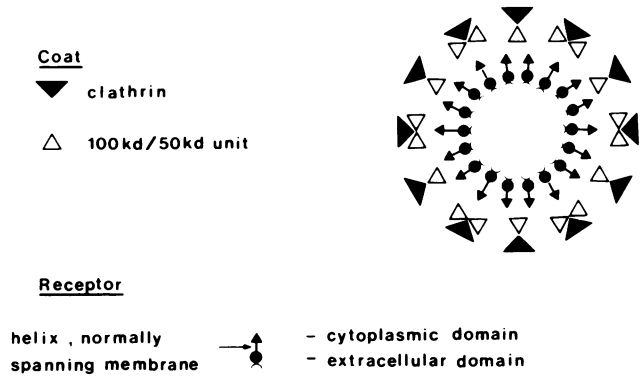

Fig. 6. Schematic diagram of a section through a small coat suggesting how receptors might be located inside. The precise organization of all the components remains to be determined. However, a model for the packing of clathrin in the outer polyhedral cage has been presented previously (Crowther and Pearse, 1981).

iniscent of fully laden coated vesicles. However, when there is not enough receptor to bind all the available 100-kd/50-kd complexes, as in this experiment, the free 100-kd/50-kd proteins tend to promote the assembly of excess clathrin to form empty coats, which are also in the mixture.

Assembly mixtures containing the transferrin receptor

In comparable assembly experiments with the transferrin receptor (derived from human placenta), no apparent interaction with the 100-kd/50-kd coat complexes was detected. Although the transferrin receptor alone remained largely soluble during the assembly procedure, the presence of either the 100-kd/50-kd complex or clathrin (or both together) did cause an appreciable increase in the amount of aggregated receptor. However, when a pellet from a transferrin receptor and the 100-kd/50-kd mixture was analyzed on an agarose gel both the receptor and the 100-kd/50-kd complexes ran in their normal positions and thus completely separated during electrophoresis. The pellet contained no spherical aggregates analogous to those formed with the mannose-6-phosphate receptor, when viewed in the electron microscope. When clathrin was included in the mixture, some coats were formed on dialysis against buffer A; however, when analyzed on a 10–90% ^2H_2O gradient, the coats sedimented away from all the transferrin receptor, which was left at the top of the gradient.

Discussion

These results show that the rat liver mannose-6-phosphate receptor binds to bullock brain 100-kd/50-kd coat complexes. This is demonstrated most strikingly by agarose gel electrophoresis of assembled mixtures of these proteins (Figures 2 and 3). The assembly products (Figure 1) appear to be spherical aggregates of variable diameter (300–1000 Å), which are about the right size to fit inside the normal range of clathrin cages. Indeed when clathrin is added to the assembly mixture, coats are formed which appear to have receptor incorporated within them (Figure 5). This is supported by the co-sedimentation of the labelled receptor with coats on the 10–90% ^2H_2O gradient (Figure 4).

That clathrin will assemble to form polyhedral cages around receptor bound to 100-kd/50-kd complexes suggests that in these particles the normal orientation of the 100-kd/50-kd proteins to clathrin is preserved. Thus, this type of reconstituted preparation, which contains a unique receptor, may be useful for determining how receptors and 100-kd/50-kd complexes are packed within the clathrin cage.

The particles obtained by co-assembly of the mannose-6-phosphate receptor with the 100-kd/50-kd complexes contained ~1 mol of receptor per mol of 100-kd polypeptide. If such aggregates bound 1 mol of clathrin heavy chain per 100-kd polypeptide, [saturation of the clathrin cage appears to require about three molecules of 100-kd polypeptide per clathrin trimer (Pearse and Robinson, 1984)], one would then expect equimolar amounts of receptor and clathrin to exist in a coated vesicle, as previoulsy suggested (Pearse and Robinson, 1984). Indeed, the electron micrographs of sections of coated pits in chicken oocytes taken by Perry and Gilbert (1979) do suggest that receptors are fairly densely packed on the extracellular surface of the membrane. A schematic diagram indicating how the receptors might be located within the coat is shown in Figure 6.

Although the transferrin receptor is known to enter coated pits in many cell types (Larrick and Cresswell, 1979), it did not package into reassembled coats. Under the conditions of reconstitution, the receptor (isolated from human placenta) did not appear to interact with bullock brain 100-kd/50-kd proteins, [which contain a subset of the heterogeneous family of 100-kd coat proteins (Pearse and Robinson, 1984; Robinson and Pearse, 1984)]. *In vivo*, receptors which recycle through coated pits and vesicles are already oriented in the membrane, making potential interaction with appropriate coat proteins much more favourable than in solution. Presumably, in the cell, the cytoplasmic regions of such receptors are available to interact, either directly or indirectly, with particular 100-kd proteins or 100-kd/50-kd units, thus forming foci for the attachment and assembly of clathrin.

The special ability of the mannose-6-phosphate receptor to bind to the 100-kd/50-kd proteins, and to pack inside coats, may correlate with its function in cells. This property might enable the mannose-6-phosphate receptor to participate in the formation of a discrete set of coated vesicles budding from the *cis* Golgi region, thus sequestering enzymes bound for lysosomes away from proteins with other destinations, as has been suggested (Brown and Farquhar, 1984b). In the absence of lysosomal enzymes bearing the mannose-6-phosphate recognition markers, coated pits containing the mannose-6-phosphate receptor appear to have a considerably prolonged half-life, as they seem to accumulate in the Golgi region (Brown and Farquhar, 1984b).

Materials and methods

Preparation of coat proteins and receptors

The coat proteins, clathrin and the 100-kd/50-kd complexes, were purified as described previously (Pearse and Robinson, 1984). Mannose-6-phosphate receptors (of ~215 000 mol. wt.) were isolated from Triton X-100 extracts of rat liver membranes by their ability to bind specifically to immobilized pentamannosyl-6-phosphate oligosaccharides (from yeast phosphomannan) essentially as described by Brown and Farquhar (1984a). Yeast phosphomannan (*Hansenula holstii* NRRL Y-2448) was obtained originally from Dr. M.E.Slodki, Northern Regional Research Laboratories, Peoria, IL. The affinity matrix was constructed by Dr. Colin Watts in this laboratory (MRC). A crude membrane fraction was obtained from rat livers homogenized in 40 mM Tris-HCl pH 7.4 containing 0.02% NaN_3 and 0.2 mM phenylmethylsulphonyl fluoride (PMSF). The homogenates were centrifuged for 15 min at 8 000 *g* and then crude microsomal membranes were pelleted from the extract by centrifugation at 100 000 *g* for 1 h at 5°C. The membranes were then extracted and washed and further extracted in 1% Triton X-100 as described previously (Brown and Farquhar, 1984a). The Triton X-100 extract, a clear reddish brown solution, was passed over the affinity column which was then washed with 200 ml of phosphate-buffered saline (PBS) containing 0.1% Triton X-100 and 0.02% NaN_3. Before elution of the receptor the column was washed with 50 ml of PBS containing 0.02% NaN_3 and 0.1% octyl glucoside to remove the Triton X-100. The receptor was then specifically eluted in 30–40 ml of PBS (+NaN_3) containing 1% octyl glucoside and 5 mM mannose-6-phosphate. The protein peak was detected by measurement of the absorbance of the eluted fractions at 280 nm using the elution buffer as the standard. Samples of the peak

fractions were analyzed by SDS-polyacrylamide gel electrophoresis showing that the eluted protein was a single species of ~215 000 kd, the mol. wt. of the mannose-6-phosphate receptor (Sahagian *et al.*, 1981). The yield was ~600 μg of receptor from ~40 rat livers (~20 g each). PMSF (0.5 mM) was added to the dilute receptor solution (~20 ml), which was then dialyzed overnight against 2 litres of 5 mM Tris-HCl pH 7.4. The receptor was then freeze-dried in 100 μg aliquots and kept at −20°C until used in the reconstitution experiments (up to ~2 months).

Transferrin receptor containing some transferrin was purified from extracts of human placental membranes by affinity chromatography using immobilized monoclonal antibody directed against transferrin, as described by Bleil and Bretscher (1982). The monoclonal antibody against transferrin was made by Ms.S.Green and Dr.J.D.Bleil in this laboratory (MRC) and the affinity matrix was kindly prepared by Dr.J.D.Bleil. After elution of the transferrin receptor (plus some transferrin) from the column in 0.1 M glycine, pH 10.0, 0.15 M NaCl, 1% octyl glucoside, the solution was brought to ~pH 7.0 with 0.5 M NaH_2PO_4 and adjusted to 0.5 mM PMSF. The receptor solution (~1 mg/ml) was then dialyzed against 1 M Tris-HCl pH 7.0, 1 mM EDTA, 0.02% NaN_3 and 0.1 mM PMSF to remove the octyl glucoside. After dialysis, the receptor solution was centrifuged for 1 h at 5°C at 100 000 *g* to remove any aggregated material. Aliquots of the receptor solution were then frozen rapidly and stored at −20°C.

Receptors were labelled with ^{125}I as described by Tejedor and Ballesta (1982) and dialyzed against 1 M Tris-HCl pH 7.0, 1 mM EDTA, 0.02% NaN_3 and 0.1 mM PMSF.

Strategy for reconstitution experiments

Before use in reconstitution experiments all the proteins involved (clathrin, 100-kd/50-kd complexes and receptors) were dissolved in 1 M Tris-HCl pH 7.0, 1 mM EDTA, 0.1% 2-mercaptoethanol, 0.02% NaN_3 and 0.2 mM PMSF (Buffer B, see Pearse and Robinson, 1984). The only exception was when the transferrin receptor was involved, when the 2-mercaptoethanol was omitted. All the proteins were then dialyzed against 200 volumes of Buffer B, and then centrifuged for 1 h at 5°C at 100 000 *g* to remove any aggregated material. The optical densities at 280 nm of the supernatants were measured against their respective dialysis buffers to estimate their protein concentrations.

The concentration of the 100-kd/50-kd complexes in the assembly mixtures was generally in the range 50 − 100 μg/ml. If clathrin was included it was added at ~50 μg/ml. The initial receptor concentrations used were generally in the range of 50 − 100 μg/ml as judged by SDS-polyacrylamide gel analysis of the mixtures. BSA was added to the assembly mixture at a concentration of 1 mg/ml. The assembly mixtures in buffer B (mostly 1 ml in volume) were dialyzed overnight against buffer A − reconstitution buffer [0.1 M MES-NaOH (pH 7.0), 0.2 mM EGTA, 0.5 mM $MgCl_2$, 0.02% NaN_3 and 0.1 mM PMSF]. Any particles formed were collected by centrifugation for 1 h at 5°C at 100 000 *g*. Typically pellets were resuspended in 20 μl of buffer A.

Agarose gel electrophoresis

Standard low mol. wt. agarose was purchased from Bio-Rad Laboratories. 1% gels (2 mm x 8 cm x 6 cm) were made up in 40 mM Tris acetae pH 7.5. The electrode buffer, which just submerged the whole gel, was 120 mM Tris acetate pH 7.5. Gels were run for 2 h at 50 V (~40 mA) using 100 μg horse spleen ferritin as a marker which traversed ~3 cm in this time. 10 − 15 μl samples containing ~10 μg of protein in buffer A plus glycerol (15%) and bromophenol blue (0.004%) were applied to the gel. After electrophoresis, the gels were generally stained with Sigma Brilliant blue R in 50% methanol, 7% acetic acid solution for 2 min and destained in 10% methanol, 10% acetic acid mixture. Alternatively a sample lane was excised from the gel and after soaking in SDS sample buffer, was applied to an SDS-polyacrylamide slab gel for electrophoresis in the second dimension. SDS-polyacrylamide gel electrophoresis on 7.5% gels was carried out as described previously (Laemmli, 1970; Pearse and Robinson, 1984).

Electron Microscopy

Samples were applied to carbon coated grids, negatively stained with 1% uranyl acetate (Huxley, 1963), air-dried, and examined in a Philips EM300 microscope operating at 80 kV.

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