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Clathrin and coated vesicles

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The discovery of clathrin

In 1973, I had never heard of coated vesicles. Beginning a new project, in Ieuan Harris's group in the Protein and Nucleic Acid Division, I was attempting to prepare tubulin from fresh pig brains by cycles of disassembly and reassembly. Although I conscientiously went early to the local slaughter house for the first few pigs' brains, the foreman gave me some of the previous day's supply from the cold room. As a result, my yield of tubulin was zero, but I did obtain a tiny pellet of material for observation in the electron microscope; I desperately hoped to see microtubules but found spherical objects like sliced tomatoes instead. As a biochemist, trained to purify enzymes, almost anything I saw on the screen was cause for excitement. Fortunately, colleagues took a few pictures for me. For the next few months, my tubulin preparations were more successful but I still remained intrigued by the curious objects which were always present amongst the microtubules on my electron micrographs. A 180-kd protein, as well as the 55-kd tubulin bands, appeared on SDS gels of samples when these particular structures were more concentrated. What were they?

Dennis Bray and Clarke Slater recognized them as the mysterious 'vesicles in a basket' described by Kanaseki and Kadota (1969), presumably the 'coated vesicles' implicated in membrane recycling at nerve synapses by Heuser and Reese (1973). Soon I learnt of the work of Roth and Porter on endocytosis of yolk proteins in the mosquito oocyte (1964).

When four different groups published results of the kind I was still trying to achieve with tubulin, I concluded the tubulin field was already overcrowded with biochemists and decided to work full time on the purification of coated vesicles. After all, I thought, a role in membrane recycling and endocytosis sounded interesting, even though it seemed to occur most dramatically in such unpleasant creatures as mosquitos. Mark Bretscher, in the Cell Biology Division, thought I should continue working on microtubules; however in the autumn of 1974 he departed to be a visiting professor at Harvard, giving a course of lectures on membrane structure. While he was away, I began to optimize the separation of coated vesicles by sucrose gradient centrifugation, using SDS gel electrophoresis to follow the protein and electron microscopy to assay for coated vesicles. Soon, it was clear that this strategy would be successful.

I visited Mark in Boston at Christmas. We met at the airport and on the platform of the tube train (the T) he started excitedly telling me about his new theory of lipid flow and its relation to the capping of surface antigens. I did not immediately understand what he was talking about but I had the intuition to see that coated vesicles were likely candidates to perform the necessary endocytosis. When we returned to the MRC laboratory, I concentrated on the purification of coated vesicles from several different tissues, while he developed his theory for eventual publication in Nature (Bretscher, 1976). Mark and Roger Kornberg, with whom I spent evenings discussing membrane and chromatin structures, revelling in Watergate and solving Herald Tribune crosswords, insisted that I produce really clean preparations of the protein, along with a purification table, and that I give it a name. Graeme Mitchison, whose knowledge of Greek surpassed ours, gave me the choice of three names of which I chose clathrin, meaning lattice-like (Pearse, 1975, 1976).

When Aaron Klug saw my pictures of coated vesicles, he realized that he had been sent some pictures of a few such structures years earlier by E.G.Gray, Professor of Anatomy at University College London (UCL), but those images had been too limited in number and too heterogeneous to study. Now using purified preparations, Tony Crowther, John Finch and I were able to obtain tilt series of fields of particles from which to identify the geometry of the clathrin shells by computed model building (Crowther *et al.*, 1976). When revisiting UCL (where I received my B.Sc. and Ph.D. degrees in the Biochemistry Department) I called on Professor Gray to tell him of my new results. Sadly, although having earlier described coated vesicles in nerve synapses (Gray and Willis, 1970), he had become convinced that they were fixation artifacts by the time of my visit. Curiously a similar sort of experience befell Mike Brown and Joe Goldstein. When querying the nature of the coated pits where low density lipoprotein (LDL) appeared to cluster, in Richard Anderson's micrographs, they too were told by experts that these were artifacts of fixation. By chance, the two manuscripts—those of Anderson *et al.* (1976) on the localization of LDL in coated pits and my paper on the purification of clathrin coated vesicles—arrived in the same household at the same time to be refereed by George Palade and Marilyn Farquhar. They, at least, had no doubt that the coated vesicles in their own electron micrographs were important structures.

Thus began a new focus of interest in endocytosis and the recycling of membrane components in cells and their consequences for maintenance of membrane specificity, the 'sorting' problem, capping and cell locomotion.

Ins and outs of coated vesicle function

As cell biologists began to search for coated pits in their electron micrographs, so the list of ligands, bound to specific receptors and observed to endocytose in coated vesicles, grew. Examples include epidermal growth factor (Gordon *et al.*, 1978), insulin (Maxfield *et al.*, 1978) asialoglycoproteins (Wall *et al.*, 1980) and transferrin (Bleil and Bretscher, 1982). Viruses too were shown to use the system and thus gain entry to subvert the cell (Helenius *et al.*, 1980).

The rate of uptake of the cell surface is enormous: estimates of this from the endocytosis of LDL (Anderson *et al.*, 1977a) or viruses (Marsh and Helenius, 1980) indicate that many cells take up the equivalent of one cell surface once every 30 min or so

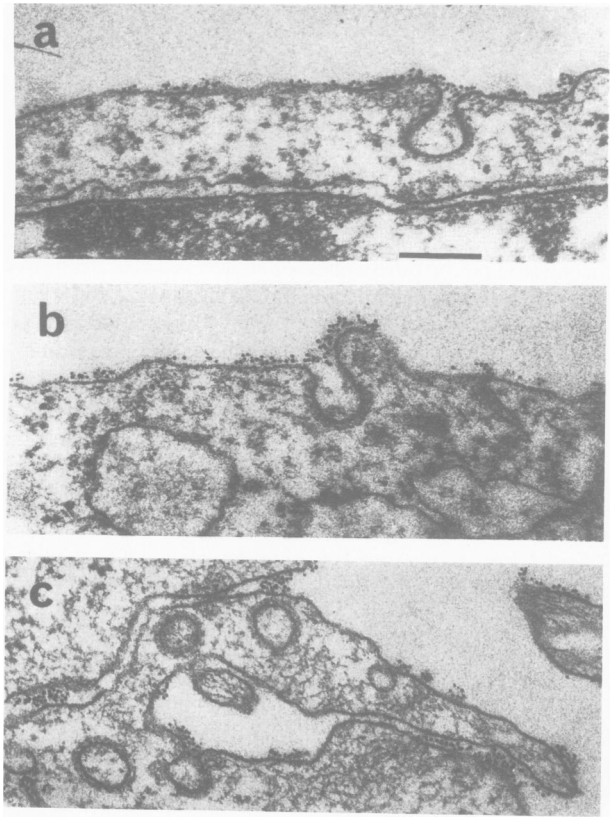

Fig. 1. Examples of coated pits on cells decorated with ferritin, at 0°C, to locate Thy 1 by immunocytochemistry. Ferritins are excluded from the coated pits. (Bar is 0.2 μm). Taken from Bretscher *et al.* (1980).

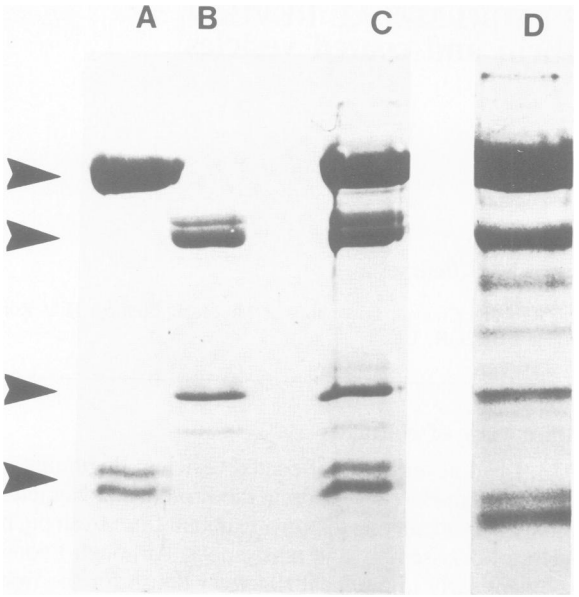

Fig. 2. Coated vesicle proteins analysed by SDS–PAGE (**A**) Clathrin cages reassembled from purified bovine brain heavy and light chains. (**B**) Purified 100-kd and 50-kd coat accessory proteins from bovine brain. (**C**) Coats reassembled from clathrin and an excess of accessory proteins. (**D**) Coated vesicle preparation containing receptors and their ligands, isolated from human placenta. The positions of the clathrin heavy chains, the accessory proteins and the clathrin light chains are marked by arrows (from Vigers *et al.*, 1986b).

and that coated vesicle uptake is the main route of fluid phase endocytosis.

Anderson *et al.* (1977b) characterized the JD mutant of the LDL receptor. This mutant receptor fails to become concentrated in coated pits as effectively as the normal receptor, leading to a reduced uptake of LDL into cells carrying this lesion. To get a quantitative estimate of the selectivity of the forming coated pit, Mark Bretscher, Nichol Thomson and I (Bretscher *et al.*, 1980) decided to look at non-recycling surface proteins. We found that resident plasma membrane proteins are efficiently excluded from coated pits. Thus, the concentration of Thy1 in coated pits is about 1% (or less) of that along the rest of the plasma membrane (Figure 1). It would be interesting to know whether the JD mutant of the LDL receptor is excluded from coated pits and if it is, how efficiently.

The selectivity of coated pits is seen in two ways: the LDL receptor concentration in them is about 100-fold higher than in the surrounding plasma membrane, whereas resident plasma membrane proteins are excluded from them. Coated pits thus act as molecular filters for transferring a subset of proteins from one membrane compartment to another.

Since coated pits seemed to have such desirable properties in selecting—and rejecting—proteins for intermembrane transfer, it seemed likely that all selective membrane transport would be initiated by coated pits (Pearse and Bretscher, 1981). Amongst the enthusiasts, Jim Rothman and Richard Fine examined the role of coated vesicles in the transport of newly synthesised proteins. Their results on the intracellular transport of newly synthesised G protein of vesicular stomatitis virus in infected cells indicated that this protein is found in coated vesicles en route from the endoplasmic reticulum to the Golgi apparatus, and later in a second transport step from there to the plasma membrane (Rothman and Fine, 1980). Rothman and his colleagues pursued this line of investigation by developing an innovative and intricate *in vitro* system to study the transport of newly synthesised proteins (Fries and Rothman, 1980). However, at present, the contention that coated vesicles mediate these transport steps remains contentious (Rothman 1986), and indeed the precise roles of intracellular coated vesicles have still to be determined. The best defined intracellular route is that of the mannose-6-phosphate receptor, which is involved in targeting lysosomal enzymes to lysosomes (Farquhar, 1985; Brown *et al.*, 1986). This receptor is sequestered into coated vesicles in the Golgi region, and is recycled on an itinerary which is being elucidated. In some cells, a proportion of it reaches the plasma membrane, where it is clearly localized in coated pits and is endocytosed along with other receptors. The problems encountered in determining the selectivity of coated pits on the plasma membrane are severe enough. The equivalent measurement on an internal membrane is much more difficult and has yet to be achieved.

Such was the excitement over coated pits in the late 1970s that, of five Roumanian cell biologists visiting the USA in one year, three went to separate laboratories and were encouraged to make antibodies against clathrin. Of course such antibodies were used to localize clathrin by immunofluorescence but one less welcome result was broadcast. Having abandoned their novel idea that receptors were cross-linked into coated pits by transglutaminase (Davies *et al.*, 1980), Willingham and Pastan then came up with another bizarre suggestion. Injection of anti-clathrin antibodies into cells had apparently failed to inhibit endocytosis (Wehland *et al.*, 1981). As is well known, images of coated pits with extended necks are frequently seen but those of discrete coated vesicles are rare, presumably because the life-time of a coated

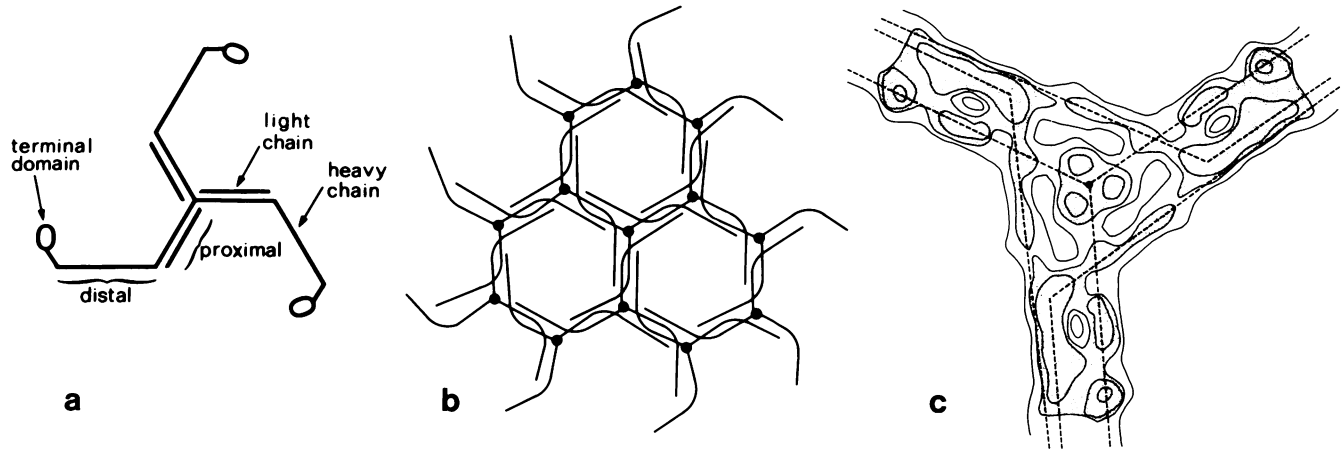

Fig. 3. (a) Schematic drawing showing the modular structure of the triskelion. (b) Packing diagram showing how triskelions form a hexagonal lattice. This packing leads to triangular vertices and a skewed arrangement of polygonal holes, as observed in negatively stained specimens (Crowther and Pearse, 1981). Formation of closed cages requires pentagons, which implies a limited degree of flexibility in the triskelion. For simplicity the terminal domains, which pack under the vertices, have been omitted. (c) Three-fold rotationally filtered image of a triangular vertex in a small fragment of cage in negative stain. (Taken from Crowther and Pearse, 1981).

vesicle—once pinched off—is short. Both observations are valid, but they interpreted the lack of action of injected antibodies to mean that coated pits never pinch off and therefore that coated vesicles do not exist. Instead, ligands bound to receptors were imagined to slide out of the coated regions into uncoated blebs, which were then endocytosed. How the specificity of uptake could be maintained in this model was never clearly explained but the existence of the model led to the tiresome post-seminar question of whether coated vesicles really exist. More recent work on the properties of antibodies raised against clathrin has proved illuminating (Blank and Brodsky, 1986). An antiserum raised against clathrin baskets may contain many antibodies, particularly against clathrin light chains, which do not inhibit clathrin assembly and disassembly *in vitro*. However, certain monoclonal antibodies against the poorly antigenic 180-kd heavy chain, which do inhibit assembly of clathrin *in vitro*, also inhibit endocytosis in cells when introduced at high concentration (Doxsey *et al.*, 1986).

Structure of coated vesicles

While discussion was generated over the function of coated vesicles, I, and others, made progress in purifying and reassembling clathrin, and in identifying further components of coated vesicle preparations, including accessory coat proteins (Figure 2; Pearse, 1978; Woodward and Roth, 1978; Schook *et al.*, 1979; Keen *et al.*, 1979), receptors and contents (Pearse, 1982). Visually dramatic were John Heuser's rapid freeze, deep etch pictures showing coated pits on the cytoplasmic surface of fibroblasts (Heuser, 1980). Ernst Ungewickell, in Dan Branton's laboratory (Ungewickell and Branton, 1981), revealed the extraordinary design of the clathrin triskelion by rotary shadowing and demonstrated the self-assembly of triskelions into cages. Intrigued, as were others (Kirchhausen and Harrison, 1981), Tony Crowther and I set about determining the packing arrangement of these extended molecules to form lattices of hexagons and pentagons. The key to our success was the ability to form fragments of the cage structure trapped on the surface of carbon coated electron microscope grids. This allowed us to see sufficient detail at the vertices to favour a particular packing model (Figure 3a,b and c; Crowther and Pearse, 1981). However, a new feature of the arrangement of triskelions in the structure was resolved only recently. Guy Vigers, Tony Crowther and I have exploited the new technique of imaging samples in vitreous ice in the electron microscope. This technique has the dual advantages that the aqueous environment preserves and supports the structure while the ice is transparent so that details of the interior of a hollow structure can be seen. Guy obtained several tilt series of individual clathrin cages in order to determine their structure. Our three dimensional maps of complete and trypsinized clathrin cages (Vigers *et al.*, 1986a) reveal that the terminal domains of the triskelion turn inwards under the vertices and form a second shell in the interior of the cage (Figure 4). Fortunately, with Margaret Robinson, I had recently achieved the purification of the accessory 100-kd and 50-kd coat proteins. These polypeptides co-assemble in stoichiometric amounts with clathrin (Zaremba and Keen, 1983; Pearse and Robinson, 1984; Figure 2). Thus we were also able to generate a map of the complete reconstituted coat structure composed of clathrin plus 100 kd/50 kd proteins, (Figure 5; Vigers *et al.*, 1986b). The crucial set of images was obtained just as water started to trickle down the microscope, which was then out of action for several months.

The coat consists of three nested shells corresponding to the outer polyhedral cage of clathrin, the inner layer of clathrin terminal domains and the innermost shell of 100 kd/50 kd proteins. These latter proteins presumably interact directly with receptors spanning the membrane of the vesicle. Plots of radial density functions of projected images of coated vesicles show three characteristic peaks corresponding to the structural layers of the coat plus a fourth larger peak in the region of the membrane. This suggests that receptors and their ligands are fairly densely packed in the vesicle.

I have been able to show a direct interaction between a receptor and the 100 kd/50 kd accessory coat proteins (Pearse, 1985). *In vitro*, the mannose-6-phosphate receptor assembles with coat proteins to form reconstituted coats with receptor molecules inside. In the absence of clathrin and in solution, the receptor interacts with the 100 kd/50 kd proteins with a k_d of $\sim 2 \times 10^{-7}$ M to form spherical aggregates. These complexes migrate as a discrete band during agarose gel electrophoresis and contain up to about one 215-kd molecule of receptor to one molecule of 100-kd polypeptide (Figure 6).

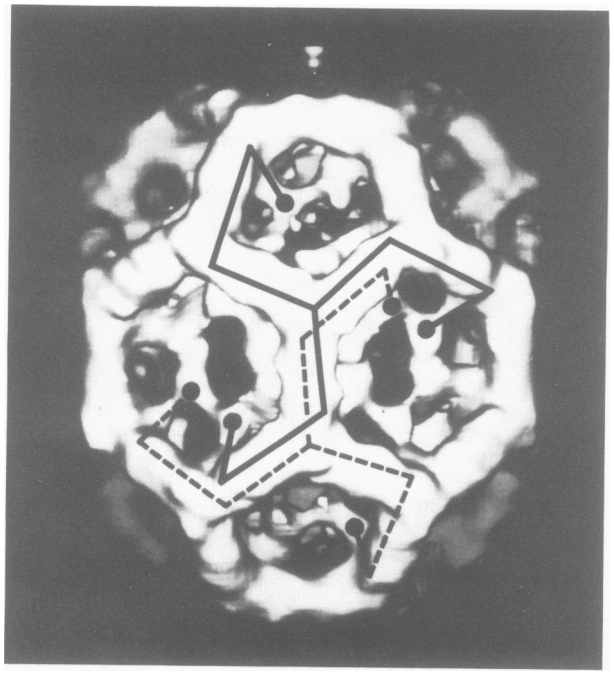

Fig. 4. Three dimensional map of a clathrin cage, computed from electron micrographs of unstained specimens embedded in vitreous ice (Vigers *et al.*, 1986a). The map shows two shells of density: the outer polyhedral lattice of the hexagonal barrel and an inner layer corresponding to the terminal domains of the clathrin heavy chains. Two triskelions have been superimposed on the map to indicate their arrangement in the structure.

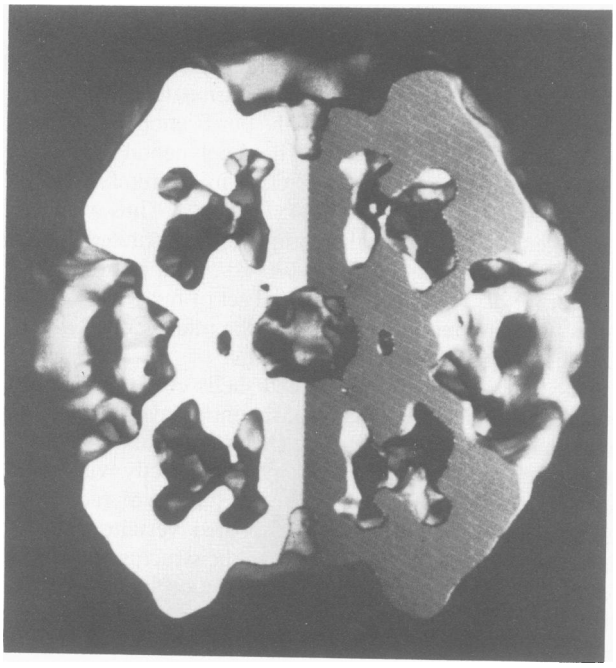

Fig. 5. Three-dimensional map of a clathrin coat, generated like that in Figure 4 (Vigers *et al.*, 1986b). A segment has been cut away to reveal the internal structure of the coat. In addition to the two outer shells formed by the clathrin (c.f. Figure 4), there is a third central shell due to the 100 kd/50 kd accessory proteins. The contacts between the clathrin and the accessory proteins appear to be made by the shell of terminal domains. The hexagonal barrel structure is too small to contain a vesicle but in a larger coat the vesicle would be enclosed within the shell of accessory proteins.

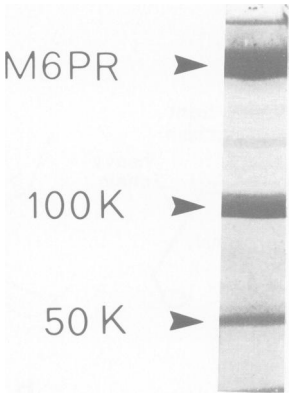

Fig. 6. Complex of mannose-6-phosphate receptor and 100 kd/50 kd coat proteins, first identified by agarose gel electrophoresis and further analysed by SDS–PAGE. The receptor and the 100-kd polypeptides are in about a 1:1 ratio in the complex.

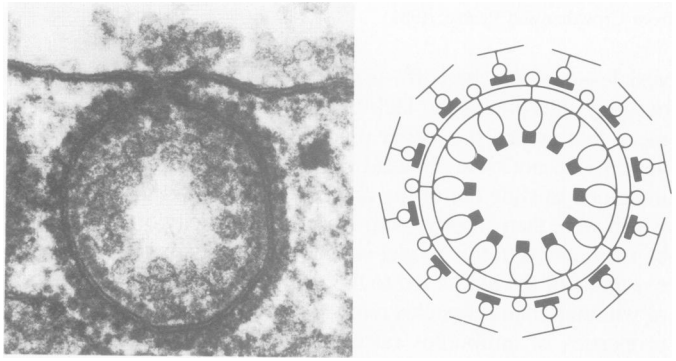

Fig. 7. (**left**) Coated pit caught in the act of budding. The micrograph is of sectioned material, taken by Perry and Gilbert (1979) showing the uptake of yolk proteins during oocyte development in the chicken. (**right**) Schematic section through a coated vesicle, showing proposed arrangement of coat components, receptors and their ligands. For simplicity, an array of identical receptors is shown, but in reality very many different receptors would be present.

If this ratio of receptors to coat proteins occurs in a coated vesicle, as seems likely, the receptors would be essentially close-packed on the inner surface of the vesicle (Figure 7). Thus other membrane proteins, with no affinity for coat proteins, would be effectively excluded as the ordered structural array of the coated pit assembles (Pearse and Bretscher, 1981; Pearse and Crowther, 1987).

Interestingly, certain members of the heterogeneous 100-kd family of coat proteins seem to be restricted to coated pits on particular membranes in the cell. An antiserum against a mixture of 100-kd coat polypeptides lights up an identical array of coated structures to those seen by an anti-clathrin antibody on immunofluorescent staining of fibroblasts (Robinson and Pearse, 1986). However, a monoclonal antibody, which recognizes a single 100-kd species in fibroblasts, stains coated pits exclusively in the plasma membrane region of the cell and not in the Golgi region (Robinson, 1987). This implies that different groups of receptors in the separate membranes of the cell are able to recruit preferentially different members of the 100-kd coat protein family. The challenge now is to distinguish the different subsets of

a cell's coated vesicles which bud from different membranes, assess their distinctive contents and identify the particular destinations of those contents.

How do coated pits work?

Recycling receptors, diffusing in the plane of the membrane, are likely to accrete at the edges of a forming coated pit; in the process more coat proteins are added so that the whole assembly grows in concert. Once incorporated into the ordered assembly, a receptor would be unable to diffuse away again. The efficiency of assembly of a receptor in a coated pit may be altered by mutation of its cytoplasmic portion. Thus deletion of the cytoplasmic portion of a normally endocytosed receptor reduces significantly the efficiency of its uptake. In the LDL receptor the retention of the first 22 amino acids of the 50 amino acid long cytoplasmic domain (residues 790–811) will suffice for rapid internalization of the receptor and the tyrosine at position 807 is especially critical (Davis *et al.*, 1987). It was substitution of this tyrosine for a cysteine residue that caused the JD mutation, resulting in a case of Familial Hypercholesterolaemia (Brown and Goldstein, 1976). However, the precise features which enable other receptors to enter coated pits are as yet unidentified, even though the primary structures of several are known. As their cytoplasmic portions are very varied in size, primary sequence and whether they represent the C-terminal or the N-terminal end of the protein, it would not be surprising if they therefore had different affinities for coat proteins. This leads to the natural conclusion that, as recycling receptors compete for sites at the edges of forming coated pits, the efficiency with which they become assembled will vary from receptor to receptor.

The picture, however, is more complicated. Receptors, like the mannose-6-phosphate receptor are concentrated in coated pits in the *trans*-Golgi network, yet newly synthesised plasma membrane receptors which are in transit there are presumably excluded. The mannose-6-phosphate receptor must therefore contain a distinct recognition site, perhaps for a specialized Golgi 100-kd coat protein, as well as a site enabling it to enter plasma membrane coated pits.

As the coat proteins have an innate tendency to form closed polyhedral shells, the assembly of the coat will naturally deform the membrane inwards making it bud into the cytoplasm. However, it seems probable that the actual pinching off of the membrane requires another discrete step. Once separated from the parent membrane, the coated vesicles appear to lose their coats rapidly. Although a putative uncoating ATPase which disassembles clathrin cages *in vitro* has been described (Schlossman *et al.*, 1984), what triggers uncoating in the cell is unclear. After all, the uncoating process has to be specific for coated vesicles, and not act on coated pits. Once released, the coat proteins are available for another round of coated pit formation.

What happens in a eukaryotic cell that has malfunctioning coated pits?

The removal of the clathrin gene from yeast cells has been performed. In some strains, one hears, this has resulted in the certifiable death of the yeast. However, a viable mutant that lacks clathrin has been constructed, though it grows slowly and fills with autophagic vacuoles characteristic of sick cells (Payne and Schekman, 1985). Unfortunately, the role of coated vesicles in yeast is not yet clear.

From what we know, animal cells lacking functional coated pits would be denied rapid selective uptake of nutrients. In the absence of membrane recycling, no forward locomotion could occur. The formation of specialized compartments such as lysosomes and storage vacuoles would be seriously disorganized and much of the newly synthesized products would be secreted, instead of efficiently packaged. Without nutrients, the cells would grow slowly, sicken and die.

Conclusions

We now have a cell biologist's LEGO model of how the structural and functional domains of the constituent proteins are assembled in the coated vesicle. Membrane proteins of a particular cell compartment can be divided into two classes: those that can and those that cannot participate in this densely packed structure. Those that have the necessary features are included in the coated pit and transferred to another membrane. Those that do not are left behind. Thus efficient, selective transfer of molecules can occur without intermixing the characteristic components of cellular compartments.

I have been lucky to find such delightful proteins to work with and very fortunate in the numerous colleagues who have contributed so much to coated vesicle study, especially Mark Bretscher, Tony Crowther, Margaret Robinson and Guy Vigers.

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