Clathrin-coated vesicle formation: a paradigm for coated-vesicle formation

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Abstract

Clathrin-coated pits are the major ports of entry into the cell and are responsible for the internalization of a variety of biologically important macromolecules. These transport intermediates form as a result of the co-ordinated assembly of a number of cytosolic proteins on to the membrane which results in specific cargo recruitment. We have used a variety of approaches including permeabilized cell assays and light and electron microscopy to identify and characterize the proteins and enzymes involved in coated vesicle formation.

Material is constantly being moved into and out of cells and also transported between the organelles within cells. For example, newly synthesized membrane proteins are translocated into the endoplasmic reticulum and move through the Golgi complex to the cell surface. They may be retained at specific locations within the secretory pathway, delivered to the cell surface or targeted to other organelles such as endosomes or lysosomes. Similarly, macromolecules from the extracellular environment required by the cell are taken up by endocytosis. This movement of material requires that there is a cellular sorting machinery which ensures that transport is vectorial and efficient. In general, intracellular transport of molecules occurs by packaging material into small transport vesicles that bud from a donor membrane and are targeted to a specific acceptor membrane where the vesicles fuse, delivering their cargo. A variety of membrane and cytosolic components control the budding, targeting and fusion of these transport vesicles ensuring that the fidelity of transport is maintained.

Endocytosis is the fundamental cellular process by which all eukaryotic cells internalize material from the extracellular medium. Material is included in endocytic vesicles that bud and pinch off from the plasma membrane. The content of the endocytic vesicle is delivered to an endosomal compartment where it is sorted and then either recycled to the cell surface, transported across the cell or targeted for degradation in lysosomes [1].

The initial internalization step may occur by several different mechanisms in mammalian cells, the best-characterized of which is via clathrin-coated pits, which are responsible for the uptake of the bulk of material [2]. Clathrin-coated pits are specialized structures that form at the plasma membrane with a striking lattice morphology that results from the polymerization of clathrin from the cytosol on to the membrane [2]. Within the clathrin lattice, AP2, a clathrin-binding adaptor complex, links transmembrane receptors destined for cellular uptake to the clathrin lattice by association with internalization motifs within the cytoplasmic tails of these receptors [3–5]. A high-molecular-mass GTPase, dynamin, is also required for coated-pit budding [6]. In addition to clathrin, AP2 and dynamin, a host of other molecules are implicated in clathrin-coated vesicle formation. These are principally binding partners for clathrin, AP2 and/or dynamin and include eps15, epsin, amphiphysin, intersectin, endophilin and rab5 [2]. Among the key issues in our understanding of the regulation of coated vesicle formation is the question of the hierarchy by which components are recruited to the coated pit and how these components interact to effect cargo recruitment, invagination and scission.

A number of years ago we established assays in permeabilized cells that reconstituted the de novo formation of clathrin-coated pits, their invagination and scission to form coated vesicles [7–9]. We use transferrin that has been biotinylated via a cleavable disulphide linkage (B-SS-Tfn) as a reporter molecule. This is incubated with permeabilized cells and other cytosolic components under conditions where endocytosis can occur. The sequestration of B-SS-Tfn into deeply invaginated coated pits and its internalization into coated vesicles is measured by a loss in accessibility of B-SS-Tfn to exogenously added avidin while the internalization of B-SS-Tfn into coated vesicles only is measured by the resistance of B-SS-Tfn to reduction by the small membrane-impermeant reducing agent, MesNa. Both the acquisition of avidin accessibility and MesNa resistance require cytosol. AP2 is limiting in bovine brain cytosol and so addition of purified AP2 stimulates the sequestration of B-SS-Tfn into newly formed clathrin-coated pits [10].

When the assays were originally set up, clathrin and AP2 were the only characterized cytosolic components required for coated-vesicle formation and so the initial aim was to use the assays to identify novel cytosolic components and ultimately to carry out characterization of the
enzymes and proteins involved in the formation of this transport intermediate. Electron-microscopy studies indicated that the transport intermediates in permeabilized cells reflect physiological intermediates observed in intact cells. Using these assays, we identified rab5GDI (Guanine nucleotide Dissociation Inhibitor) as an essential component required for B-SS-Tln sequestration into clathrin-coated pits in vitro and by quantitative electron microscopy we were able to show that rab5 is required after new coated pits form [10]. This suggested either that rab5 might have a role in cargo recruitment into coated pits, or in their invagination. Given that rab5 has a very well-established role in fusion, a third possibility was that the requirement for rab5 might reflect a role in quality control, i.e. that a functional transport vesicle would not bud off until it had ensured that it had recruited all of the components that it would require for subsequent fusion. It is important to note that we do not consider these possibilities to be mutually exclusive, particularly since rab5 in its GTP conformation has been shown to interact with greater than 20 cytosolic proteins [11]. Characterization of these proteins indicates that they are bona fide effectors of rab5 with defined functions on the endocytic pathway, including endosome recruitment and motility [12]. It is therefore a reasonable prediction that rab5 may interact with specific effectors that are crucial to its role at the plasma membrane. We have, however, investigated the role of rab5 in the invagination of coated pits in permeabilized cells using quantitative electron microscopy and our results to date indicate that rab5 does not appear to have a significant role in this process (A. Osborne, E. Eskelien and E. Smythe, unpublished work). This suggests that rab5 is more likely to have a role in cargo recruitment and there are precedents for this in the formation of other transport vesicles. For example, rab9 is involved in the transport of material from the late endosome to the trans-Golgi network and it forms a ternary complex with the mannose-6-phosphate receptor (a cargo molecule) and TIP47, a characterized effector of rab9 [13]. Furthermore, studies in Saccharomyces cerevisiae have implicated Ypt1p (the yeast homologue of rab1) in the selection of glycosylphosphatidylinositol-linked cargo into vesicles that bud from the endoplasmic reticulum [14] (see also [15]). Our current studies are aimed at exploring the connection between rab5 and cargo recruitment.

Apart from allowing the identification of novel components required for coated vesicle formation, our assay systems allow us also to look at the regulation and temporal requirements for coated vesicle formation. In particular we have studied how phosphorylation of AP2 regulates coated vesicle formation. It had been known for many years that AP2 is phosphorylated both in vivo and in vitro but the available assays did not point to an obvious physiological role for these post-translational modifications. We therefore decided to address what would be the effect of interfering with any of these phosphorylation events, using our functional assays as a read-out. Specifically, we focused on the phosphorylation of the medium µ2 subunit of AP2 because, when AP2 is incubated with [32P]ATP, almost all of the 32P that is incorporated into protein is found in µ2. To interfere with the phosphorylation of µ2 we made use of fluorosulphonylbenzoyl adenosine (FSBA), an ATP analogue that binds irreversibly to ATPases and kinases. Treatment of AP2 with FSBA results in a marked (>90%) inhibition of µ2 phosphorylation as a result of inactivation of a kinase that co-purifies with AP2. Addition of FSBA-treated AP2 to the permeabilized cell assay also strongly inhibited AP2-dependent stimulation. As expected, if the FSBA treatment was carried out in the presence of an excess of ATP, AP2 was protected from inactivation but, strikingly, if the AP2 was first phosphorylated and then treated with FSBA, it was completely protected from inactivation. This indicated that phosphorylation of µ2 is essential for clathrin-coated-pit function in vitro. By transfection of HEK293 cells with mutant forms of µ2 where Thr-156 (the site phosphorylated on µ2) was changed to an Ala residue, we also demonstrated that µ2 phosphorylation was essential in vivo [16].

Coated vesicle formation is a constitutive process that involves continuous cycling of the coat proteins from the cytosol to the membrane. Reversible phosphorylation has therefore long been a plausible and attractive mechanism for how this might be regulated [17]. Our aim is to understand where phosphorylation is occurring and precisely what it is doing. From our permeabilized cell assay we know that phosphorylation of µ2 is required after the assembly of a new coated pit and the formation of a deeply invaginated pit (Figure 1). The most likely role for µ2 phosphorylation is in cargo recruitment since the µ2 subunit has been shown to interact with tyrosine-containing internalization motifs in the cytoplasmic tails of receptors such as the transferrin receptor [3–5]. The affinity of phosphorylated µ2 for peptides containing these motifs has been shown to be much greater than that of unphosphorylated µ2 [18]. Most recently, the structure of the entire complex of AP2 (lacking only the appendage domains of the large α- and β-2-adaptin subunits of the AP2 complex) has been solved at atomic resolution [19]. What is striking about the structure is that it appears that the binding site for cargo on µ2 is blocked by β2-adaptin, suggesting that the AP2 complex needs to undergo a substantial conformational change to allow cargo access to µ2. Intriguingly, the phosphorylation site on µ2 (Thr-156) is located on an exposed area of the µ2 molecule where it is ideally placed to induce a major conformational change [19, 20]. However, proof of this model will require crystallization of the phosphorylated AP2 complex.

To date there is little information about where and when dephosphorylation occurs during the coated pit cycle, although it has been suggested that µ2 needs to undergo cycles of phosphorylation before a deeply invaginated coated pit is formed [21]. One possibility is that dephosphorylation occurs after coated-vesicle scission. At this stage, the vesicle needs to shed its bulky coat to facilitate subsequent fusion with the endosome and hence it might be beneficial to minimize interactions with cytosolic components such as that between AP2 and transmembrane cargo.
Figure 1 | The role of phosphorylation of the \( \mu_2 \) subunit of AP2 in clathrin-coated-vesicle formation

Phosphorylation of \( \mu_2 \) is required between the assembly of a new coated pit and the formation of a deeply invaginated pit. The most likely role for this phosphorylation is in cargo recruitment. Currently, there is no clear evidence to indicate where dephosphorylation occurs.

Our studies on rab5- and AP2-dependent endocytosis indicate that the permeabilized cell system allows us to determine the temporal order of events in coated vesicle formation and we have also used this system to examine the role of the high-molecular-mass GTPase, dynamin, in clathrin-coated vesicle formation [22]. Specifically, we have been able to show that dynamin, in addition to its well-established role in the pinching off of coated vesicles, has a role in the invagination process. Our data are most consistent with dynamin acting as a ratchet, as has been suggested by yeast two-hybrid analysis and also by the three-dimensional electron microscopic reconstruction of dynamin [23]. Endophilin is a binding partner of dynamin which appears to promote curvature of coated pits and also to be involved in invagination and scission [24,25]. Based on inhibition and rescue studies in the permeabilized-cell system we have proposed that, after scission, endophilin undergoes a conformational change, allowing it to activate the inositol-5'-phosphatease synaptojanin, whose major \textit{in vivo} substrate is PtdIns(4,5)P_2. It is believed that reduction in PtdIns(4,5)P_2 levels may facilitate the uncoating process [26] (much as proposed for the dephosphorylation of \( \mu_2 \) described above), as many coat proteins are recruited to the membrane as a result of interactions with PtdIns(4,5)P_2 [27]. A similar model has been proposed based on studies complementary to ours, using peptide injection into the lamprey synapse [28].

Our studies to date have provided insight into some of the temporal requirements for coated vesicle formation and in the future we hope to extend this work to understand further the spatial and temporal control of coated pit assembly. We believe that this information will be important not only in our understanding of the endocytic pathway but may also serve as a paradigm for the formation of other transport vesicles. There is increasing evidence to suggest that, although the individual molecules involved may be different, there are strong mechanistic similarities in the way in which transport vesicles are formed on other intracellular trafficking pathways.

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References

The Behaviour of Enzymes in Cells


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