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Snap-shots of clathrin-mediated endocytosis

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Clathrin-mediated endocytosis is one of the major entry routes into a eukaryotic cell. It is driven by protein components that aid the selection of cargo and provide the mechanical force needed to both deform the plasma membrane and detach a vesicle. Clathrin-coated vesicles were first observed by electron microscopy in the early 1960s. In subsequent years, many of the characteristic intermediates generated during vesicle formation have been trapped and observed. A variety of electron microscopy techniques, from the analysis of sections through cells to the study of endocytic intermediates formed *in vitro*, have led to the proposition of a sequence of events and of roles for different proteins during vesicle formation. In this article, these techniques and the insights gained are reviewed, and their role in providing snap-shots of the stages of endocytosis in atomic detail is discussed.

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Endocytosis is the process of vesicle formation from the plasma membrane. It has many functions, including bringing nutrients into the cell, regulating the number of signalling receptors on the cell surface and recycling synaptic vesicles at nerve terminals. The endocytic machinery can also be hijacked by pathogens for entry into the cell. Snap-shots of the engulfment of a variety of substrates, including yolk proteins [1], low density lipoproteins [2] and influenza virus [3], have been taken using the electron microscope.

The clathrin-mediated pathway (Box 1) is one of the most commonly used, and the most intensively studied, mechanisms of endocytosis. Clathrin-coated pits and vesicles were first observed by electron microscopy. The characteristic 'bristle-like' appearance of the coat in cross sections of forming vesicles, or the presence of lattices of hexagons and pentagons on the surface of the plasma membrane, makes them easily discernible (Fig. 1). Therefore, although dynamic changes occurring during endocytosis can be studied using fluorescent labelling and confocal microscopy, detailed visualization of endocytic processes is greatly aided by use of the electron microscope. In this review, we will examine how electron microscopy has given insight into the different stages of clathrin-mediated endocytosis and the proteins involved in these stages. We will review the techniques available and look forward to the future when we hope that a combination of three-dimensional reconstruction of electron microscope images, and the docking of atomic structures into these models, will reveal 'snap-shots' of the different stages of endocytosis in atomic detail.

Looking into the cell

Several invasive approaches have been used to study the formation of coated vesicles within a cell. By attaching fibroblasts to coverslips, breaking open the cells, freeze-drying and generating a carbon-platinum replica of the exposed cytoplasmic surface (reviewed in Ref. [4]), highly ordered polygonal arrays of clathrin can be seen by electron microscopy [5] (Fig. 1a). These arrays can be removed from the membrane surface with alkali, and reassembled from the components of purified clathrin-coated vesicles [6].

An alternative approach is to fix a cell with formaldehyde, embed it in resin and take sections through the resin block. In negatively stained thin-sections through a nerve terminal, different intermediates of endocytosis can be seen.

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Box 1. Clathrin-mediated endocytosis

Endocytosis is the process of vesicle budding from the plasma membrane. It is one way in which materials can enter the cell from the extracellular environment. The many functions of endocytosis include nutrient uptake, regulation of the number of signalling receptors at the cell surface and recycling of synaptic vesicles. Clathrin-mediated endocytosis (Fig. I) begins when cytoplasmic factors associate with the plasma membrane.

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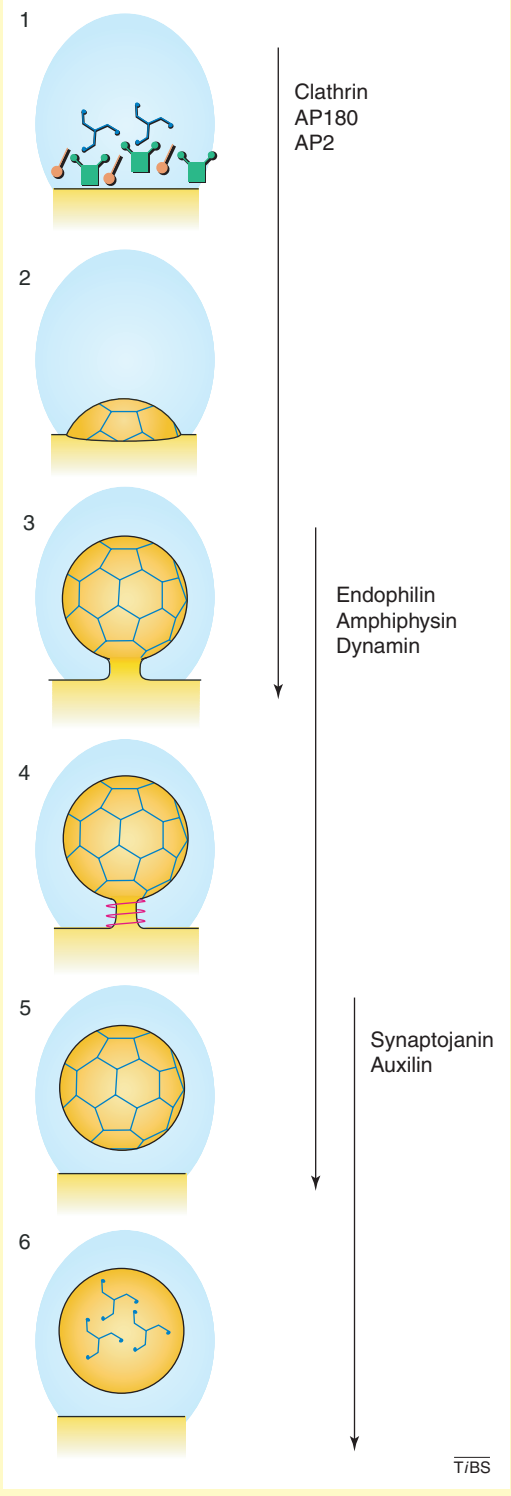

Fig. I. Clathrin-mediated endocytosis. AP180 [a] (red) interacts with membrane phospholipids. The AP2 adaptor complex (green) binds both to lipids [b] and to the intracellular parts of membrane proteins that are destined for internalization [c]. Both AP180 and AP2 interact with the clathrin that constitutes the vesicle coat [d] (blue). A clathrin lattice assembles on the surface of the membrane (1). The natural curvature of this lattice leads to the formation of invaginated structures known as coated pits (2). Through the action of factors that include endophilin [e], amphiphysin [f] and dynamin [g], these coated pits become further deformed, resulting in a vesicle that is attached to the plasma membrane by a narrow neck (3). A helical array of dynamin and amphiphysin forms at the vesicle neck [f] (4) and a GTP-dependent conformational change in this apparatus causes scission and release of the vesicle into the cytoplasm [g–i] (5). The clathrin lattice is removed by the action of factors that include synaptojanin [j] and auxilin [k] (6) and the uncoated vesicle is targeted to its final destination, where it fuses with an acceptor membrane and releases its cargo.

This approach has been used to great effect with the giant reticulospinal axon of the lamprey (reviewed in Ref. [7]). When these cells are depleted of Ca^{2+}, endocytosis is inhibited. At progressive time points after the reintroduction of Ca^{2+}, different relative proportions of endocytic intermediates are observed and the order in which these intermediates form can be inferred [8] (Fig. 1b). Shallow coated pits predominate soon after endocytosis is initiated with Ca^{2+} whereas, at later times, the proportions of invaginated pits and pits with narrow necks increase.

The analysis of thin sections has proved particularly effective when endocytosis has been frozen by the expression of dominant negative

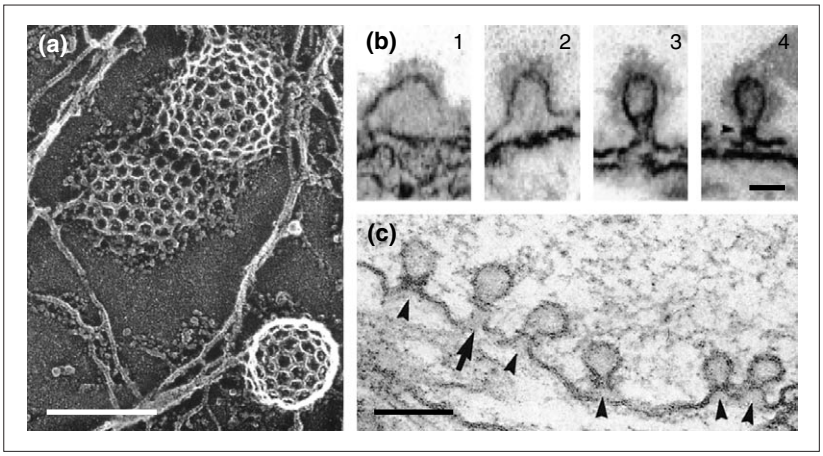

Fig. 1. The formation of clathrin-coated vesicles. (a) Different stages in clathrin coat formation have been captured in a carbon platinum replica of the inner surface of a chick fibroblast. Scale bar = 200 nm. (b) Stages in clathrin-coated vesicle formation can also be observed in sections through a fixed, stained giant axon. Scale bar = 100 nm. (c) In the nerve terminal of *Drosophila melanogaster* with the *shibire* mutant of dynamin, invaginated coated pits with narrow necks accumulate under temperatures in which dynamin is not functional. Scale bar = 100 nm. Figure is reproduced, with permission, from Refs [7], [9] (copyright of the Society for Neuroscience) and [44] (copyright of The Rockefeller University Press).

proteins that interfere at various stages of the process. Analysis of intermediates that accumulate in a blocked terminal reveals the stage at which the inhibited protein operates. An early example of this was seen in nerve terminals from flies with the temperature sensitive *shibire* mutation of dynamin [9]. Under conditions in which dynamin is not functional, collared pits with narrow necks accumulate in *shibire* nerve terminals [9] (Fig. 1c). This led to the suggestion that dynamin is involved in scission of the neck of a coated bud and in vesicle release. A similar strategy involves the generation of mice with mutations in the gene for an endocytic protein and examination of the intermediates that accumulate in the nerve terminals of these knockout animals. For example, clathrin-coated vesicles accumulated in the nerve terminals of synaptojanin-1 deficient mice [10], suggesting that synaptojanin plays a role in the uncoating process.

Endocytosis can also be blocked at a particular stage by injecting an antibody, peptide or protein domain into the cell under study. The injection of peptides that contain parts of the clathrin adaptor proteins, AP180 [11] and AP2 [12], into the giant axon of the squid led to the depletion of synaptic vesicles from these terminals, without noticeable appearance of coated pit intermediates. This might be caused by competition for clathrin binding between the injected peptide and the endogenous protein, thereby preventing endogenous AP180 and AP2 from promoting clathrin assembly at the plasma membrane. Therefore, these adaptor proteins play a role in the early stages of clathrin coat formation. By contrast, injection of an antibody that interacts with endophilin into a lamprey axon blocked endocytosis at a later stage, leading to the accumulation of shallow coated pits without necks [13]. Therefore, inhibition of endophilin does not prevent initial coat formation, but does inhibit invagination of the clathrin lattice. A later block comes by injecting peptides that prevent the interactions between dynamin and amphiphysin. In these injected synapses, invaginated coated pits with narrow necks accumulate, suggesting that the dynamin–amphiphysin interactions are not necessary for invagination and vesicle neck formation, but are required for fission of the vesicle [14]. Finally, a block of interactions between the SH3 domain of endophilin and proteins including dynamin and synaptojanin leads to accumulation of both invaginated coated pits and coated vesicles, suggesting that the interactions are important both in fission of the vesicle from the membrane and in the subsequent uncoating process [15]. Therefore, by blocking an endocytic protein *in vivo*, and determining which budding intermediates accumulate, the stage at which that protein acts can be determined.

Clathrin-mediated endocytosis on liposomes

An alternative to analysing disrupted whole cells is to study the behaviour of purified proteins from the endocytic apparatus *in vitro*. Indeed, a long-term goal is to reconstitute clathrin-coated vesicle formation *in vitro* using purified components. As the proteins involved in endocytosis interact with the head-groups of membrane lipids, artificial liposomes provide a useful substrate on which to analyse their roles. Liposomes are easy to prepare [16] and can be readily studied in sedimentation assays or with the electron microscope. Indeed, when brain cytosol was incubated with synthetic liposomes in the presence of ATP and GTPγS, and the liposomes were fixed and studied by electron microscopy, both clathrin-coated buds (Fig. 2a) and dynamin-coated helices (Fig. 2b) were observed [17]. These structures are similar in appearance to those observed in the inhibited nerve terminals of Fig. 1b,c, with coated buds resembling the forming vesicle, and dynamin-coated helices the vesicle necks. Therefore, the formation of intermediates of clathrin-mediated endocytosis only requires soluble proteins and a lipid surface.

The location of different endocytic proteins within these structures has been investigated by specific labelling with gold-conjugated antibodies. The gold particles can be seen by electron microscopy, showing the presence and locations of clathrin, dynamin, amphiphysin and synaptojanin [17], and endophilin [13], in these structures. A further development of this technique is to immunodeplete one component from brain cytosol before incubation with liposomes. Depletion of endophilin inhibits formation of the dynamin-coated spirals in this *in vitro* assay, supporting its role in generating invaginated structures that elongate to form the neck of the vesicle [13].

Subsequent work has focused on reconstituting different stages of clathrin-mediated endocytosis on

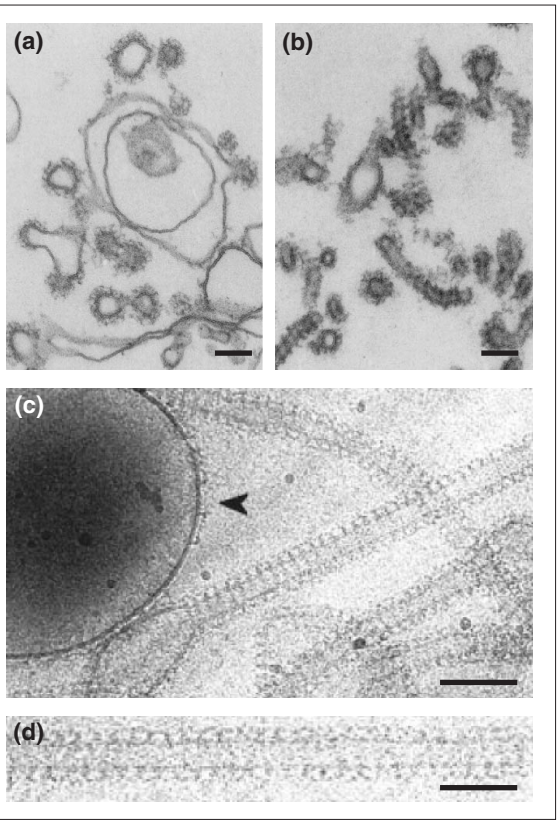

Fig. 2. Studying coated vesicle formation on liposomes. When synthetic liposomes are incubated with clathrin coat proteins, clathrin-coated vesicles (a) and dynamin-coated helical arrays (b) can be observed. The incubation of liposomes with dynamin alone leads to tubulation of liposomes and the formation of regular helical arrays (c,d). Scale bars = 100 nm. Figure is reproduced, with permission, from Refs [17,18].

liposomes. Incubation of phosphatidylserine liposomes with purified, recombinant dynamin leads to the formation of helical structures [17,18] (Fig. 2c,d). These have a similar appearance to the necks of vesicles in nerve terminals inhibited by the *shibire* mutation (Fig. 1c), suggesting that dynamin is part of the machinery at the vesicle neck. When these dynamin tubes are incubated with GTP they fragment [18], suggesting that dynamin forms a helix around the neck of a vesicle that can drive fission of the neck.

The interactions of dynamin with amphiphysin [18] and endophilin [19] have also been investigated using liposomes. All three proteins are able to tubulate liposomes independently. Tubes formed from a combination of dynamin and amphiphysin fracture more completely upon incubation with GTP than those formed from dynamin alone. They also appear to interact more readily with clathrin. Therefore, a combination of these proteins appears to be involved in fission of the neck of the vesicle *in vivo* [20]. By contrast, the presence of endophilin appears to inhibit dynamin-mediated vesiculation [19].

In addition to formation of vesicles from the plasma membrane, vesicles form on intracellular membranes and move between intracellular compartments. Clathrin-coated vesicles containing adaptor proteins AP1 and AP3 form on the surface of the Golgi apparatus and move to locations such as the lysosome and the endosome. By contrast, the very different COPI and COPII coats are involved in vesicular movement between the Golgi apparatus and the endoplasmic reticulum. In each of these cases, purified soluble factors and synthetic liposomes are sufficient to reconstitute coat formation [21–24]. However, as yet, the formation of clathrin-coated vesicles from purified plasma membrane components has not been reconstituted *in vitro*.

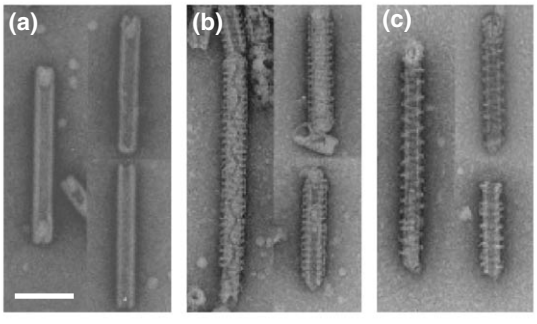

Fig. 3. Studying vesicle fission on lipid nanotubes. (a) Lipid nanotubes form from lipid mixtures that contain ≥40% of the lipid, nonhydroxyacyl-galactosylceramide. These nanotubes were incubated with dynamin in the presence of (b) 1 mM GTPγS or (c) 1 mM GDP, before negative staining and electron microscopy. Scale bar = 100 nm. Figure is reproduced, with permission, from Ref. [26]; copyright © 1999 Macmillan Magazines Ltd.

Studying vesicle budding using lipid nanotubes

The monounsaturated, nonhydroxyacyl-galactosylceramide lipid (NFA-GalCer) is a minor component of most plasma membranes. However, when resuspended in aqueous buffer it forms 'nanotubes' [25] (Fig. 3). These lipid tubules are relatively uniform, with lengths of 250–400 nm and diameters of 25–30 nm. Nanotubes also form readily from lipid mixtures that contain 40% of NFA-GalCer together with other lipids, allowing tubules to be generated that have a composition closer to that of the plasma membrane. As dynamin is able to bind to and tubulate liposomes [17,18], its ability to assemble on lipid tubules has been investigated [26]. Indeed, when the ligand lipid of dynamin, phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P_2], is included in a lipid tubule, dynamin binds to form a clear helix (Fig. 3b).

Electron microscopy has been used to investigate the conformational changes undergone by dynamin on lipid tubules [26,27]. Dynamin is a GTPase, but its mode of action is under considerable debate. It has been suggested to act as a classical G protein that senses the state of the bound nucleotide to regulate a downstream factor that causes vesicle scission [28]. An alternative suggestion is that dynamin is a mechanochemical enzyme that undergoes a conformational change to cause vesicle scission. Studies of lipid tubules with dynamin bound have supported this idea. Whereas the GTP-bound state of dynamin forms a tightly spaced helix (Fig. 3b), in the GDP-bound state, the spacing between adjacent turns of the helix increases nearly twofold [26] (Fig. 3c). This led to

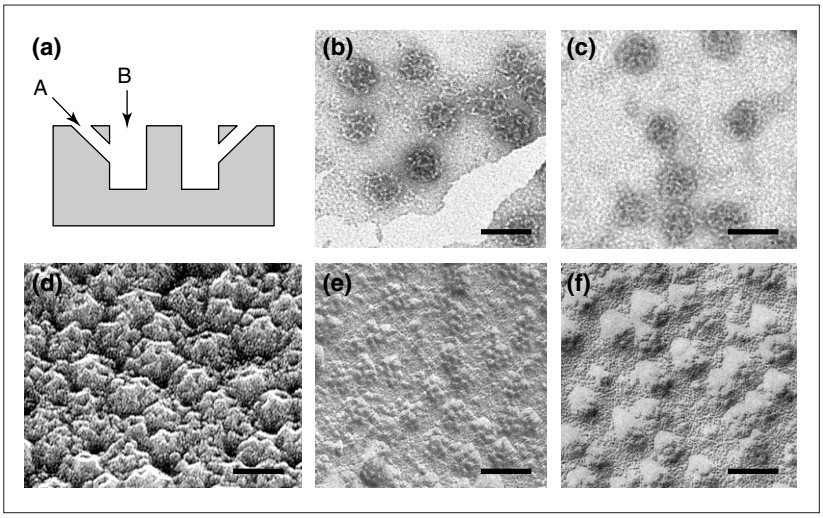

Fig. 4. Studying clathrin coat formation of a lipid monolayer. Lipid monolayers form when solvent dissolved lipid is placed onto the surface of a droplet of aqueous buffer. (a) A cross-section through a teflon block used to generate a monolayer [45]. The wells are filled with aqueous buffer and a chloroform-dissolved lipid mixture is placed onto well A. The smaller opening (B) is used to inject protein underneath the pre-formed monolayer. (b) In the presence of AP180, clathrin lattices form on the surface of a monolayer containing phosphatidylinositol-4,5-bisphosphate. (c) In the presence of both AP180 and AP2, these lattices appear more electron dense. (d) Rotary platinum shadowing of the lattices studied in (c) shows the presence of invaginated clathrin-coated pits. Indeed, single angle platinum shadowing reveals that, in the presence of AP180 alone, flat clathrin lattices form (e) whereas, in the presence of AP180 and AP2, these lattices were curved (f). Scale bar = 100 nm.

the suggestion that dynamin acts as a mechanochemical spring that undergoes a length-wise conformational change as it hydrolyses GTP, thereby popping a vesicle from the membrane. An alternative suggestion is that dynamin at the vesicle neck acts as a 'constrictase', reducing the diameter of the neck and releasing the vesicle. Indeed, the diameters of dynamin-coated PtdSer tubules decrease upon the addition of GTP [18,29]. Analysis of various mutants of dynamin in the different nucleotide-bound states supports a mechanochemical role for dynamin in which a conformational change accompanying GTP hydrolysis is necessary for the function of dynamin in endocytosis [27], but the exact nature of the conformation change is an area of intense study.

Formation of clathrin lattices on lipid monolayers

Although studies of the interactions of proteins with liposomes and lipid nanotubes have provided a wealth of information, lipid monolayers were chosen to investigate early stages of clathrin cage assembly (Fig. 4). When a droplet of solvent-dissolved lipid is placed onto an aqueous droplet, the solvent evaporates and leaves a single layer of lipid molecules, oriented with their head groups in contact with the aqueous mixture and their hydrophobic tails extending into the air.

Recently, monolayers have been used to study the roles of AP180 and AP2 in the nucleation of clathrin lattices [30]. A lipid monolayer containing PtdIns(4,5)P_2 was used to mimic the internal leaflet of the plasma membrane. In the presence of AP180 and clathrin, lattices form on the surface of such a lipid monolayer (Fig. 4b). The addition of the AP2 complex leads to the formation of more electron-dense areas of clathrin assembly (Fig. 4c). Rotary platinum shadowing suggests that the structures formed in the presence of AP2 and AP180 are invaginated half cages of clathrin (Fig. 4d). To more reliably compare the degree of invagination of clathrin lattices generated in the presence and absence of AP2, single angle platinum shadowing can be used (Fig. 4e,f) as the lengths of the shadows reveal the degree of invagination of the different structures present. Therefore, whereas AP180 nucleates the formation of a predominantly flat clathrin lattice (Fig. 4e), the addition of AP2 stimulates curvature of these lattices to generate coated pits (Fig. 4f). The structures formed in the presence of AP2 have a similar diameter to clathrin-coated vesicles found in a nerve terminal. Therefore, lipid monolayers provide a means of mimicking the internal surface of the plasma membrane and provide a valuable tool for study of the early stages of clathrin lattice formation and the interplay between clathrin assembly proteins. However, the presence of only one leaflet of the membrane makes it difficult to envisage how this system can be used to study the entire process of vesicle budding.

Photoconversion

To understand vesicle formation, it is necessary to study the movement of both protein and lipid during the different stages of endocytosis. Fluorescent dyes such as FM1-43 and FM2-10 allow lipid to be visualized. They partition into the outer leaflet of the plasma membrane of a cell, but cannot penetrate through to the inner leaflet because of the charged head group of the dye. When in the membrane environment, these dyes fluoresce strongly. However, they can be removed from the outer surface of a cell and, after this washing, only dye that has been internalized by endocytosis remains to label the cell. By using fluorescent microscopy, the rates of uptake and release of the dye can be used to investigate the movement of vesicles through a nerve synapse (reviewed in Ref. [31]).

To get a closer look at where internalized dyes are located within a nerve terminal, and to identify which vesicles have been internalized during the labelling process, photoconversion techniques can be used. When FM1-43 or FM2-10 are illuminated with blue light (at 430 nm) in the presence of diaminobenzidine, they are converted into electron-dense reaction products that can be visualized by electron microscopy. Examination of sections of stained, labelled, photoconverted cells show clearly which vesicles have taken up the dye and their location within the cell [32]. The different kinetics of uptake of FM1-43 and FM2-10, combined with their locations following uptake, have reinforced the idea that there are two different pools of vesicles, which show

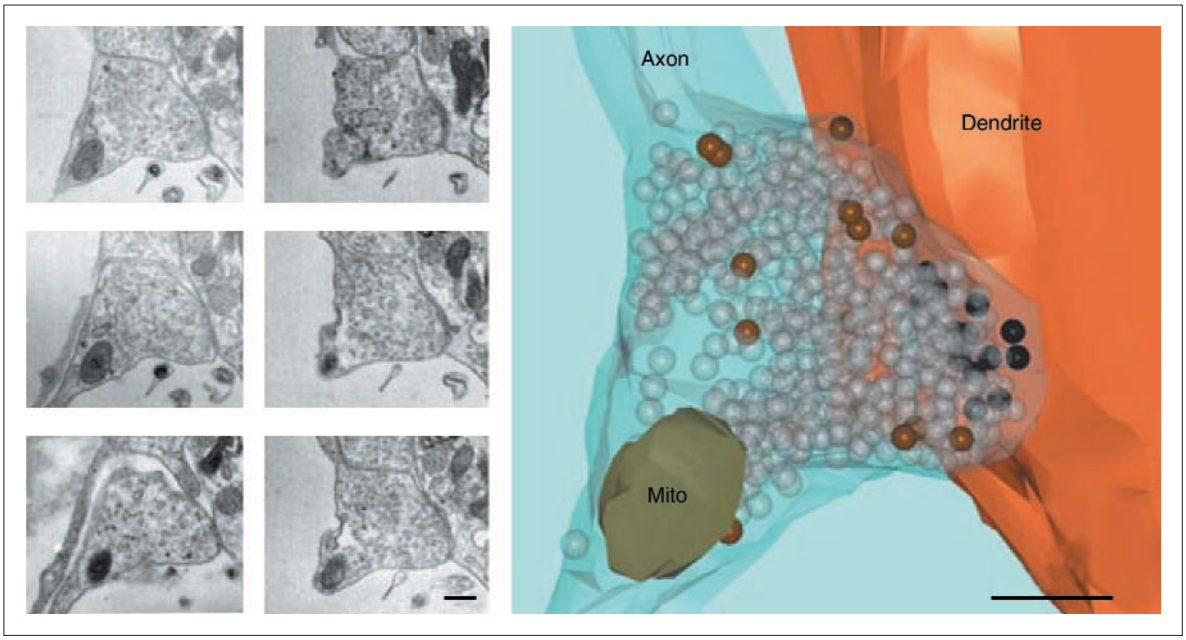

Fig. 5. Observation of internalized vesicles using fluorescent dyes. Photo-conversion of the fluorescent dye FM1-43 generates an electron-dense substrate that can be visualized in the electron microscope. In this case, a nerve terminal has been stimulated in the presence of FM1-43 and then photo-converted. Successive thin sections show the locations of labelled vesicles and can be used to generate a three-dimensional reconstruction of the vesicles in the terminal. Scale bar = 100 nm. Figure is reproduced, with permission, from Ref. [35].

different recycling properties [33]. Successive sections through photoconverted terminals have been used to generate three-dimensional reconstructions of vertebrate nerve-muscle synapse [34] and hippocampal synapses [35] (Fig. 5), in which recently internalized vesicles can be distinguished from those at different stages of vesicle recycling. These reconstructions show that the labelled synaptic vesicles are, on average, located closer to the active zone than unlabelled vesicles. The ability to identify recently endocytosed vesicles, perhaps combined with electron tomography, could enable us to pinpoint differences between the vesicles that are participating in cycles of exo- and endocytosis in the nerve terminals and those that are not.

Electron tomography

Whereas electron microscopy of thin-sections enables visualization of slices through the cell, electron tomography can be used to generate three-dimensional reconstructions from two-dimensional projection images [36]. The object to be imaged can be prepared in several different ways. Thick sections of fixed, embedded, stained material can be studied. Alternatively, to reduce artefacts caused by sample preparation, the specimen can be suspended in holes on the surface of an electron microscope grid and fast-frozen by plunging the grid into liquid ethane. Successive images can then be taken with the sample tilted by 1° between each imaging event. Image processing of these data generates a three-dimensional reconstruction of the object. Tomography of ice-embedded specimens has been used to generate three-dimensional models of a prokaryotic cell [37], a phage captured during the process of injecting DNA into a liposome [38], and a mitochondrion [39]. The resolution of the reconstruction of a mitochondrion [39] is in the region of 70 Å and is sufficiently high to see ATP synthase protruding from the surface of the mitochondrial membrane. Therefore, tomography of ice-embedded specimens enables reconstructions of sufficient resolution for observation of individual protein complexes.

Electron tomography has also been used to investigate stained sections of the active-zone material of the frog neuromuscular junction, generating a three-dimensional model of docked vesicles and the network in which they dock [40]. A reconstruction of a clathrin-coated vesicle has also been generated [15], showing that the achievable resolution of tomography is sufficient for the generation of three-dimensional reconstructions of intermediates in vesicle budding. As electron tomography of fast-frozen samples avoids the potential artefacts of fixation, sectioning and negative staining, it allows us to look into a cell with a minimum of perturbation. In addition, techniques are available that allow atomic structures determined by X-ray crystallography to be 'docked' into low resolution structures derived from electron microscope images [41]. Similar techniques are under development to allow structures to be docked into structures derived from electron tomography [42]. Already, the structures of many protein domains implicated in clathrin-mediated endocytosis have been determined [43]. By docking these structures into electron tomography models of the different stages of endocytosis, it should be possible to derive snap-shots of the intermediates of endocytosis at atomic resolution.

Summary and future directions

By studying the characteristic intermediates of endocytosis in the electron microscope, much has been learned about the machinery that assembles to drive coated vesicle formation. *In vitro* assays have shown the roles of different protein components of this machinery and lead towards the reconstitution of vesicle budding in a cell-free system. In addition, the study of thin sections of axons has allowed different intermediates to be visualized *in vivo*, and microinjection of peptides and antibodies has revealed the stages in vesicle budding at which many protein components act. Finally, photoconversion of lipophylic dyes has allowed the destination of internalized vesicles to be studied in the context of the cell. A combination of these techniques is allowing us to see the stages of endocytosis by electron microscopy. With the introduction of electron tomography, it should soon be possible to limit artefacts caused by fixing, sectioning and staining, and to generate three-dimensional reconstructions of the different stages of vesicle budding. Antibody labelling will reveal locations of different proteins within these assemblies, and the docking of atomic structures of protein domains into tomography reconstructions will result in atomic resolution models. By combining these methods, we hope to soon see a series of three-dimensional 'snap-shots' of endocytosis in atomic detail.

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