Sequential steps in clathrin-mediated synaptic vesicle endocytosis
Lennart Brodin*, Peter Löw† and Oleg Shupliakov‡

Synaptic vesicles are recycled with remarkable speed and precision in nerve terminals. A major recycling pathway involves clathrin-mediated endocytosis at endocytic zones located around sites of release. Different ‘accessory’ proteins linked to this pathway have been shown to alter the shape and composition of lipid membranes, to modify membrane–coat protein interactions, and to influence actin polymerization. These include the GTPase dynamin, the lysophosphatic acid acyl transferase endophilin, and the phosphoinositide phosphatase synaptojanin. Protein perturbation studies in living nerve terminals are now beginning to link the actions of these proteins with morphologically defined steps of endocytosis.

Overview of proteins in clathrin-mediated endocytosis
Clathrin-mediated endocytosis depends on two sets of proteins: those comprising the clathrin coat, and an array of other proteins often referred to as ‘accessory’ proteins. Many of these proteins have now been characterized in considerable detail — work has included studies of their crystal structure and of their interactions with other proteins and membrane phospholipids [11–15].

The basic building block of the clathrin coat is the three-legged clathrin triskelen (Figure 1), of which each leg contains a heavy and a light chain of clathrin [1,11]. The triskelia assemble into a lattice of hexagons and pentagons (for a live model, see http://www.hms.harvard.edu/news/clathrin), which attaches to the plasma membrane via the tetrameric adaptor protein (AP) complex AP2 (Figure 1). The coat also contains a neuron-specific form of a monomeric adaptor protein, AP180, which can stimulate clathrin assembly and which appears to be critical for the generation of synaptic vesicles with an homogenous size. In Drosophila and C. elegans lacking AP180-like proteins, nerve terminals still contain synaptic vesicles, but their average size is larger and the size variability is increased when compared with that of vesicles in wild-type controls [5,6]. Moreover, microinjection studies in the squid giant synapse [7] have shown that a peptide corresponding to the clathrin assembly domain of AP180 causes a marked depletion of synaptic vesicles, while the average size of the remaining vesicles is increased [7].

The formation of an endocytic clathrin coat is thought to begin with binding of AP2 to the membrane [1,15,16]. One candidate membrane receptor for AP2 is the synaptic vesicle protein synaptotagmin, which binds AP2 via one of its cytoplasmic C2 domains (C2B; for references see [2]). Via a distinct binding site, AP2 also binds tyrosine-based sequence motifs, which are known as sorting signals in the context of receptor-mediated endocytosis [16] and which occur in synaptic vesicle proteins [17••,18••]. Peptides containing a tyrosine-based motif have been shown to strengthen the synaptotagmin–AP2 interaction [17••], suggesting a functional link between the two binding sites. The formation of the clathrin coat also involves interactions between coat proteins and membrane phospholipids. Phosphorylated inositol phospholipids (phosphoinositides) appear to be particularly important [13,14]. For instance, the plasma membrane targeting of AP2 depends on its phosphoinositide binding site [19], and stimulation of
phosphoinositide synthesis can enhance coat formation [20], while ‘capping’ of phosphoinositides by overexpressing phosphoinositide-binding proteins has an inhibitory effect ([21]; see also the section on Uncoating, below). Clathrin coat formation can also be enhanced by phospholipase D, which may at least partly involve stimulation of phosphoinositide synthesis [13,14,20].

Among the accessory proteins (Figure 1), dynamin has so far been the most extensively studied. It was originally linked to endocytosis through the temperature-sensitive *Drosophila* mutant *shibire*, in which endocytosis is blocked at the restrictive temperature (for references, see [22]). Dynamin is a multi-domain protein (Figure 1) with a GTPase domain, a phospholipid-binding pleckstrin homology (PH) domain, and a so-called GTPase effector domain (GED; [22]; see also the section on Fission, below). The carboxy-terminal consists of a proline-rich domain (PRD), which interacts with src-homology (SH3) domains of other accessory proteins including amphiphysin, endophilin, dap160/intersectin and synaptojanin/pacin ([22,23,24•]. Dynamin has a tendency to assemble into tetramers, which can polymerize into ring-like structures. When dynamin is incubated with spherical liposomes these can be turned into narrow tubules surrounded by polymerized dynamin ([22,25,26]. The structure of dynamin polymers is altered by GTP hydrolysis ([25,27] which can even cause vesiculation of the lipid tubules [25]. Recently, amphiphysin was also shown to be capable of tubulating liposomes — either alone, or together with dynamin [28•]. Two of the accessory proteins, synaptojanin and endophilin, have been found to act as lipid-metabolizing enzymes. Synaptojanin is a polyphosphoinositide phosphatase that dephosphorylates

---

**Figure 1**

Diagram of proteins implicated in clathrin-mediated synaptic vesicle endocytosis. Clathrin occurs as a triskelion consisting of three heavy and three light chains of clathrin. Triskelia assemble together into a lattice of pentagons and hexagons [1,11], which are connected to the plasma membrane via the adaptor complex AP2. The amino-terminal domain of the clathrin heavy chain (N) binds to the hinge domain in the AP2 α2 and β2 subunits. The ‘ear’ (or appendage) domain of AP2 interacts with a number of proteins including AP180, Eps 15, amphiphysin and auxilin. AP2 interacts with synaptojanin, with tyrosine-based sequence motifs via the μ2 subunit, and with phosphoinositides via the ε2 subunit (for further details and references see [1,2,11,12,19]). AP 180 is a monomeric adaptor-like protein, which interacts with AP2 and stimulates clathrin assembly [2,5–7]. It also interacts with phosphoinositides [13,14]. The remaining proteins (i.e. the accessory proteins) are usually not detectable in preparations of clathrin-coated vesicles; this suggests that they interact transiently with the coat. Only one form is represented, although each protein is, as a rule, found in multiple isoforms. Here, each protein is represented by boxes indicating the approximate size of the different domains. Domains with enzymatic activity include the GTPase domain in dynamin and the 5′-phosphatase and Sac1 homology domains of synaptojanin [22,29••]. The LPAAT activity of endophilin is likely to reside in the conserved amino-terminal region of the protein [8••,31•]. Modular binding domains mediating protein–protein interactions include SH3 domains which interact with distinct binding sites within proline-rich domains (PRD), and EH domains which interact with NPF (Asn-Pro-Phen) motifs. Multiple NPF repeats occur in the carboxy-terminal of epsin. NPF repeats are also present in synaptojanin and in one isoform of synaptojanin. ENTH indicates a novel domain termed the epsin amino-terminal homology domain, CC indicates coiled-coil domains, which may be involved in heteromerisation. Distinct binding sites for AP2 and clathrin are present in amphiphysin and Eps 15. Alternative names are as follows; endophilin, SH3p4; synaptojanin, pacsin (see [54]); intersectin, Dap160 [23]; epsin, lbp (for further details and references see [1,2,11,12,15,47]).
phosphoinositides at positions 3, 4 and 5 of the inositol ring (see [13]), and it may thus regulate interactions of endocytic proteins with the plasma membrane [2,29•,30]. Endophilin acts as a lysophosphatidic acid acyl transferase (LPAAT; [31••]). By converting lysophosphatidic acid into phosphatidic acid, it may alter the biophysical properties of the lipid bilayer (see below). Some accessory proteins have been shown to interact with actin-regulating proteins. For instance, dynamin binds profilin [32], and syndapin/pacsin binds N-WASP [24•].

Localization of clathrin-mediated endocytosis at the synapse

The proteins mentioned above are highly concentrated in nerve terminals. Studies of terminals in Drosophila and lamprey indicate that, within a terminal, different endocytic proteins are enriched in a plasma membrane region surrounding the active zone [3,23,33,34]. This region appears to define an ‘endocytic zone’ which typically extends about a micron from the edge of an active zone.

Actin polymerization can be induced in this region by activation of GTPases with GTPyS [34]. The rho-type GTPase-activating protein ‘still life’ has also been localized to this zone [35].

Clathrin-coated pits appear in the endocytic zone (see below and Figure 2) shortly after exocytosis [2], suggesting that the synaptic vesicle membrane and its protein components quickly move to this region. The targeting of clathrin coat formation to the endocytic zone is, however, not absolute, as coated pits occasionally occur within the active zone. Their number can increase after manipulations which cause massive depletion of synaptic vesicles, suggesting that docked synaptic vesicles may limit coat formation in the active zone (O Shupliakov, P Löw, L Brodin, unpublished observations). The extent of the endocytic zone in the plasma membrane does not appear to be fixed. After certain manipulations that inhibit endocytosis, coated pits can occur in an area extending several microns from the active zone [4].
Clathrin-coated pits can also appear at invaginations of the plasma membrane that occur after inhibition of endocytosis. In this case their features may differ from those formed in the intact endocytic zone (see section on Fission, below), consistent with a functional specialization of the latter. The synaptic endocytic zone may correspond to the endocytic hot spots identified at the plasma membrane of non-neuronal cells [36•]

**Onset of endocytosis**

Clathrin-mediated synaptic vesicle endocytosis is normally coupled with exocytosis, but the two processes can be separated experimentally. In the experiment shown in Figure 2, intense action-potential stimulation was first applied to deplete synaptic vesicles (not shown in the figure), and endocytosis was then blocked by removal of extracellular Ca$^{2+}$ [37]. Under these conditions, coated structures are virtually absent (Figure 2a). Addition of Ca$^{2+}$ induces the synchronous formation of coated pits in the endocytic zone (Figure 2b). The onset of clathrin coat formation thus requires Ca$^{2+}$ (low micromolar concentrations are sufficient [37]), but spike-evoked influx is not needed. It also appears to depend on ATP, as lowering of ATP levels causes vesicle depletion and an appearance of plasma membrane invaginations ([38]; O Shupliakov, L Brodin, unpublished observations). In contrast, clathrin coat formation in vitro is not ATP-dependent [26].

After addition of Ca$^{2+}$, sequential stages of coated-pit formation (Figure 2c) can be identified by their relative abundance at different time points ([37]; see also references in [1,2]). The first stage (Figure 2c1) consists of a coated membrane patch with slight curvature. The second (Figure 2c2) is an invaginated coated pit with a broad base. The third (Figure 2c3) is an invaginated coated pit with a narrow neck. A ring-like structure is occasionally seen around the narrow neck which probably defines a fourth stage (Figure 2c4), although its low abundance makes the location in the sequence somewhat uncertain. The next stage is likely to be represented by a free coated vesicle but, as further discussed below, this stage appears to be very transient. Data from protein perturbation studies have shown that each stage (Figure 2c1–4) can be retarded by interfering with specific proteins, suggesting that these distinct morphological states correlate with intermediates in a molecular cascade.

**Intermediates in coated-pit formation: invagination of the coated membrane**

Microinjection studies in the lamprey giant synapse suggest that the proteins of the clathrin coat cannot alone generate an invaginated coated pit, but that accessory factors, including endophilin, appear to be required. After presynaptic microinjection of anti-endophilin antibodies [8••], stimulation causes depletion of synaptic vesicles, along with a massive accumulation of shallow coated pits in the endocytic zone (Figure 3a,b). The invagination process appears to be inhibited in a concentration-dependent manner, as the depth of the coated pits decreases with increasing antibody concentration (Figure 3c,d1–3). The precise mechanism underlying this effect is not yet clear. **In vitro** formation of clathrin coats from brain cytosol is not affected by immunodepletion of endophilin [8••], indicating that endophilin acts on the membrane, rather than on coat assembly. One
may be indirectly influenced by endophilin, perhaps via the cytoskeleton. Moreover, binding partners of the endophilin SH3 domain, including dynamin and synaptojanin, could also be involved (see below).

**Narrowing of the neck region**

The process subsequent to invagination — the narrowing of the neck of the invaginated coated pit (Figure 1c2–3) — also appears to depend on mechanisms extrinsic to the clathrin coat. Several factors may be involved. For instance, perturbation of SH3-domain interactions in permeabilized cells can affect the narrowing of the neck of the coated pit, as judged from the altered accessibility to large, but not to small, tracer molecules [41]. Microinjection of actin toxins can increase the abundance of coated pits with a wide neck at stimulated synapses (O Shupliakov et al., unpublished data). Actin disruption has been found to affect the formation of clathrin-coated pits at the plasma membrane of other cell types, but the effects are variable [1,2], and the exact role of actin in coated pit formation remains unclear. A general involvement of actin in endocytosis has, however, been supported by imaging studies of GFP-coupled actin in mast cells, which indicate that actin polymerization occurs at emerging endocytic membrane invaginations [42•]. The polymerization of actin in the endocytic zone of synapses appears to be coupled with synaptic activity, possibly through signalling via GTPases [34] and phosphoinositides [29•]. Both types of signal can regulate N-WASP/arp2,3-mediated actin polymerization ([43,44•]; see also Transport of the newly retrieved vesicle, below).

**Fission**

The fission of the neck of the coated pit is sensitive to a variety of perturbations. In nerve terminals of the shibire mutant, invaginated endocytic pits with narrow necks surrounded by an electron-dense ring accumulate at the restrictive temperature (see [22] for references). The localization of the shibire mutation to dynamin’s GTPase domain, along with observations that GTP hydrolysis can alter the structure of dynamin polymers, suggested that GTP hydrolysis by dynamin may drive fission [22,25–27]. However, recent studies of dynamin argue against this model. The GED of dynamin appears to mediate the increased GTPase activity which occurs during oligomerization [45•]. When the assembly-stimulation of the GTPase activity is perturbed by mutations in the GED, receptor-mediated endocytosis is found to be enhanced rather than inhibited [45•]. While this finding does not explain the role of dynamin’s GTPase activity, it shows that the GTPase activity is not rate-limiting for endocytosis. It is possible that dynamin, like ‘conventional’ GTPases, acts as a regulator which interacts with downstream effectors in its GTP-bound state [45•].

Another dynamin-related intermediate — a clathrin-coated pit with an elongated neck surrounded by multiple electron-dense rings (Figure 4a) — can be trapped with
the slowly hydrolysable GTP analog GTPγS. This intermediate was first observed in vitro [22], but it also occurs in stimulated synapses after microinjection of GTPγS [34]. Although the mechanisms underlying the induction of this intermediate are unclear (other GTPases than dynamin may be involved), it has provided insight into the composition of the fission machinery. Immunocytochemical studies suggest that the GTPγS-dependent rings contain both dynamin, amphiphysin [26,28•], and endophilin (Figure 4b; [8••]). The interaction between dynamin and amphiphysin appears to be essential for fission [4,11], as microinjection of proteins or peptides which inhibit this interaction causes synaptic vesicle depletion along with a massive accumulation of invaginated coated pits with narrow necks (Figure 4b). Electron-dense rings are not seen after this perturbation, indicating that SH3 domain interactions contribute to ring formation [4].

A trapping of similar deeply invaginated coated pits can occur also after perturbation of endophilin [8••]. In the antibody-injection studies discussed above, plasma membrane invaginations sometimes extended outside the endocytic zone. Tracing of these invaginations showed that they sometimes contain invaginated coated pits with narrow necks (Figure 4c) differing from the shallow coated pits in the endocytic zone. These observations appear to converge with studies of synaptic-like vesicle formation in permeabilized PC12 cells ([31••]; see also [41]). In this assay, both endophilin and dynamin are required for vesicle formation. Endophilin is only active when its SH3 domain is intact, consistent with a role of dynamin–endophilin interactions in fission. This possibility, however, remains to be tested experimentally. Vesicle formation in vitro was also found to be sensitive to treatments interfering with lipid metabolism catalyzed by the LPAAT activity [31••]. The Golgi trafficking protein CtbP/BARS (carboxy-terminal-binding protein/brefeldin A-ADP-ribosylated substrate) has also been shown to possess LPAAT activity. Golgi tubules incubated with increasing concentrations of CtbP/BARS exhibited an increased occurrence of invaginations which appeared to proceed to vesiculation [46••].

Thus, recent studies indicate that fission of clathrin-coated pits depends on a coordinated action of a set of proteins that includes dynamin, amphiphysin, endophilin, and perhaps others. The exact sequence of events during fission remains to be elucidated.

Uncoating
It has not yet been possible to track the exact path of the endocytic vesicle after it has left the plasma membrane in the endocytic zone. Most likely, a rapid uncoating occurs, as free coated vesicles are rarely seen in stimulated synapses. The uncoating reaction involves disassembly of the clathrin coat by the uncoating ATPase heat shock cognate protein 70 kD (hsc 70) and auxilin [12]. Synaptojanin 1 has also been proposed to contribute to uncoating [29•]. Using an in vitro coating assay, cytosol from synaptojanin 1-deficient mice was shown to support coat formation more effectively than cytosol from wild-type mice, suggesting that synaptojanin 1 can act as a negative regulator of membrane–coat protein interactions. Electron microscopic analysis showed...
that nerve terminals of synaptojanin 1-deficient mice contained synaptic vesicles, and comparatively few coated vesicles were present. The relative proportion of coated vesicles versus synaptic vesicles was, however, higher in knockout mice than in wild-type mice, consistent with a partially retarded vesicle uncoating [29*].

Transport of the newly retrieved vesicle
The newly uncoated endocytic vesicle may return directly to the release site as a fully functional synaptic vesicle, or it may pass through a secondary endosomal fusion-and-budding step [2]. While evidence for synaptic vesicle formation from both endosomal and plasma membrane compartments have been obtained, the single budding step pathway has recently been favored as the main physiological route (for discussion of this topic see [2,5,15,37,47,48]). Actin-based transport has been implicated in the transport of endocytic vesicles in other cell types [42*,49*], and it may well play a similar role in the transport of synaptic vesicles between the endocytic zone and the synaptic vesicle cluster.

Conclusions
The results obtained so far in mutation and microinjection studies have allowed a first glimpse of the sequential actions of endocytic proteins in living nerve terminals. The continued use of these approaches, and their combination with high resolution immunolabeling, should help to clarify the temporal aspects of the endocytic cascade. It is already evident, however, that endocytosis cannot be described as a simple chain of proteins acting in a strict order (see Figure 5). For instance, endophilin appears to act at more than one endocytic stage, and this may well apply to other accessory proteins such as dynamin and amphiphysin [22,25*,50]. Some proteins, like synaptotagmin, appear to play critical roles in both exo- and endocytosis [2,17**]. Moreover, the efficient sorting of synaptic vesicle proteins at each vesicle cycle is likely to depend on a close functional coupling between exo- and endocytic proteins [6,15,18**,47,51]. As well as the clarification of the sequence of protein actions, fundamental problems such as the spatial and temporal reorganization of membrane phospholipids during endocytosis remain to be addressed. Another important problem concerns the regulation of the endocytic molecular machinery [52], which can now be addressed with dynamic protein imaging methods using GFP [36,42*,53*]. These methods should also allow investigators to test the possible role of alternative pathways in synaptic vesicle recycling.

Acknowledgements
We are indebted to Dr P De Camilli for discussions, and to Dr O Kjaerulff for comments on the manuscript.

References and recommended reading
Papers of particular interest, published within the annual period of review, have been highlighted as:
- of special interest
- of outstanding interest


24. Qualmann B, Roos J, DiGregorio PJ, Kelly RB: Syndapin I, a synaptic • dynamin-binding protein that associates with the neural Wiskott-Aldrich syndrome protein. *Mol Biol Cell* 1999, 10:501-513. This study shows that syndapin (also termed pacsin [34]) binds endocytic proteins, including dynamin and synaptophysin, and that it also binds the actin regulator N-WASP. These observations suggest a molecular link between the endocytic budding machinery and the regulation of the actin cytoskeleton (see also [32,44]).


28. Takei K, Slepenev V, Haucke V, De Camilli P: Functional partnership • between amphiphysin and dynamin in clathrin-mediated endocytosis. *Nat Cell Biol* 1999, 1:33-39. This study shows that amphiphysin 1 can bind lipid membranes, that it can transform spherical liposomes into narrow tubules, and that it can co-assemble with dynamin 1 and enhance the lipid-fragmenting activity of dynamin 1 in the presence of GTP. The results indicate a close functional relationship between amphiphysin and dynamin (see also [4,11]), and they suggest that both proteins may contribute to the generation of bilayer curvature during endocytic membrane budding.


Mice lacking synaptojanin 1 are shown to exhibit neurological deficits and die shortly after birth. The level of phosphatidylinositol-4,5-bisphosphate is enhanced when normal cytosol is replaced with cytosol from knockout mice. The generation of phosphatidylinositol-4,5-bisphosphate is required for endocytic coated vesicle formation. *Curr Biol* 1999, 9:1411-1414. Syndapin I, a synaptic •• terminal protein, is shown to associate with GDP–GTP exchangers. Syndapin I is also shown to associate with Clathrin-coated vesicles. The localization of syndapin I is shown to be limited, but increases after disruption of the actin cytoskeleton. Coated pits are found to form repeatedly at defined sites, while other regions are excluded.


The study provides evidence for a role of sphingolipid–cholesterol rafts as preferred platforms for membrane-linked actin polymerization, and for an involvement of actin tails in the transport of endocytic and Golgi-derived vesicles (see also [42]).


53. Sankaranarayanan S, Ryan TA: Real-time measurement of vesicle SNARE recycling in synapses of the central nervous system. Nat Cell Biol 2000, 2:197-204. A pH-sensitive GFP linked to synaptobrevin (also known as vesicle-associated membrane protein, or VAMP) was used to monitor vesicle recycling in cultured hippocampal neurons. The measured time courses are consistent with a single, presumably clathrin-mediated, endocytic pathway.
