An endophilin–dynamin complex promotes budding of clathrin-coated vesicles during synaptic vesicle recycling

Anna Sundborger1, Cynthia Soderblom1, Olga Vorontsova1, Emma Evergren1,*, Jenny E. Hinshaw2,‡ and Oleg Shupliakov1,‡

1Department of Neuroscience, DBRM, Karolinska Institutet, 17177 Stockholm, Sweden
2Laboratory of Cell Biochemistry and Biology, NIDDK, NIH, Bethesda, MD 20892, USA

*Present address: MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 0QH, UK
‡Authors for correspondence (jennyh@helix.nih.gov; oleg.shupliakov@ki.se)

Summary

Clathrin-mediated vesicle recycling in synapses is maintained by a unique set of endocytic proteins and interactions. We show that endophilin localizes in the vesicle pool at rest and in spirals at the necks of clathrin-coated pits (CCPs) during activity in lamprey synapses. Endophilin and dynamin colocalize at the base of the clathrin coat. Protein spirals composed of these proteins on lipid tubes in vitro have a pitch similar to the one observed at necks of CCPs in living synapses, and lipid tubules are thinner than those formed by dynamin alone. Tubulation efficiency and the amount of dynamin recruited to lipid tubes are dramatically increased in the presence of clathrin-coated intermediates (Dickman et al., 2005; Schuske et al., and in an increase in the number of free and membrane-attached clathrin-coated intermediates (Granseth et al., 2007; Jung and Haucke, 2007; Rodal and Littleton, 2008). At the final step of this process, clathrin-coated vesicles bud from the presynaptic membrane and shed their clathrin coat. The accessory protein endophilin binds to two endocytic molecules implicated in the scission and uncoating events in synapses – the GTPase dynamin and the polyphosphoinositide phosphatase synaptojanin (Liu et al., 2009; Ferguson et al., 2007; Heymann and Hinshaw, 2009; Mettlen et al., 2009). Dynamin plays the key role in synaptic vesicle budding, as a complete depletion of all dynamin isoforms blocks fission of clathrin-coated pits (CCPs) (Ferguson et al., 2007). The interaction of endophilin with synaptojanin serves mainly to facilitate uncoating of vesicles after fission (Cremona et al., 1999; Gad et al., 2000; Ringstad et al., 1999; Ringstad et al., 2001; Schuske et al., 2003; Song and Zinsmaier, 2003; Verstreken et al., 2003), although recent studies in budding yeast suggest a possible involvement of synaptojanin in the actual fission reaction. This property of endophilin implies that it functions as a molecular switch linking fission and uncoating during recycling of synaptic vesicles. The molecular mechanisms underlying this ‘switch’ are unknown, and in particular the role of the endophilin–dynamin interaction remains to be clarified.

Loss of endophilin in both fly and nematode resulted in a slowdown of the endocytic process in neuromuscular junctions and in an increase in the number of free and membrane-attached clathrin-coated intermediates (Dickman et al., 2005; Schuske et al., 2003; Verstreken et al., 2002; Verstreken et al., 2003). Additionally, in lamprey giant synapses, one of the effects of acute perturbation of the endophilin Src-homology 3 (SH3) domain interaction was an increase in the number of constricted CCPs (Gad et al., 2000). These results indicate that endophilin interactions are not only involved in uncoating but also play a role in a stage dealing with membrane-bound constricted CCPs before the fission step and might thus involve dynamin.

There are three dynamin isoforms in neurons. Dynamin-1 is the neuron-specific isoform required during high levels of neuronal activity (Ferguson et al., 2007; Lou et al., 2008). All dynamin proteins contain a pleckstrin-homology (PH) domain, which allows the protein to interact with membrane phospholipids, and a proline-rich domain (PRD), which enables them to bind to SH3 domains of accessory proteins (Ferguson et al., 2007). The SH3 domain of endophilin 1 has been shown to bind to the PRD of dynamin 1 at two sites in vitro (Anggono and Robinson, 2007; Heymann and Hinshaw, 2009). Endophilin also contains an N-terminal BAR domain with membrane curvature-generating and curvature-sensing properties (Gallop et al., 2006; Masuda et al., 2006). Together with dynamin, endophilin is present on tubules formed by liposomes after addition of brain cytosol (Farsad et al., 2001; Ringstad et al., 1999). Taken together, these studies suggest that endophilin might regulate fission, although it still remains unclear how the complex of endophilin and dynamin, which is not modified by GTP (Farsad et al., 2001), might aid this process.

Here, we utilized the lamprey reticulospinal synapse to elucidate the role of endophilin–dynamin interactions in the regulation of the fission mechanism during recycling of synaptic vesicles. Using immuno-electron microscopy, we localized endophilin to the sites of synaptic vesicle recycling in relation to dynamin and studied the
effects of acute perturbations of endophilin SH3 domain interactions on endocytosis in lamprey synapses. To model the effects observed in living synapses in vitro, we used recombinant proteins and lipid templates. Our experiments indicate that endophilin accumulates at a restricted part of the neck of the CCP. We propose that endophilin provides a template for dynamin assembly and forms a complex with dynamin at the neck. This complex increases dynamin recruitment to the neck and structurally promotes dynamin-mediated fission and thus aids sustaining a high rate of synaptic vesicle recycling during neurotransmitter release.

Results

Endophilin localizes to the rim of the clathrin coat of CCPs and colocalizes with dynamin at a restricted part of the neck

To study the relative distribution of endophilin and dynamin at sites of synaptic vesicle recycling in intact stimulated synapses, we used immunogold labeling (Evergren et al., 2004). Both endophilin and dynamin accumulate in the synaptic vesicle pool at rest (supplementary material Fig. S1A) (Evergren et al., 2007). During stimulation, an accumulation of both proteins is observed at CCPs in the periactive zone (Fig. 1A–C,E,F; supplementary material Fig. S1B,C) (see also Evergren et al., 2007). This is in contrast to synapsin, which has also been shown to reside in the synaptic vesicle cluster at rest but does not associate with CCPs in the periactive zone of stimulated synapses (Evergren et al., 2004). Interestingly, endophilin is localized at the edge of the clathrin coat of shallow constricted pits (Fig. 1B1,2) and at later stages is concentrated at the necks of constricted coated pits (Fig. 1B3). Localization of endophilin at the rim of the coat was also confirmed by post-embedding immunogold labeling (supplementary material Fig. S1B,C). Dynamin immunoreactivity is also detected at early endocytic stages. Contrary to endophilin, dynamin is found associated with the entire coat at early endocytic stages (Fig. 1C1,2), and, at late stages, dynamin is detected on both the coat and the neck of CCPs (Fig. 1C3). Gold particles signaling for another endophilin binding partner, synaptojanin, were also associated with the entire coat at early endocytic stages. At late stages, however, gold particles were found preferentially concentrated at the lower half of CCPs (upper:lower CCP area labeling ratio=0.33, n=35; supplementary material Fig. S1D–F). Only 40% of CCPs were labeled, which was in agreement with earlier observations (Haffner et al., 1997). By contrast, all CCPs investigated were labeled with antibodies against dynamin and endophilin. These experiments show that, during formation of coated vesicles in the periactive zone, endophilin and dynamin are in position to interact at the necks of constricted CCPs.

Perturbation of endophilin–SH3 domain interactions prevents dynamin localization at the neck of constricted CCPs and inhibits fission in a living synapse

PP19, a peptide derived from the PRD of synaptojanin binds to rat endophilin 1 and blocks interactions of the endophilin SH3 domain selectively (Ringstad et al., 2001). Previous studies showed that microinjection of PP19 into living lamprey synapses blocks interactions of endophilin with its endocytic binding partners – synaptojanin and dynamin – which results in an inhibition of clathrin-mediated endocytosis at late stages (Gad et al., 2000) (see also supplementary material Fig. S2A). One of the striking effects was an accumulation of free clathrin-coated vesicles. An accumulation of clathrin-coated vesicles was also observed in synaptotxin-1-knockout mice. These data led to the conclusion that endophilin serves as a recruiter of the polyinositolphosphatase synaptojanin to CCPs to promote the uncoating reaction (Cremona et al., 1999). In the present study, we sought to investigate further the origin of another, upstream, effect of PP19 microinjection – the accumulation of constricted CCPs – which possibly involves dynamin (Gad et al., 2000).

We first tested whether PP19 blocks SH3 domain interactions of other endocytic proteins interacting with dynamin in lamprey and found that PP19 competes efficiently with dynamin for binding to the GST–SH3 domain of lamprey endophilin but does not affect the interactions between dynamin and the lamprey amphiphysin SH3 domain or the whole cassette of five SH3 domains of lamprey intersectin (supplementary material Fig. S2B). Additionally, coupled to beads, PP19 selectively affinity-purifies the lamprey endophilin orthologue but not amphiphysin or intersectin from lamprey brain extracts (supplementary material Fig. S2C).

We then microinjected PP19 into lamprey giant axons to test whether perturbations of the endophilin SH3-domain interactions affect the localization of dynamin at the necks of constricted CCPs. Ultrastructural effects induced by microinjection of PP19 in giant axons (n=4) are similar to those described earlier (Gad et al., 2000). Synaptic vesicle recycling is perturbed and an accumulation of constricted CCPs is observed in synaptic periactive zones (Fig. 2A,G and supplementary material Fig. S2D). The fission of clathrin-coated vesicles, however, is not completely blocked as free clathrin-coated vesicles are observed in the axoplasm (Fig. 2A2 and supplementary material Fig. S2D).

To determine whether the localization of dynamin to constricted CCPs is affected by PP19, we labeled endocytic intermediates with antibodies against dynamin. While labeling of the upper part of the coat is unaffected, labeling at the lower areas of CCPs is decreased significantly (Fig. 2C vs 2D,H–I). The localization of endophilin to lower parts of CCPs remains unchanged (Fig. 2E vs Fig. 2F,H), indicating that the interaction between endophilin and dynamin is important for the localization of dynamin at the neck of the coated pit.

Endophilin and dynamin form a complex on the necks of constricted CCPs in living synapses

To investigate further the organization of dynamin and endophilin on the necks of CCPs just before fission, we microinjected GTPγS into living reticulospinal axons (Evergren et al., 2007). Endocytic intermediates formed in the presence of GTPγS have elongated necks decorated with a striation pattern. Tilting of sections in an electron microscope allowed us to define these striations as protein spirals (Fig. 3B and Fig. 4B,C). We labeled these structures with antibodies against endophilin, dynamin, intersectin and amphiphysin and compared the distributions of gold particles (Fig. 3A,C–G and supplementary material Fig. S1G,H). Interestingly, endophilin localizes predominately to the area of the neck proximal to the coat [Fig. 3A (region I), Fig. 3C,D,G]. Dynamin and amphiphysin, however, are distributed evenly along the spiral (Fig. 3E–G and supplementary material Fig. S1G), and intersectin immunolabeling is confined to the coat region (supplementary material Fig. S1H). The distribution of gold particles signaling for synaptojanin on protein spirals formed in synapses in the presence of GTPγS using the same technique has been reported previously (Haffner et al., 1997). Clusters of synaptojanin 1 immunogold, which have been observed, are clearly different from the heavy, continuous labeling produced by antibodies against dynamin and
the restricted signal produced by immunogold labeling for endophilin observed in our experiments. Thus, before fission, endophilin is concentrated at the base of the coat of the CCPs, where it colocalizes with dynamin. When the dynamin spiral continues to form in the presence of GTP\textsuperscript{\gamma}S, other SH3-domain-containing proteins come into interaction with the PRD of dynamin. One such protein appears to be amphiphysin. As dynamin and endophilin interact directly, this suggests that, in synapses, they assemble into a complex in a restricted part of the spiral proximal to the coat.

**Endophilin and dynamin form a structurally distinct complex, which promotes dynamin lipid binding in vitro**

To investigate further the assembly and structure of an endophilin–dynamin complex, we employed an in vitro model. Previous negative-stain electron microscopy studies have shown that endophilin and dynamin together tubulate liposomes and assemble into spirals (Farsad et al., 2001). When adding dynamin and endophilin to 1,2-dioleoyl-sn-glycero-3-(phospho-l-serine) (hereafter referred to as PS) liposomes, we find that lipid tubules are decorated by protein complexes similar to those observed on the necks of constricted CCPs in vivo, as shown in previous figures (Fig. 3B). Importantly, these endophilin–dynamin complexes are structurally different from those formed by either dynamin or endophilin alone (Fig. 4A,D vs 4F,G). This is further visualized by cryoelectron microscopy (Fig. 4H,I vs 4J,K). Dynamin-decorated tubes have an average diameter and pitch of 43.8±0.42 nm and 13.3±0.26 nm, respectively (mean ± s.e.m.; n=100 tubes; Fig. 4F,J,L,M). Together, endophilin and dynamin assemble into distinct spirals with significantly smaller diameters, 32.1±0.26 nm, as compared with the spirals formed by dynamin alone, and the pitch of the endophilin–dynamin complex spirals is larger, 25.7±0.44 nm (mean ± s.e.m.; n=100 tubes; Fig. 4D,I,L,M). Also, the average inner diameter of endophilin–dynamin-decorated tubes is 8.1 nm.
(Fig. 4L,M), similar to that of the tubes decorated by endophilin alone (Fig. 5G,K). Immunogold labeling of the tubes confirms that both proteins are components of these structures (Fig. 4N,O).

By measuring the protein–lipid tube diameter, and the pitch of the protein spirals, we further confirmed that the endophilin–dynamin complex assembles on liposomes of different lipid compositions, including PS, PS:PC (PC, 1-acyl-2-acyl-sn-glycero-3-phosphocholine), brain total lipid extract plus PIP2 (hereafter referred to as BTLEPIP2) and PS:PL [Plx, 1,2-dioctanoyl-sn-glycero-3-phosphate (1′-myo-inositol-4′-phosphate)] (Fig. 5B,D, and data not shown). Also, after addition of endophilin to dynamin, many ring-like structures are observed in solution at a salt concentration of 150 mM, indicating that the mixed proteins form distinct complexes even in the absence of lipids (supplementary material Fig. S3A).

To investigate whether endophilin aids in the recruitment of dynamin to lipid membranes, we changed the composition of the lipid templates by decreasing the amount of PS, which dramatically reduces the lipid binding and tubulation properties of dynamin (Table 1). After testing different PS:PC ratios, we found that neither dynamin nor endophilin binds or tubulates PS:PC liposomes mixed at a 30:70% ratio as efficiently as PS liposomes (Fig. 5A and Table 1). Mixing the two proteins together, however, increases the amount of dynamin bound to PS:PC lipids by an average of 1.8-fold under these conditions (Fig. 5H; n=8, P<0.05; supplementary material Fig. S4A). The number of tubulated liposomes decorated with endophilin–dynamin complexes is also increased (Fig. 5A vs 5B; supplementary material Fig. S4B). The structural organization of the spirals assembled on this template is similar to that observed in Fig. 4. To test whether a similar improvement of dynamin recruitment might occur under more physiological conditions, we used brain total lipids plus 10% PIP2 (BTLEPIP2) (Fig. 5C vs 5D). A 6.6-fold average increase in dynamin recruitment to liposomes is observed after addition of endophilin (Fig. 5H; n=2; P<0.05).

To determine whether the interaction between the SH3 domain and PRD is important for endophilin–dynamin complex formation on lipid templates, we added PP19 to the endophilin–dynamin mixture. When endophilin and dynamin are pre-incubated with PP19 and then added to PS:PC (30:70% ratio) or BTLEPIP2 liposomes, a significant reduction in dynamin lipid binding is observed. The presence of PP19 results in an average 2.5-fold reduction in dynamin binding to PS:PC liposomes (Fig. 5I; n=4; P<0.05) and an average 4.7-fold reduction in dynamin binding to total brain lipid extract liposomes (Fig. 5I; n=2; P<0.05). Furthermore, electron microscopy (EM) analysis shows that endophilin–dynamin complexes are disrupted in the presence of PP19 and large gaps in the protein decoration of lipid tubes are visible, whereas addition of 1 mM GTPγS has no effect (Fig. 5E vs 5F; supplementary material Fig. S5D). Furthermore, addition of PP19 has no effect on lipid binding and tubulation properties of the
individual proteins (supplementary material Fig. S5A–C) and does not have any effect on endophilin dimerization in solution, as revealed by size-exclusion chromatography and crosslinking of endophilin (supplementary material Fig. S5E). Recombinant ΔPRD-dynamin fails to form the distinct dynamin–endophilin spirals seen on lipids incubated with wild-type dynamin. Additionally, endophilin does not facilitate the lipid binding of ΔPRD-dynamin (Fig. 5G,I). Thus, the interaction between the endophilin SH3 domain and dynamin PRD mediates assembly of the proteins into a structurally distinct complex on lipids that leads to the formation of protein–lipid tubes of a smaller diameter and facilitates further recruitment of dynamin to lipids.

Blocking endophilin SH3 domain interactions in a living synapse perturbs the assembly of an endophilin–dynamin complex

To investigate in vivo how blocking of endophilin SH3 domain interactions affects the assembly of the endophilin–dynamin complex, which promotes fission in intact synapses, we microinjected GTPγS into lamprey giant axons in the presence of PP19. GTPγS, microinjected alone (n=10), strongly blocks endocytosis at the fission step. Numerous constricted coated pits with long necks decorated with protein spirals are observed at the periaactive zone of these synapses (Fig. 6A1–D).

In three isolated spinal cord preparations, PP19 and GTPγS were microinjected into the same axon at distances of 200 μm to 2 mm from each other, as shown in Fig. 6C. The distance between the two injection sites was adjusted to allow analysis of each compound at separate sites (which served as a control) and to achieve mixing of the two compounds to investigate the effect of the double-injection in the same axon. To monitor the diffusion of the reagents within the axon, Texas Red was added as an injection marker. Extracellular stimulation at 5 Hz was initiated to induce cycling of synaptic vesicles. PP19 was injected first, to block selectively interactions of the endophilin SH3 domain, followed by stimulation at 5 Hz to increase the number of accumulated constricted coated pits at periaactive zones before introduction of GTPγS (Farsad et al., 2001), which was injected 10 minutes after the onset of stimulation. The stimulation was maintained for an additional 20 minutes before fixation. For controls, neighboring axons were microinjected with Texas Red alone or Texas Red and GTPγS to allow comparison of the morphology of endocytic intermediates at the same distance from the site of injection in the same spinal cord preparation (Fig. 6A; supplementary material Fig. S2F). Ultrastructural analysis of clathrin-coated intermediates was performed at the site of injection of each compound (Fig. 6C, ‘area 1’ and ‘area 2’) and at several levels (up to eight) where PP19 and GTPγS mixed (Fig. 6C, ‘area 3’). Fig. 6B1–4 shows the effects of double-injection of PP19 and GTPγS. A massive accumulation of constricted CCPs with short necks is observed (Fig. 6D,E). This is in clear contrast to adjacent axons microinjected with GTPγS alone at the same level (‘area 3’), where only long necks decorated by dynamin spirals were found (Fig. 6A). Thus, the average neck length of the CCPs is significantly lower in PP19–GTPγS double-injected axons as compared with the
ones found in axons injected with GTPγS alone, as well as in control non-injected axons (Fig. 6E). Thin spirals, as compared with those observed in control non-injected axons, are occasionally detected at high magnification decorating the short necks (Fig. 6B4, arrow). These spirals are also thinner than the ones induced by GTPγS alone (Fig. 3B and Fig. 6A2). We also found that the amount of dynamin detectable at these necks is reduced by 84% (1.9±0.24 vs 0.3±0.12 gold particles per lower CCP area, in control synapses compared with synapses in axons injected with PP19 and GTPγS; mean ± s.e.m., n=35 CCPs, P<0.001). Moreover, free clathrin-coated vesicles, observed in PP19-only microinjected synapses, are no longer present, suggesting that PP19 and GTPγS together completely blocked synaptic vesicle recycling at the fission step. Injection of Texas Red alone has no effect on the ultrastructure of stimulated synapses (supplementary material Fig. S2E vs Fig. S2F).

These experiments further indicate that endophilin recruits dynamin to the CCP neck and that the assembly of an endophilin–dynamin complex is a crucial step in the fission reaction in a living synapse.

Discussion

Our experiments show that, in living synapses, endophilin 1 is one of the proteins selectively recruited to the neck of clathrin-coated vesicles during recycling of synaptic vesicles. The inhibition of
endophilin SH3-domain interactions by PP19 reduces the amount of dynamin at the neck and inhibits budding of synaptic vesicles. This further implies that the dynamin–endophilin interaction is of functional importance at this location. PP19 also inhibits the formation of CCPs with elongated necks in the presence of GTP$\gamma$S. Instead, an accumulation of constricted CCPs with short necks is observed in these synapses. Also, no free clathrin-coated vesicles were found, which is indicative of a complete block of fission. Taken together, these data indicate that endophilin and dynamin are important players in the membrane budding-reaction in synapses.

The organization of the endophilin–dynamin interaction at the neck of the CCPs seems to be dictated by endophilin. Our experiments show that endophilin localizes in the synaptic vesicle pool at rest and accumulates in the periactive zone during recycling of synaptic vesicles (Shupliakov, 2009). Endophilin is probably recruited to CCPs by means of its BAR domain (Andersson et al., 2010; Ringstad et al., 1999). Recent studies in non-neuronal cells have shown that formation of an actin matrix around CCPs is required for efficient endophilin recruitment (Ferguson et al., 2009).

Interestingly, a number of endophilin-interacting proteins – for example, CD2AP or CIN85 (Ferguson et al., 2009) and oligophrenin-1 (Nakano-Kobayashi et al., 2009) – interact with the actin cytoskeleton, suggesting that endophilin might be guided to the periactive zone by one of these proteins as the actin matrix assembles. The role of the endophilin BAR domain seems to be unique. Supporting this, Drosophila endophilin-null mutants cannot

| Table 1. Endophilin and dynamin lipid binding and tubulation |
|----------------------------------------------------------|
|              | Dynamin | Endophilin | Dynamin plus endophilin |
| PS:PC (70:30) | ++      | ++         | +++                    |
| PS:PC (30:70) | ++      | ++         | +++                    |
| PS:PC (20:80) | +       | +          | ++                     |
| BTLE         | –       | –          | –                      |
| BTLEPIP$_2$  | –       | –          | ++                     |

+: Some tubes and <50% of the protein in the pellet after sedimentation.
++: Tubes and ≥50% of the protein in the pellet after sedimentation.
+++: Many tubes and >90% of the protein in the pellet after sedimentation.
be rescued with endophilin constructs containing F-BAR domains of other proteins or the N-BAR domain of amphiphysin (Jung et al., 2010).

The interaction between endophilin and dynamin results in the formation of a complex, which can be visualized as a spiral at the bottom of the coat after microinjection of GTP\(_\gamma\)S. The organization of this protein spiral has strong similarities with the complex between endophilin 1 and dynamin 1 observed in vitro. To form this complex, the interaction between the endophilin SH3 domain and the dynamin PRD is required, as perturbations of this interaction...
both in vitro and in situ reduce dynamin recruitment to lipid templates and to presynaptic membranes in living synapses, respectively.

In vitro studies have shown that the endophilin–dynamin complex is stable after addition of GTP (Farsad et al., 2001). This stable complex might form a non-constrictable spiral, similar to the one formed by Rvs proteins in budding yeast, which precedes the fission reaction (Liu et al., 2009). Imaging and molecular modeling experiments suggest that, in budding yeast, fission is triggered by lipid phase separation achieved by hydrolysis of PIP2 driven by the phosphatase sgl2 (synaptojanin-like protein 2), which enhances curvature, and the assembly of the actin cytoskeleton (Liu et al., 2009). Although it cannot be excluded that hydrolysis of PIP2 might promote fission during recycling of synaptic vesicles, there is strong evidence that dynamin plays the major role (Ferguson et al., 2009). Dynamin depletion in non-neuronal cells results in the formation of coated pits with elongated necks decorated with spirals containing endophilin and actin (Ferguson et al., 2009). In these cells, fission cannot occur in the presence of the phosphatase synaptojanin, thus supporting the idea that regulation of fission in eukaryotic cells is more complex than in yeasts (Conibear, 2010).

Endophilin is not the only protein that might recruit synaptojanin in nerve terminals. It has been shown recently that one mechanism of synaptojanin recruitment to the clathrin coat in neurons involves intersectin 1, and the binding of synaptojanin to intersectin is regulated by the adaptor protein complex AP2 (Peichstein et al., 2010). This allows for a possible synaptojanin–endophilin interaction, which is crucial for efficient uncoating of synaptic vesicles, to occur directly after fission, following disassembly of the endophilin–dynamin complex. Further studies are needed, however, to verify this hypothesis.

How might the formation of a non-constrictable complex of endophilin–dynamin promote fission in synapses? Spontaneous polymerization of dynamin can be initiated on tubes with radii ranging between 10 and 30 nm, but not on larger tubes (Roux et al., 2009). We show that the diameter of the endophilin–dynamin-decorated lipid tube is within this range. In the presence of endophilin, dynamin is efficiently recruited to lipid templates, including total brain lipids, which allows us to propose a model for the sequence of events leading to fission of the neck of CCPs in vertebrate synapses (Fig. 7): endophilin accumulates at the base of the coat, where it serves as a template for dynamin. Together, they assemble into a ‘pre-fission complex’. This complex shapes the lipid neck to an average inner diameter of 8.1 nm (3.8–14.8 nm) and brings the neck to the hemi-fission state (Lenz et al., 2009). More dynamin is recruited to the neck and oligomerizes into a ‘constrictable’ spiral. The smaller diameter of the pre-fission complex might accelerate membrane fission by conformational changes in the constrictable dynamin spiral below the pre-fission complex. The pre-fission complex might also function as a template for rapid spiral disassembly. Synaptojanin, recruited after, or possibly during, the fission reaction might compete with dynamin for binding to endophilin and thus aid the disassembly of the pre-fission complex.

Materials and Methods

Proteins

Full-length cDNA encoding human neuronal dynamin (dynamin-1) (obtained from A. van der Bliek, UCLA, CA) was subcloned into the pBlueBac baculovirus expression vector (Invitrogen) and expressed in TN5-JE cells (modified from Warnock et al. (Warnock et al., 1995)). Cells were harvested 48 hours after infection, pelleted and frozen in liquid nitrogen before purification. Cell pellets were thawed in 25 ml of HCB100 (20 mM Hepes (pH 7.2), 2 mM EGTA, 1 mM MgCl2, 100 mM NaCl) with protease inhibitor cocktail (Roche), calpain inhibitor (85 μM) and 25 μl of pepabloc (4-2-amioethyl benzenesulfonyl fluoride hydrochloride, Sigma, 100 mg/ml). Cells were broken open by nitrogen cavitation at ~3400 kPa for 25 minutes in a cold room, and centrifuged at 20,000 g in a 70.1 Ti rotor for 1 hour at 4°C. Supernatant was collected and saturated by adding ammonium sulfate (in HCB100) drop-wise while stirring until a concentration of 35% (ammonium sulfate) was reached. The solution was incubated for 1 hour at 4°C before centrifugation at 10,000 g for 15 minutes at 4°C. The ammonium sulfate pellet was resuspended in 30 ml HCB50 and centrifuged at 8000 g for 10 minutes to remove aggregates. The supernatant was applied to a High Q ion exchange column, washed with HCB50 followed by HCB100 and eluted with HCB250. The peak fractions were pooled, loaded onto a HAP column, washed with HCB250 until flow-through reached baseline, washed with 200 mM KH2PO4 (pH 7.2), and protein was eluted in 400 mM KH2PO4. Peak fractions were pooled, pepabloc was added and aliquots were frozen in liquid nitrogen and stored at ~80°C. APRD-dynamin construct, obtained from Sandy Schmid (The Scripps Research Institute, CA), was produced by introducing a stop codon at residue 751 into the wild-type dynamin sequence. The construct was cloned into the pBlueBac baculovirus expression vector (Invitrogen), expressed and purified from TN5-JE cells as described above for full-length dynamin.

pGEX-2 constructs of rat full-length endophilin or its SH3 domain were received from Volker Haucke. Full-length endophilin was subcloned into the pQE-6P-2 vector before both constructs were transformed into competent Escherichia coli B21(DE3) cells (Invitrogen). The cells were plated onto 100 μg/ml ampicillin plates and incubated at 37°C overnight. Colonies were resuspended in 5 ml LB media with 100 μg/ml ampicillin and grown at 37°C overnight on a shaker. The bacteria suspension was diluted 1:100 in YTG media with 10% glucose and 100 μg/ml ampicillin and grown at 37°C with O2 until the OD590 was 0.6–0.8. Isopropyl β-D-1-thiogalactopyranoside (IPTG; 0.1–0.3 mM) was added and the cells were grown for another 1.5 hours at 30°C. The suspension was centrifuged at 5500 g for 10 minutes. Cell pellets were frozen in liquid nitrogen and stored at ~80°C. Cell pellets were thawed and resuspended in 40 ml PBS with a protease inhibitor cocktail (Sigma). The suspension was frozen in liquid nitrogen and thawed twice before sonication at 4°C. 1% Tween-20 was added, and the suspension was incubated at 4°C for 30 minutes on a shaker following centrifugation at 18,000 g for 30 minutes. Supernatant was filtered and applied to 1 ml Fastflow GST-ovalbumin (GE Healthcare). The column was washed with PBS until baseline OD280 level was regained. GST-endophilin or GST-SH3 domain of endophilin was eluted in 10 mM glutathione in 50 mM Tris, pH 8. The GST-tag was cleaved with PreScission protease enzyme (GE Healthcare) according to the manufacturer’s instructions. Protein was aliquoted, frozen in liquid nitrogen and stored at ~80°C.

Antibodies and reagents

All lamprey antibodies were protein A and affinity purified and stored in stocks at concentrations of 1–2 mg/ml. Antibodies against lamprey dynamin GTGase domain (Evergreen et al., 2007; Evergreen et al., 2004) were diluted 1:100 in blocking solution containing 1% BS A and 1% HSA in Tris–PBS (pH 7.2) for electron microscopy and 1:10,000 for western blotting. Antibodies against the lamprey endophilin SH3 domain, rat endophilin and synaptojanin have been characterized previously and were diluted 1:100 for electron microscopy and 1:1000 for western blotting.
In injection buffer to a final concentration of 30 mM in the electrode. GTP was injected into the same four axons (injected by PP19). GTP

was treated according to the Swedish Animal Welfare Act (SFS 1988: 534), as approved by the Local Animal Research Committee of Stockholm. All efforts were made to minimize animal suffering and to reduce the number of animals used. The Microinjection experiments

Lampreys (Lampetra fluviatilis) were kept in aquaria at 4°C. All animals were treated according to the Swedish Animal Welfare Act (SFS 1988: 534), as approved by the Local Animal Research Committee of Stockholm. All efforts were made to minimize animal suffering and to reduce the number of animals used. The

Microinjection experiments

Lampreys (Lampetra fluviatilis) were kept in aquaria at 4°C. All animals were treated according to the Swedish Animal Welfare Act (SFS 1988: 534), as approved by the Local Animal Research Committee of Stockholm. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Microinjection experiments

Lampreys (Lampetra fluviatilis) were kept in aquaria at 4°C. All animals were treated according to the Swedish Animal Welfare Act (SFS 1988: 534), as approved by the Local Animal Research Committee of Stockholm. All efforts were made to minimize animal suffering and to reduce the number of animals used.
(10 mM HEPES buffer, pH 7.4, 100 mM KCl, 2 mM MgCl2, and 1% Triton X-100) plus Pefablock (Sigma), E-64, leupeptin, aprotinin (Sigma) or 1 mM PMSF and 10–300 μM PP19 for 1–2 hours at 4°C on a rotating wheel. Samples were washed three times with buffer A and three times with buffer A without Triton X-100, eluted with sample buffer and analyzed by SDS-PAGE, followed by detection with antibodies against dynamin, synaptojanin and synapsin.

**Pull-down experiment**

PP19 peptide was bound to SulfoLink beads (Pierce), as directed by the manufacturer, and incubated subsequently with 10% lamprey brain extract in buffer B (20 mM HEPES pH 7.4, 150 mM KCl, 10 mM EDTA, 5 mM MgCl2, 0.5% Triton X-100) for 2 hours at 4°C on a rotating wheel. The supernatant was removed and beads were resuspended in fresh brain extract. Samples from both pull-downs were washed four times with PBS, eluted with sample buffer and analyzed by SDS-PAGE, followed by detection with antibodies against dynophilin, amphiphysin and intersectin.

This study was supported by the Swedish Research Council grants 13473 and 20587, Linne Center for Developmental Biology and Regenerative Medicine, Wallenberg’s Stiftelse and Hjärnfonden (O.S.); Intramural NIDDK, NIH (J.E.H.). A.S. received stipends and support from KI-NIH exchange program and Fernström’s Stiftelse. We thank V. Haucke for endophilin constructs, N. Tomilin for help with electron microscopy, Peter Löw and Arndt Pechstein, Karolinska Institutet, for expert advice and comments, and Pampa Ray, NIH, for help with the purification of dynamin. Deposited in PMC for release after 12 months.

**Supplementary material available online at**

http://jcs.biologists.org/cgi/content/full/124/1/133/DC1

**References**

Andersson, F., Löw, P. and Brodin, L. (2010). Selective perturbation of the BAR domain of endophilin impair synaptic vesicle endocytosis. *Synapse* 64, 556-560.

Anggono, V. and Robinson, P. J. (2007). Syndapin 1 and endophilin I bind overlapping proline-rich regions of dynamin I: role in synaptic vesicle endocytosis. *J. Neurochem.* 102, 931-943.

Cookhead, E. (2010). Converging views of endocytosis in yeast and mammals. *Curr. Opin. Cell Biol.* 22, 513-518.

Cremona, O., Di Paolo, G., Wenk, M. R., Luthi, A., Kim, W. T., Takei, K., Daniell, L., Nemoto, Y., Shears, S. B., Flavell, R. A. et al. (1999). Essential role of phosphoinositide metabolism in synaptic vesicle recycling. *Cell* 99, 179-188.

Dickman, D. K., Horne, J. A., Meinertzhagen, I. A. and Schwarz, T. L. (2009). Coordinated actions of actin and dynamin 1 in synaptic vesicle endocytosis. *J. Cell Biol.* 155, 169-174.

Evergren, E., Tomilin, N., Vasilyeva, E., Simen, D. A., Dabis, I., Deckers, S. et al. (2008). The mechanochemistry of endophilin-BAR domain-mediated membrane curvature. *FEBS Lett.* 583, 3839-3846.

Liu, J., Sun, Y., Drubin, D. G. and Oster, G. F. (2009). The mechanochemistry of endophilin. *Proc. Natl. Acad. Sci. USA* 106, 17555-17560.

Metselaar, J. A. and Hinshaw, J. E. (2009). Dynamins at a glance. *J. Cell Sci.* 122, 3427-3431.

Mettlen, M., Stoebner, M., Loerke, D., Antonescu, C. N., Danuser, G. and Schmidt, S. L. (2009). Endocytic accessory proteins are functionally distinguished by their differential effects on the maturation of clathrin-coated pits. *Mol. Biol. Cell* 20, 3251-3260.

Nakano-Kobayashi, A., Kasi, N. K., Newey, S. E. and Van Aelst, L. (2009). The Rh-linked mental retardation protein OPHN1 controls synaptic vesicle endocytosis via endophilin A1. *Curr. Biol.* 19, 1131-1139.

Pechstein, A., Baejic, J., Vaheedi-Faridi, A., Gromova, K., Sundborger, A., Tomilin, N., Krainer, G., Vorontsova, O., Schafer, J. G., Owe, S. G. et al. (2010). Regulation of synaptic vesicle recycling by complex formation between intersectin 1 and the clathrin adaptor complex AP2. *Proc. Natl. Acad. Sci. USA* 107, 4206-4211.

Ringstad, N., Gad, H., Low, P., Di Paolo, G., Brodin, I., Shupliakov, O. and De Camilli, P. (1999). Endophilin/SH3p4 is required for the transition from early to late stages in clathrin-mediated synaptic vesicle endocytosis. *Neuron* 24, 143-154.

Ringstad, N., Nemoto, Y. and De Camilli, P. (2001). Differential expression of endophilin 1 and 2 dimmers at central nervous system synapses. *J. Biol. Chem.* 276, 40424-40430.

Rodal, A. A. and Littleton, J. T. (2008). Synaptic endocytosis: illuminating the role of clathrin assembly. *Curr. Biol.* 18, R259-R261.

Roux, A., Koster, G., Lenz, M., Sorre, B., Manneville, J. B., Nasoy, P. and Bassereau, P. (2009). Membrane curvature controls dynamin polymerization. *Proc. Natl. Acad. Sci. USA* 107, 4141-4146.

Schuske, K. R., Richmond, J. E., Matthias, D. S., Davis, W. S., Runz, S., Rube, D. A., van der Blik, A. M. and Jorgensen, E. M. (2003). Dynamin is required for synaptic vesicle endocytosis by localizing synaptojanin. *Neuron* 40, 749-762.

Shupliakov, O. (2009). The synaptic vesicle cluster: a source of endocytic proteins during neurotransmitter release. *Neuroscience* 158, 204-210.

Song, W. and Zinsmaier, K. E. (2003). Endophilin and synaptojanin hook up to promote synaptic vesicle endocytosis. *Neuron* 40, 665-667.

Verstrekken, P., Jauroulff, O., Lloyd, T. E., Atkinson, R., Zhou, Y., Mezini, I. A. and Bellen, H. J. (2002). Endophilin mutations block clathrin-mediated endocytosis but not neurotransmitter release. *Cell* 109, 101-112.

Verstrekken, P., Koh, T. W., Schulze, K. L., Zhai, R. G., Hiesinger, P. R., Zhou, Y., Mehta, S. Q., Cao, Y., Roos, J. and Bellen, H. J. (2003). Synaptojanin is recruited by endophilin to promote synaptic vesicle uncoating. *Neuron* 40, 733-748.

Warnock, D. E., Terlecky, L. J. and Schmid, S. L. (1995). Dynamin GTPase is stimulated by crosslinking through the C-terminal proline-rich domain. *EMBO J.* 14, 1322-1328.