

Generation of high curvature membranes mediated by direct endophilin bilayer interactions

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Endophilin 1 is a presynaptically enriched protein which binds the GTPase dynamin and the polyphosphoinositide phosphatase synptotjanin. Perturbation of endophilin function in cell-free systems and in a living synapse has implicated endophilin in endocytic vesicle budding (Ringstad, N., H. Gad, P. Low, G. Di Paolo, L. Brodin, O. Shupliakov, and P. De Camilli. 1999. *Neuron*. 24:143–154; Schmidt, A., M. Wolde, C. Thiele, W. Fest, H. Kratzin, A.V. Podtelejnikov, W. Witke, W.B. Huttner, and H.D. Soling. 1999. *Nature*. 401:133–141; Gad, H., N. Ringstad, P. Low, O. Kjaerulff, J. Gustafsson, M. Wenk, G. Di Paolo, Y. Nemoto, J. Crun, M.H. Ellisman, et al. 2000. *Neuron*. 27:301–312). Here, we show that purified endophilin can directly bind and evaginate lipid bilayers into narrow tubules similar in diameter to the neck of a clathrin-coated bud, providing new insight into the

mechanisms through which endophilin may participate in membrane deformation and vesicle budding. This property of endophilin is independent of its putative lysophosphatidic acid acyl transferase activity, is mediated by its NH₂-terminal region, and requires an amino acid stretch homologous to a corresponding region in amphiphysin, a protein previously shown to have similar effects on lipid bilayers (Takei, K., V.I. Slepnev, V. Haucke, and P. De Camilli. 1999. *Nat. Cell Biol.* 1:33–39). Endophilin cooligomerizes with dynamin rings on lipid tubules and inhibits dynamin's GTP-dependent vesiculating activity. Endophilin B, a protein with homology to endophilin 1, partially localizes to the Golgi complex and also deforms lipid bilayers into tubules, underscoring a potential role of endophilin family members in diverse tubulovesicular membrane-trafficking events in the cell.

Introduction

A major pathway of synaptic vesicle retrieval after exocytosis is clathrin-mediated endocytosis (Heuser and Reese, 1973). This specialized version of a general endocytic pathway is necessary for the maintenance of the synaptic vesicle pool, and perturbation of this process results in rapid depletion of synaptic vesicles (Koenig and Ikeda, 1989; Shupliakov et al., 1997; Gad et al., 2000). The 100-kD GTPase, dynamin, has been implicated in the “fission” stage of clathrin-mediated endocytosis (Koenig and Ikeda, 1989), and disruption of dynamin function results in an accumulation of electron-dense “collars” at the tubular neck of deeply invaginated clathrin-coated buds (Koenig and Ikeda, 1989; Takei et al., 1995). These collars are enriched with dynamin, as well as two of the major SH3 domain-containing dynamin binding

partners at the synapse: endophilin 1 (endophilin) and amphiphysin 1 (amphiphysin) (David et al., 1996; de Heuvel et al., 1997; Ringstad et al., 1997). Recombinant dynamin 1 and amphiphysin have been shown to deform artificial lipid bilayers into narrow tubules, both independently as well as cooperatively in a complex, likely reflecting a role for these proteins in membrane binding and deformation at the neck of clathrin-coated pits (Takei et al., 1998, 1999).

Endophilin has been implicated in many stages of clathrin-mediated synaptic vesicle endocytosis, from early events generating membrane curvature, to later stages such as vesicle fission and uncoating (Ringstad et al., 1999; Gad et al., 2000). Selective depletion of endophilin from rat brain cytosol inhibited the generation of synaptic-like microvesicles (SLMV)* in broken PC12 cells, and markedly reduced the formation of dynamin-coated tubules on synaptic membranes in the presence of ATP/GTPγS (Ringstad et al., 1999; Schmidt et al., 1999). Therefore, we tested whether endophilin plays a direct role in the generation of membrane

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Key words: SH3P4; dynamin; endocytosis; RICH; endophilin B1

*Abbreviations used in this paper: AT, acyl transferase; GST, glutathione S-transferase; LPA, lysophosphatidic acid; PH, pleckstrin homology; SMLV, synaptic-like microvesicle.

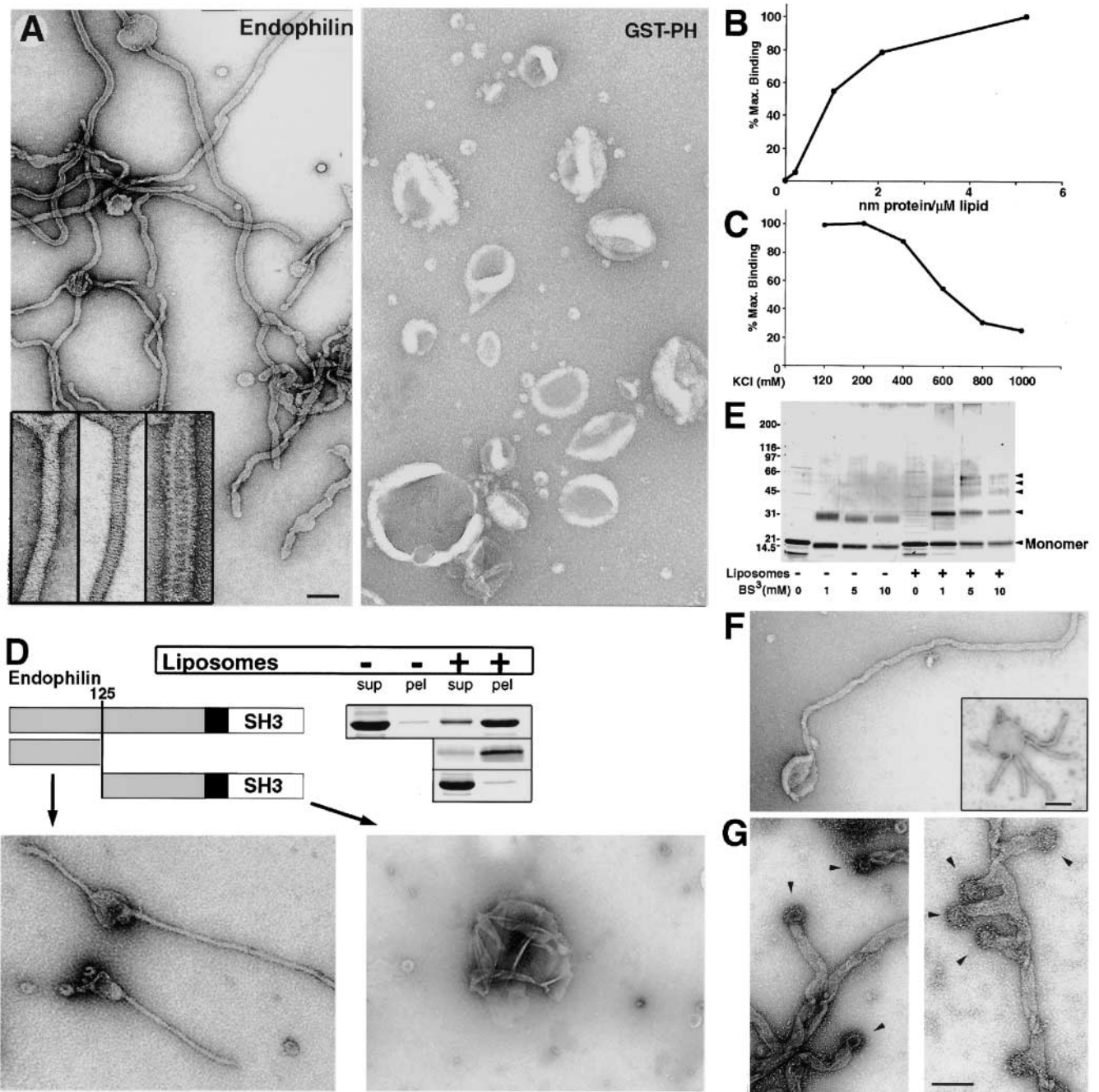


Figure 1. Endophilin directly binds and deforms lipid bilayers. (A) Negative stain EM of liposomes composed of a brain lipid extract after addition of recombinant endophilin 1 or of the PH domain of PLC δ (GST-tagged) as a control, respectively. Liposomes were incubated for 15 min at 37°C with the purified proteins. Endophilin deformed the spherical liposomes into long tubules with 20–100-nm diameters. Inset shows a comparison of the protein coats observed on the tubules generated by endophilin (left), amphiphysin (center), and dynamin (right). (B) Saturable binding of endophilin to liposomes with increasing concentrations of endophilin. (C) Salt sensitivity of endophilin binding to liposomes with increasing [KCl]. (D) The NH₂-terminal region of endophilin is necessary and sufficient for lipid binding and tubulation. Deletion constructs were incubated with or without liposomes as shown, and cosedimentation with liposomes was determined. In the absence of liposomes, full length endophilin remains in the supernatant. The grey area in the domain cartoon of endophilin represents the portion of the protein most highly conserved amongst endophilin isoforms. Amino acids 1–125 within this region bind and tubulate liposomes. (E) The NH₂-terminal 125 amino acid fragment was incubated with or without liposomes as shown, with increasing concentrations of the chemical cross-linker BS³ for 20 min at 37°C. Samples were then prepared for SDS-PAGE analysis on a 4–12% gradient gel. In the absence of liposomes, the endophilin construct crosslinks into a dimer, while in the presence of liposomes higher molecular weight products are formed suggestive of oligomerization (see arrowheads). (F) The lipid tubulation activity of endophilin does not require LPA-AT activity. Endophilin was incubated with synthetic liposomes lacking both LPA and arachidonoyl coA, the two substrates for its reported LPA-AT activity. The inset shows a liposome composed of brain lipids after incubation with endophilin for 20 min at 4°C. (G) Clathrin-coated buds are observed on endophilin tubules when liposomes are incubated with a mixture of purified clathrin-coat proteins and recombinant endophilin 1. Bars: (A) 100 nm; 70 nm for inset; (F) 100 nm; (G) 200 nm.

curvature in a similar fashion to dynamin and amphiphysin. Furthermore, we tested whether this property was shared by other proteins with homology to endophilin.

Results and discussion

Endophilin directly binds and deforms lipid bilayers

Using a liposome-based assay previously shown to support generation of coated intermediates of clathrin-mediated endocytosis (Takei et al., 1998, 1999), purified recombinant endophilin efficiently and robustly deformed lipid bilayers into tubules with outer diameters ranging between 20–100 nm (Fig. 1 A). The tubule surfaces were decorated by tightly packed thin transverse striations, reminiscent of the coat observed on tubules generated by recombinant amphiphysin (Fig. 1 A, see inset for a comparison of the endophilin, amphiphysin, and dynamin coats on tubules) (Takei et al., 1999). The recombinant pleckstrin homology (PH) domain of PLC δ , a known phospholipid binding domain, did not deform the lipid bilayers into tubes, thereby distinguishing this activity from nonspecific changes due to protein membrane interactions (Fig. 1 A). Endophilin binding to liposomes appeared to saturate at a molar ratio of under 1:200 (protein to lipid) in a liposome sedimentation assay (Fig. 1 B), and displayed a nonlinear loss of binding with increasing salt (Fig. 1 C).

The NH₂ terminus of endophilin is necessary and sufficient for tubulation and lipid binding

Deletion constructs of endophilin defined amino acids 1–125 as both necessary and sufficient for tubulation (Fig. 1 D). Correspondingly, this 125–amino acid fragment was also seen to cosediment with liposomes, whereas a construct that lacked this fragment stayed in the supernatant (Fig. 1 D). In the presence of a chemical cross-linker, bis(sulfosuccinimidyl) suberate (BS³), the endophilin NH₂-terminal fragment cross-linked into higher order oligomers in a liposome-stimulated manner, whereas in the absence of liposomes, the fragment cross-linked only to a dimer (Fig. 1 E). Similar results were obtained using a second cross-linker, EDC, and with the full length protein (unpublished data). The presence of coated striations on the tubules and the cross-linking data together suggest that endophilin generates bilayer curvature by oligomerizing on the surface of liposomes.

Membrane tubulation by endophilin is independent from LPA-AT activity

The property of endophilin to deform the lipid bilayer is independent from its reported lysophosphatidic acid (LPA)–acyl transferase (AT) activity (Schmidt et al., 1999). Endophilin tubulated synthetic liposomes devoid of the putative substrates for the LPA-AT reaction, arachidonoyl-CoA and LPA (Schmidt et al., 1999) (Fig. 1 F). Furthermore, endophilin tubulated liposomes in a reaction incubated on ice, minimizing any potential enzymatic contribution (Fig. 1 F, inset). Thus, bilayer deformation may represent a thermodynamically favorable transition mediated by endophilin polymerization at the liposome surface, rather than by the active enzymatic generation of specific membrane microdomains.

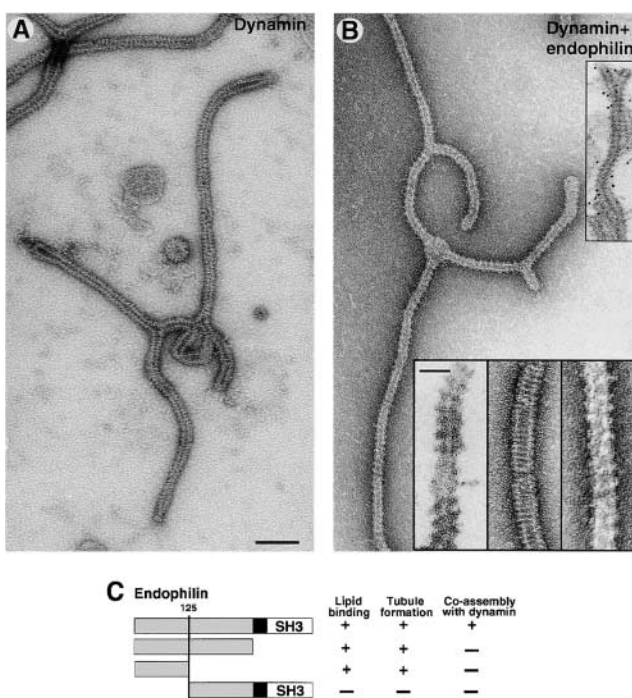


Figure 2. Complex of endophilin with dynamin along membrane tubules. (A and B) The tubular coat produced by dynamin is modified by the presence of endophilin. Liposomes were incubated for 15 min at 37°C with affinity-purified dynamin (A) or with dynamin plus recombinant endophilin (B). The top inset of (B) shows the immunogold localization of endophilin on dynamin-coated tubules formed by incubating synaptic membranes with brain cytosol, ATP, and GTP γ S. See also Ringstad et al. (1999). The bottom inset of (B) shows a comparison of the tubules generated on liposomes by brain cytosol, ATP, and GTP γ S (left, thin section), with those generated by purified dynamin (center, negative stain) and dynamin plus recombinant endophilin (right, negative stain). Note the similarity of the electron-dense rings produced by brain cytosol with those formed by the dynamin–endophilin complex. (C) Both the NH₂-terminal lipid binding region and the SH3 domain of endophilin (which binds dynamin) are required to form a complex of endophilin and dynamin on the lipid bilayer. Bars: (A) 200 nm; (B) 50 nm.

Incubation of liposomes, purified clathrin coat proteins, and endophilin-generated tubules associated with clathrin-coated buds, consistent with a potential role for this protein in generating tubular membrane curvature at the neck of the nascent clathrin-coated pit (Fig. 1 G). Similar observations were made for amphiphysin (Takei et al., 1999). Of note, amphiphysin has binding sites for clathrin and the endocytic clathrin adaptor, AP2 (Slepnev et al., 2000), but we have not observed binding to clathrin or to clathrin adaptors by endophilin.

Endophilin and dynamin form a protein complex on bilayer tubules

Since endophilin, like amphiphysin, interacts with dynamin via its SH3 domain, we investigated whether dynamin and endophilin could form a complex on liposomes, similar to what has been described for dynamin and amphiphysin (Takei et al., 1999). The coat generated by affinity-purified dynamin 1 on bilayer tubules was represented by rings with an average spacing of 132 ± 5 Å (Fig. 2 A), in agreement

with the previously reported ring spacing determined by electron diffraction (Sweitzer and Hinshaw, 1998). Liposomes incubated with both recombinant endophilin and purified dynamin created a different ring morphology (Fig. 2 B, and lower inset). The rings were thicker, and had an average spacing of $202 \pm 15 \text{ \AA}$, a difference of $>50\%$ compared with the rings formed by dynamin alone (Fig. 2, A and B). This is similar to the ring morphology generated by the dynamin–amphiphysin complex (Takei et al., 1999) and by brain cytosol with ATP/GTP γ S (Takei et al., 1995, 1998). Such a finding supports the presence of a multiprotein complex in the thick electron-dense rings observed at the neck of clathrin-coated pits on synaptic membranes, and explains the colocalization of endophilin with dynamin on coated tubules formed by incubating synaptic membranes with brain cytosol and ATP/GTP γ S (Fig. 2 B, upper inset) (Ringstad et al., 1997, 1999). Both the NH₂-terminal lipid binding domain of endophilin and its COOH-terminal dynamin-binding SH3 domain were necessary for the formation of the complex with dynamin on the membranes, since neither domain alone could reproduce the phenomenon (Fig. 2 C).

Lipid binding and tubulation by endophilin requires an amphipathic stretch homologous to a corresponding region in amphiphysin

We were intrigued by the similarities in the membrane deformation properties of endophilin and amphiphysin (Fig. 3 D), and in the tubular coats which they form (Fig. 1 A, inset). The two proteins have an overall similar domain structure, each with a phylogenetically highly conserved NH₂-terminal region comprising predicted α -helices and coiled-coils, and a COOH-terminal SH3 domain which binds dynamin and synaptojanin (Fig. 3 B). Furthermore, the membrane-tubulating properties of both proteins map to their respective NH₂-termini (Takei et al., 1999; and this study). We explored whether endophilin and amphiphysin share any primary sequence similarity within this region. A BLAST alignment of full-length endophilin 1 and amphiphysin 1 (both rat) identified the highest scoring region to be a 29 amino acid stretch (41% identity and 75% similarity) within the first 35 amino acids of endophilin and the first 41 amino acids of amphiphysin (Fig. 3 A). Deleting most of this region of homology abolished binding to liposomes (Fig. 3 C) and tubulation (unpublished data) for both endophilin and amphiphysin. Secondary structure algorithms (nnpredict and PSIPRED) for this 29 amino acid stretch predict α -helices and random coils. When plotted on a helical wheel, a similar amphipathic pattern emerged for both proteins, with a hydrophobic patch and an opposite hydrophilic face consisting of several basic residues (Fig. 3 A). This feature may allow this domain to interact with phospholipid headgroups via the hydrophilic face, and then to partially embed into the bilayer via the hydrophobic patch in a manner which favors membrane deformation. A point mutation of a conserved hydrophobic residue to an acidic residue (phenylalanine to glutamate) within this putative hydrophobic patch in endophilin abolished both lipid binding and tubulation, whereas a conservative hydrophobic to hydrophobic point mutation (phenylalanine to tryptophan) of the same residue preserved both lipid binding and tubulation (Fig. 3, E and F).

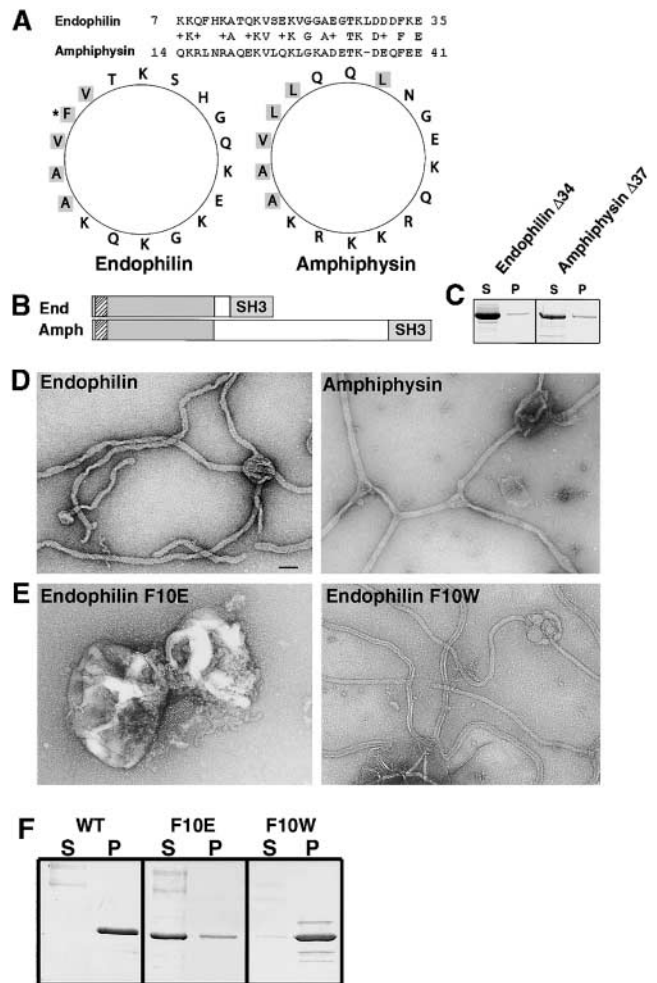


Figure 3. An endophilin amino acid stretch homologous to a corresponding region in amphiphysin is required for lipid binding and tubulation. (A) BLAST alignment of rat endophilin 1 and amphiphysin 1 reveals a region of homology within the NH₂ terminus of both proteins. Plotting this region, which is predicted to form α -helices and random coils, along a helical wheel reveals a similar amphipathic pattern. Hydrophobic residues are highlighted in grey. Asterisk indicates phenylalanine targeted for point mutations. (B) Domain structure of endophilin and amphiphysin. The grey area corresponds to a conserved region of predicted α -helices and coiled-coils in each protein, respectively, along the NH₂ terminus. The hatched region corresponds to the stretch of homology between endophilin and amphiphysin within their NH₂-terminal domains. (C) Endophilin and amphiphysin deletion constructs missing most of this region of homology no longer cosediment with liposomes (S, supernatant; P, pellet), and also fail to tubulate liposomes (unpublished data). (D) Incubation of liposomes with endophilin or amphiphysin generates membrane tubules with similar morphology. (E and F) A point mutation (F10E) in endophilin of a hydrophobic residue to an acidic residue within the putative hydrophobic patch in the endophilin–amphiphysin homology region prevents liposome binding and tubulation, whereas a conservative hydrophobic to hydrophobic mutation (F10W) preserves liposome binding and tubulation (WT, wild-type; F10E and F10W, mutant endophilins). Bar, 100 nm.

Endophilin and amphiphysin have different effects on dynamin vesiculation of bilayer tubules

Given the similar morphology of amphiphysin- and endophilin-coated tubules both in the presence and absence of dynamin, we compared their individual effects on dynamin-mediated

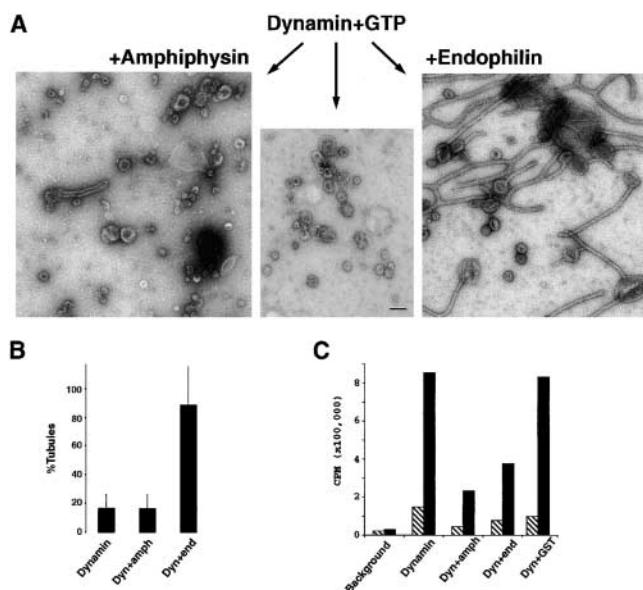


Figure 4. Different effects of endophilin and amphiphysin on dynamin properties in vitro. (A) Liposomes incubated with buffer, GTP, and either purified dynamin (center), or dynamin–amphiphysin (left) vesiculate liposomes. In contrast, addition of endophilin to dynamin (right) inhibits this phenomenon. (B) Morphometric analysis of the reactions shown in A. (C) Phosphate release from γ -³²P-labeled GTP incubated with dynamin and dynamin interactors, or GST as a control, in the absence (hatched bars) or presence (solid bars) of liposomes. Note the decrease in phosphate release by dynamin in the presence of endophilin and amphiphysin. Bar, 100 nm.

ated membrane fragmentation. Dynamin-coated membrane tubules readily fragment into small vesicles in the presence of GTP (Sweitzer and Hinshaw, 1998; Takei et al., 1999). As reported previously, amphiphysin supports this fragmentation (Takei et al., 1999). Accordingly, after an incubation of liposomes with dynamin-GTP, or dynamin with amphiphysin-GTP, a large number of small vesicles were seen as evidenced by electron microscopy (Fig. 4, A and B). Any tubules which remained were generally much shorter. In contrast, when liposomes were incubated with dynamin and endophilin-GTP, fragmentation was inhibited, with a large number of long tubules persisting (Fig. 4, A and B). This result suggests that the presence of endophilin stabilizes the lipid tubule against the structural changes in dynamin which lead to vesiculation in the presence of GTP. Both endophilin and amphiphysin inhibited phosphate release by dynamin-GTP in the presence and absence of liposomes (Fig. 4 C). These results may reflect unique dynamics of dynamin oligomers in coassembly with endophilin versus amphiphysin: one which allows for a mechanochemical transduction productive for vesiculation, and another which hinders this. Therefore, despite important similarities, the dynamin–endophilin interaction is qualitatively different from the dynamin–amphiphysin interaction on lipid bilayers. This difference may have significant ramifications for the in vivo dynamics of these complexes at the neck of clathrin-coated pits.

An endophilin-like protein localizes to the Golgi complex and also tubulates lipid bilayers

We searched the database for other proteins which have sequence homology to the NH₂ terminus of endophilin 1 to de-

termine whether they also deform lipid bilayers. The search revealed the recently named endophilin B family of proteins (Huttner and Schmidt, 2000). This protein family shares a common domain structure with endophilin, including a COOH-terminal SH3 domain (EMBL/GenBank/DDBJ accession nos. AF263364 and AF263293) (Fig. 5 A). Antibodies were raised against the recombinant endophilin B1 protein. By Western blot analysis, this antibody recognized a 41-kD band mainly in heart, brain, spleen, and lung, with lower expression in liver and testis (Fig. 5 C). Additional higher molecular weight bands were also detected, specifically in brain, possibly representing other splice isoforms or post-translational modifications (EMBL/GenBank/DDBJ accession no. AF263364) (Fig. 5 C). By immunofluorescence of brainstem frozen sections, endophilin B was detected in a synaptic-like pattern as shown by colocalization with synaptic markers such as amphiphysin (which also shows diffuse cytosolic staining) (Fig. 5 D). In addition, endophilin B staining was detected on intracellular membranes in the perikaryon-dendritic region of neurons (Fig. 5 D). Further analysis of this staining showed partial colocalization with the Golgi complex marker GM-130 (Fig. 5 E). In CHO cells, endophilin B localized to a perinuclear compartment strongly positive for the Golgi complex marker GM-130, in addition to a more diffuse reticular staining pattern throughout the cell (Fig. 5 F). Brefeldin A caused dispersion of the perinuclear staining by endophilin B, consistent with localization to the Golgi complex (unpublished data). There was no colocalization to endosomal compartments labeled with transferrin (unpublished data).

The purified recombinant human endophilin B1 protein tubulated liposomes in a manner morphologically indistinguishable from endophilin A1 and amphiphysin (Fig. 5 G). The amphipathic region of homology between endophilin (now referred to as endophilin A1) and amphiphysin is also conserved in endophilin B1, and deletion of this region in endophilin B1 also blocks tubulation (unpublished data). Thus, endophilin B1 may be involved in tubulovesicular dynamics of the Golgi complex and potentially of other intracellular membrane compartments, underscoring a diverse role for proteins which share this membrane tubulating property.

A lipid-interacting/deforming domain for tubulovesicular dynamics in membrane trafficking?

Collectively, our results provide evidence that endophilin directly binds and deforms lipid bilayers into narrow tubules within the size range of the tubular neck of clathrin-coated pits. This activity of endophilin is a protein-mediated phenomenon which likely occurs by oligomerization at the surface of the bilayer. Endophilin tubulates membranes at 4°C, and also tubulates synthetic bilayers devoid of the putative substrates of its reported LPA-AT activity, showing that formation of high curvature membranes by endophilin can be independent of lipid-modifying enzymatic activity. We did observe that endophilin binds and tubulates liposomes made from a brain lipid extract more efficiently than synthetic liposomes, thus indicating some role for the composition of the bilayer (unpublished data). However, elucidation of this finding requires further experimentation. We hypothesize that the tubulating activity of endophilin reflects one possible function for this protein in clathrin-mediated endocyto-

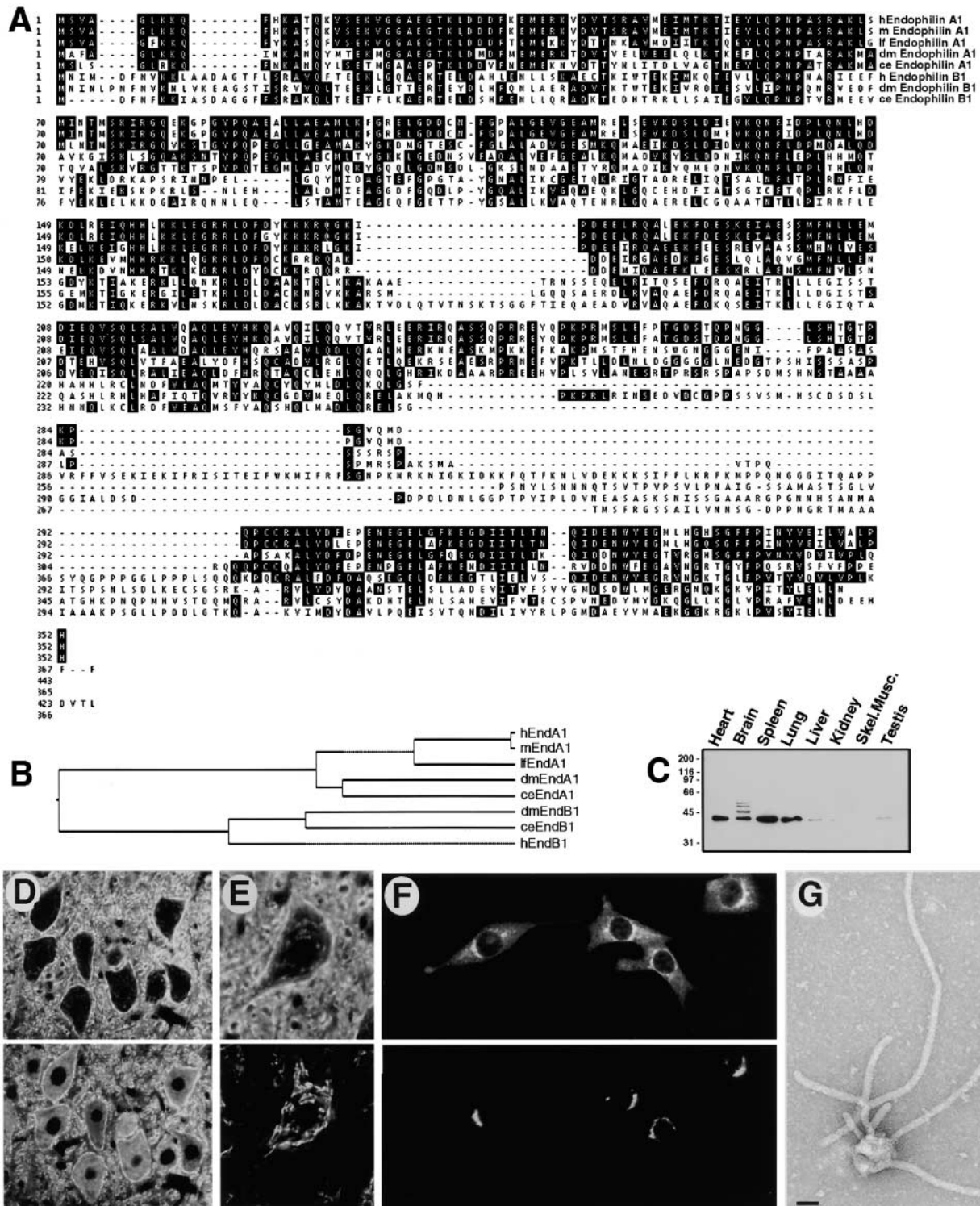


Figure 5. The endophilin B class of proteins is found on intracellular membrane compartments and also tubulates liposomes. (A) Cross-species sequence alignment (Clustal method) of the endophilin A1 and endophilin B1 proteins showing broad homology. h, human; m, mouse; lf, lamprey (*L. fluviatilis*); dm, *D. melanogaster*; ce, *C. elegans*. (B) Phylogenetic tree of the endophilin A1 and endophilin B1 proteins. (C) Multiple tissue Western blot using a polyclonal rabbit antibody raised against recombinant endophilin B1 showing expression primarily in heart, brain, spleen, and lung, with lower expression evident in liver and testis. Note the presence of brain-specific higher molecular weight bands. (D) Immunofluorescence of a rat brainstem frozen section using antibodies against either endophilin B1 (top) or amphiphysin 1 (bottom). Note the localization of endophilin B in a synaptic-like pattern at the cell periphery similar to the localization of amphiphysin. In addition to this synaptic staining, endophilin B localizes to intracellular particles, while amphiphysin shows a diffuse cytosolic staining pattern. (E) Endophilin B (top) partially colocalizes with a Golgi-specific marker, GM-130 (bottom), in a large brainstem neuron. (F) Endophilin B (top) partially colocalizes with a Golgi-specific marker, GM-130 (bottom), in CHO cells. (G) Purified recombinant endophilin B1 deforms liposomes into tubules similar to those seen with endophilin and amphiphysin. Bar, (D–F) 10 μ m; (G) 100 nm.

sis. Along with dynamin and amphiphysin, endophilin may be part of a protein complex governing aspects of membrane deformation and fission.

Our findings provide a rationale for the observed requirement for endophilin in the tubulation of synaptic membranes by brain cytosol in the presence of GTP γ S (Ringstad et al., 1999). These observations may also partially explain the arrest of clathrin-mediated synaptic vesicle retrieval at shallow stages of invagination upon perturbation of endophilin function in lamprey (Ringstad et al., 1999). Endophilin membrane binding and protein-protein interactions may serve to localize and functionally orient the protein at the clathrin-coated pit. Amphiphysin and endophilin tubulate lipid bilayers both independently and as a complex with dynamin; however, their distinct effects on dynamin *in vitro* suggest a potential differential modulatory role on dynamin function *in vivo* which needs to be explored further.

We find that endophilin B1, a member of a newly discovered family of proteins, localizes to both synaptic and Golgi/intracellular membrane compartments, and displays a membrane tubulating activity similar to that of endophilin A1 and amphiphysin. All three proteins share a short NH₂-terminal amphipathic region which we show to be required for membrane tubulation. This may have implications for a shared function for this motif in tubulo-vesicular dynamics in cellular trafficking events. Recently, RICH (RhoGAP interacting with CIP4 homologues), a novel RhoGAP containing protein, was found in a yeast two-hybrid screen for CIP 4 (CDC-42-interacting protein 4) interacting proteins (Richnau and Aspenstrom, 2001). RICH has an NH₂-terminal domain with homology to endophilin A1, and the amphipathic motif of endophilin A1 is also conserved. A splice isoform of RICH which only contains the endophilin homology domain (RICH 1B) localizes to intracellular punctate compartments (Richnau and Aspenstrom, 2001). It will be interesting to see whether RICH also has a membrane-interacting/tubulating function, further expanding the diversity of proteins with this motif in membrane dynamics.

Materials and methods

Liposomes

Liposomes composed of brain lipid extract (type 1, Folch fraction 1; Sigma-Aldrich) were made as described (Takei et al., 1999). Synthetic liposomes were made using HPLC-purified DOPC (40%), DOPS (40%), and DOPE (20%) from Avanti Polar Lipids, Inc.

Purified proteins

Dynamin was affinity purified from rat brain cytosol using amphiphysin 1-SH3 domain as described (Owen et al., 1998). This material, as assessed by SDS-PAGE, yielded ~90–95% dynamin, and 5–10% synaptojanin. All data shown use this material. Synaptojanin was immunodepleted from this affinity-purified material using a monoclonal antibody to yield a more highly purified dynamin preparation, and similar results were obtained.

Recombinant rat endophilin A1, human amphiphysin 1, human endophilin B1, and PLC δ -PH were cloned in pGEX (Amersham Pharmacia Biotech), and purified as glutathione S-transferase (GST) fusion proteins according to standard methods (Amersham Pharmacia Biotech). For endophilin, amphiphysin, and endophilin B1, the GST tag was subsequently cleaved by PreScission Protease (Amersham Pharmacia Biotech).

Endophilin deletion constructs were prepared by PCR to yield the following fragments: amino acids 1–125, 1–261, 126–352, and 34–352. The constructs were subcloned and purified as above. Point mutants were generated using mutant primers and plasmid extension by PCR.

Clathrin-coat fraction was purified from bovine calf brain as described (Takei et al., 1999).

Electron microscopy

Liposomes (0.1 mg/ml final) were incubated at 37°C for 10–20 min in buffer A (25 mM Hepes-KOH, pH 7.4, 25 mM KCl, 2.5 mM Mg²⁺ acetate, 150 mM K-glutamate) with various proteins and nucleotides at the following final concentrations: dynamin 0.1 mg/ml, amphiphysin 0.1 mg/ml, endophilin 0.1 mg/ml, endophilin deletion constructs 0.1–0.2 mg/ml, coat proteins 0.5 mg/ml, PLC δ -PH 0.1 mg/ml, GTP 1 mM, and GTP γ S 0.5 mM (Takei et al., 1999). At the end of the incubation, aliquots were adsorbed onto 200–400 μ m formvar- and carbon-coated copper EM grids for 3–5 min at room temperature, washed in 0.1 M Hepes, pH 7.4, stained in 1–2% uranyl acetate, blotted, and then allowed to air dry. For incubations at 4°C, all of the above was performed on ice.

Average ring spacing was determined by counting the number of rings in 10 350-nm tubules, and then dividing by the tubule length. To quantify the number of tubules seen under various conditions, 6 13- μ m² areas of the grid were scored. Each independent tubule and each branch of a tubule was counted as a tubule.

Liposome binding

Liposome sedimentation was performed using 100 μ g sucrose-loaded liposomes incubated with 5–10 μ g protein (1–25 μ g for saturable binding) in 400–500 μ l buffer A or in Hepes-KCl, pH 7.4 (to determine salt sensitivity) for 10–20 min at 37°C. Liposomes were sedimented at 100,000 g in a Beckman Coulter TLA 100.3 rotor for 20 min, the supernatant was thoroughly removed, and sedimented liposomes were solubilized in 2% SDS. To monitor recovery, liposomes were labeled with 0.5% NBD-phosphatidyl choline and absorbance was measured at 460 nm. In some cases, proteins in the pellet and supernatant were concentrated by chloroform precipitation and methanol extraction. Samples were subjected to SDS-PAGE and analyzed by either Coomassie staining or by slot blot analysis using affinity-purified polyclonal endophilin antibodies and ¹²⁵I. Band intensities were quantified by optic densitometry or phosphorimaging.

For the crosslinking assay, 4 μ g of the endophilin 125 amino acid fragment (with or without 8 μ g liposomes) was preincubated in Hepes, pH 7.4, 100 mM KCl for 20 min at 37°C, then an equal volume of 2 \times buffer with the heterobifunctional chemical cross-linker bis(sulfosuccinimidyl) suberate (BS³) was added to achieve final concentrations of 0, 1, 5, and 10 mM cross-linker. The mixture was incubated for an additional 30 min at 37°C, and the samples were then prepared for SDS-PAGE analysis.

Miscellaneous procedures

Immunogold labeling of endophilin was performed by standard agarose-embedding/labeling protocols as described (Ringstad et al., 1999) followed by thin sectioning. The GTPase activity of dynamin was determined by phosphate release as described (Takei et al., 1999). Western blotting and immunofluorescence was performed using standard procedures as described (Ringstad et al., 1997).

We are most grateful to Drs. Graham Warren and Vinzenz Unger for many helpful discussions and critical reading of the manuscript. We also wish to thank Drs. Gilbert Di Paolo, Lorenzo Pellegrini, Markus Wenk, Vladimir Slepnev, and Gianluca Cestra for technical advice and helpful discussions.

This work was supported in part by National Institutes of Health grants NS36251 and CA46128 to P. De Camilli. K. Farsad and S.R. Floyd are supported in part by a Medical Scientist Training Program fellowship from the National Institutes of Health. N. Ringstad was supported in part by a predoctoral training grant (T32GM07527) from the National Institutes of Health to the Department of Cellular and Molecular Physiology, Yale University School of Medicine.

Submitted: 23 July 2001

Revised: 10 September 2001

Accepted: 13 September 2001

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