Endophilin I mediates synaptic vesicle formation by transfer of arachidonate to lysophosphatidic acid

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Endophilin I is a presynaptic protein of unknown function that binds to dynamin, a GTPase that is implicated in endocytosis and recycling of synaptic vesicles. Here we show that endophilin I is essential for the formation of synaptic-like microvesicles (SLMVs) from the plasma membrane. Endophilin I exhibits lysophosphatidic acid acyl transferase (LPAAT) activity, and endophilin-I-mediated SLMV formation requires the transfer of the unsaturated fatty acid arachidonate to lysophosphatidic acid, converting it to phosphatidic acid. A deletion mutant lacking the SH3 domain through which endophilin I interacts with dynamin still exhibits LPAAT activity but no longer mediates SLMV formation. These results indicate that endophilin I may induce negative membrane curvature by converting an inverted-cone-shaped lipid to a cone-shaped lipid in the cytoplasmic leaflet of the bilayer. We propose that, through this action, endophilin I works with dynamin to mediate synaptic vesicle invagination from the plasma membrane and fission.

Membrane traffic mediated by vesicular carriers is a fundamental process in all eukaryotic cells. Any given trafficking step requires two processes of membrane fusion: that leading to the pinching-off of the vesicle from its donor membrane, called fission, and that resulting in the merging of the vesicle with its acceptor membrane. With regard to membrane fission, two principal models can be distinguished. These are not necessarily mutually exclusive but may reflect basic differences between the various types of intracellular donor membranes and vesicles.

One model views the process of membrane fission as the ultimate consequence of membrane budding. Here, the continuation of the change of shape of the donor membrane by a proteinaceous coat machinery that mediates budding is thought to constrict the lipid bilayer at the edge of the polymerizing coat, that is, the neck of the nascent vesicle, sufficiently for membrane fission to occur. Studies on vesicle formation in the early secretory pathway have provided considerable evidence in support of this model, notably the demonstration that the coat proteins (COP I and COP II) are sufficient to generate small lipid vesicles from liposomes, if the liposomes bear an integral membrane constituent (lipid or membrane-anchored protein motif) that is appropriate for coat recruitment⁴,⁵.

The other model views membrane budding and membrane fission as being separate, though often linked, processes that are mediated by distinct proteinaceous machineries with the potential, but not the need, to interact. Here, following the coat-mediated formation of a membrane bud, membrane fission results from the action of a separate set of proteins that mediate the constriction of the narrow membrane tube, connecting the nascent vesicle with the donor membrane. Evidence supporting this model has largely been obtained in studies on endocytosis, in particular the recycling of synaptic vesicles from the plasma membrane⁶-⁸. Whereas the clathrin coat mediates bud formation⁹, a ring around the neck of the nascent vesicle, formed upon polymerization of the GTPase dynamin¹⁰,¹¹, is thought to be central in membrane constriction and fission⁴,⁵,⁸.

It has been proposed that this membrane constriction and fission is driven by a change in the conformation of dynamin that occurs upon GTP hydrolysis¹²-¹⁴. However, the view that dynamin itself acts as a "pinchase" has been questioned¹⁵. In fact it appears that dynamin does not function as a force-generating GTPase but rather, like other members of the GTPase superfamily, activates an as yet unknown effector system when in the GTP state, and it is this effector system that is essential for fission¹⁶. If so, dynamin-interacting proteins, such as the endophilin I (originally called SH3p4, ref. 18) that is studied here, are obvious candidates for this effector system.

The shape of a membrane is influenced not only by proteins but also by lipids. The shape of membrane lipids is a key determinant of membrane curvature¹⁷-²¹. Inverted-cone-shaped lipids favour positive curvature, which characterizes the outer leaflet of a membrane bud, and cone-shaped lipids favour negative curvature, as found in the inner leaflet of a bud. Examples of inverted-cone-shaped lipids and cone-shaped lipids include lysophospholipids and unsaturated fatty acids, respectively²²-²³. Indeed, in one of the paradigms of membrane fusion, that of the influenza virus envelope with the plasma membrane mediated by the viral haemagglutinin²⁴, the differentially shaped lipids lysophosphatidylcholine (inverted cone) and oleic acid (cone) affect the fusion process in an opposite (negative and positive, respectively) manner²⁵. The shape of these lipids is thought to affect critically the transition between the intermediates in lipid bilayer organization that have been hypothesized to exist during the fusion process²⁶,²⁷.

This raises two questions. First, do differently shaped lipids also have a role in membrane fission? Second, how is lipid shape controlled? Here we report on the role of endophilin I in the control of lipid shape and changes in membrane curvature underlying the formation of synaptic vesicles from the plasma membrane.
Endophilin I is required for SLIMV formation

To identify rate-limiting factors for SLIMV formation, we used a previously established perforated-cell system, derived from the rat neuroendocrine cell line PC12, which reconstitutes this process34. This perforated-cell system is based on cell-surface biotinylation of the synaptic vesicle membrane protein synaptophysin, and its accumulation in a specialized domain of the plasma membrane at 18°C (ref. 26), followed by its incorporation into SLIMVs upon cell perforation, addition of brain cytosol and ATP, and a return of the temperature to 37°C. In investigating whether the dynamin-interacting35, actin-binding protein profilin was involved in SLIMV formation, we noted that cytosol depleted of profilin by passage over poly(L-proline) Sepharose36 was much less efficient than control cytosol but that addition of recombinant profilin to the depleted cytosol did not fully restore SLIMV formation (Fig. 1a). This indicated that passage of cytosol over poly(L-proline) Sepharose led to the depletion of a protein (or proteins) other than profilin that is required for SLIMV formation.

Elution of the proteins bound to poly(L-proline) Sepharose with 8 M urea revealed four major bands with relative molecular masses (Mr) of 45K, 40K, 28K and 15K (Fig. 1b). Immunoblotting (data not shown) revealed the identity of the 45K and the 15K bands as actin and profilin, respectively. Selective elution of the 40K protein and the 28K protein, along with some actin but without profilin, was achieved using 30% dimethyl sulphoxide (DMSO) (Fig. 1b). Addition of the DMSO eluate to depleted cytosol supplemented with recombinant profilin fully restored SLIMV formation (Fig. 1a).

High-accuracy matrix-assisted laser desorption/ionization (MALDI) peptide-mass mapping revealed that the 40K protein was endophilin I (originally called SH3p4, refs 8, 18, 29), with 51%, 48% and 44% of the complete human (accession no. Q99962). complete mouse (accession no. Q62420) and partial rat (accession no. AF009603) sequence, respectively, being covered. Similarly, the 28K protein was unambiguously identified as the SH3- and SH2-domain-containing protein Grb2 (data not shown).

Endophilin I interacts with dynamin37, synaptotagmin38 and amphiphysin39, which have been implicated in synaptic vesicle recycling34. The identification of the 40K protein as endophilin I indicated that this protein might be the relevant component in the DMSO eluate with regard to SLIMV formation. We therefore investigated the effect of recombinant endophilin I on SLIMV formation in the perforated-cell system using low-protein cytosol (cytosol that is suboptimal for SLIMV formation34). SLIMV formation in the presence of low-protein cytosol that had been completely depleted of endogenous endophilin I by passage over poly(L-proline) Sepharose (as revealed by immunoblotting; data not shown) was almost as low (Fig. 1c) as the background value obtained in the absence of added cytosol. Addition to depleted cytosol of a concentration of recombinant endophilin I corresponding to that in high-protein cytosol increased SLIMV formation in the perforated-cell system to that observed with high-protein cytosol (Fig. 1c). This showed not only that endophilin I is the critical cytosolic component depleted by passage over poly(L-proline) Sepharose, but also that this protein is rate-limiting for SLIMV formation. Confirming the latter conclusion, addition of increasing amounts of recombinant endophilin I (up to 16 μg ml−1) to low-protein cytosol resulted in a progressive stimulation of SLIMV formation, which approached the level obtained with high-protein cytosol (data not shown). SLIMV formation was also stimulated by recombinant endophilin I in a modified cell-free system, in a similar manner to the perforated-cell system (data not shown). In this system, PC12 cells were biotinylated at 18°C and homogenized (rather than only perforated), and the homogenate was subjected to cell-free reaction followed by isolation of SLIMVs without further homogenization (analogous to the cell-free system of ref. 33).

Endophilin I has LPAAT activity

An independent line of investigation originated from the observations34–36 that stimulation of exocytosis in parotid acinar cells is associated with an increase in the activity of lysophosphatidic acid acyl transferase (LPAAT), which converts lysophosphatidic acid (LPA) to phosphatic acid. The increase in LPAAT activity upon stimulation of secretion is due to phosphorylation by cyclic-AMP-dependent protein kinase or Ca2+/calmodulin-dependent protein kinase II (refs 35, 36). To identify this phosphorylation-sensitive LPAAT, we purified it (Table 1). Bovine brain tissue was used as the source because LPAAT activity increases upon addition of either protein kinase to a brain-derived total post-nuclear membrane fraction38. Q-Sepharose ion-exchange chromatography gave two peaks of LPAAT activity, one (peak I) of which also contained lysophosphatidylcholine (LPC) acyl transferase activity and was not significantly stimulated by phosphorylation, and the other (peak II) of which did not exhibit significant LPC acyl transferase activity but was stimulated by phosphorylation (data not shown). LPAAT in peak II was purified to apparent homogeneity (Table 1). The final purification step consisted of preparative, continuous elution SDS-polyacrylamide gel column electrophoresis (ESPACE). The fractions obtained were examined for protein content by analytical SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 2a) and for LPAAT activity after protein renaturation (Fig. 2b). This revealed a single ~40K protein band (Fig. 2a), which coincided with LPAAT activity (Fig. 2b), in fractions 18–30. Two-dimensional (2D) PAGE showed that the 40K protein band consisted of a single protein with
Figure 2 Identification of purified bovine brain LPAAT as endophilin I. a, b, ESPACE, the final step in the purification. Aliquots of some of the ~150 fractions obtained after ESPACE were subjected to analytical SDS-PAGE on three gels followed by Coomassie-Blue staining (a, entire separating gels are shown), and the remainder of the indicated fractions was rerun and aliquots of equal volume (containing 3.3 μg protein in the case of fraction 24) analysed for LPAAT activity (b). c, 2D PAGE followed by Coomassie-Blue staining of the pooled fractions containing the peak of LPAAT activity (5 μg protein).

an acidic isoelectric point (Fig. 2c). The sequence of five tryptic peptides obtained from the purified 40K protein (AVMEIMTKit ELRQKqGPGYQQAe, GPGPqQAEALAEAEML, QGKpED EELRQALE, B1AEEMFNLLEMD) was identical to that of predicted tryptic fragments of endophilin I. This, together with the finding that the reported molecular mass (40K, refs 30, 31) and predicted isoelectric point (pI 5.33) of endophilin I are, within the range of experimental accuracy, identical to those of the 40K band that comigrates with LPAAT activity upon purification, indicates that LPAAT activity is intrinsic to endophilin I.

When oleoyl-CoA was used as a co-substrate, the purified bovine brain enzyme exhibited a high specificity for LPA as substrate as compared to other lysophospholipids (Table 2). Moreover, like the enzyme studies in tissue25-28 and crude subcellular fractions30,31, the activity of the purified enzyme was stimulated by phosphorylation with cAMP-dependent protein kinase (Table 2) or Ca2+/calmodulin-dependent protein kinase II (data not shown). Activation of the purified enzyme was accompanied by 32P) phosphate incorporation into the 40K band that approached a value of 1 mol of phosphate per mol of protein (data not shown).

To confirm that endophilin I has LPAAT activity, recombinant endophilin I was expressed in bacteria as either His6-tagged or glutathione S-transferase (GST)-fusion protein and analysed for LPAAT activity. To exclude a contribution of endogenous bacterial LPAAT, which is a transmembrane protein20, the bacteria were lysed by freezing-thawing in the absence of detergent, followed by ultracentrifugation. The high-speed supernatant obtained from bacteria transformed with vector containing the insert encoding His6-tagged endophilin I exhibited LPAAT activity in the presence of LPA and arachidonoyl-CoA as exogenous substrates, whereas that obtained after transformation with vector lacking the insert did not (Fig. 3a). The same results were obtained when oleoyl-CoA was used instead of arachidonoyl-CoA (data not shown).

Recombinant His6-tagged endophilin I was purified from the bacterial high-speed supernatant by affinity chromatography on poly(L-proline) Sepharose (Fig. 3b). Purified recombinant endophilin I exhibited LPAAT activity (Fig. 3c). In contrast, a poly(L-proline) eluate obtained from the high-speed supernatant of bacteria transformed with vector lacking insert, which did not contain endophilin I (Fig. 3b), did not show detectable LPAAT activity (Fig. 3c). Similar results were obtained with recombinant His6-tagged endophilin I purified by metal chelate affinity chromatography, which indicated that endophilin I has LPAAT activity.

Table 2 Lysophospholipid specificity of purified and recombinant endophilin I and its activation by phosphorylation

<table>
<thead>
<tr>
<th>Protein</th>
<th>LPA</th>
<th>PKA</th>
<th>LPC</th>
<th>LPS</th>
<th>LPI</th>
<th>LPE</th>
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<tr>
<td>Pure endophilin</td>
<td>100</td>
<td>204</td>
<td>3.3</td>
<td>3.3</td>
<td>3.3</td>
<td>3.3</td>
</tr>
<tr>
<td>H6-tag endophilin</td>
<td>100</td>
<td>205</td>
<td>3.3</td>
<td>3.3</td>
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Specific LPAAT activity was expressed as a percentage of control, using LPA as substrate. Specific LPAAT activities for the control condition were 4.96, 10.80 and 5.46 for endophilin I purified from bovine brain, H6-tag endophilin I and GST-endophilin I, respectively. PKA, cyclic AMP-dependent protein kinase.
Endothelin I binds fatty acyl-CoA and LPA

Endothelin I was depleted from brain cytosol upon passage over a palmitoyl-CoA agarose column, in contrast to the bulk of the cytosolic proteins and Grb2 (Fig. 4a), another SH3-domain-containing protein with an isoelectric point similar to that of endothelin I. Binding of endothelin I to palmitoyl-CoA agarose was prevented when cytosol was loaded onto the column in the presence of free palmitoyl-CoA (Fig. 4b). Likewise, endothelin I bound to palmitoyl-CoA agarose was eluted from the column by free palmitoyl-CoA (Fig. 4a). These results indicate that endothelin I binds fatty acyl-CoA.

To investigate the binding of endothelin I to LPA, we prepared a radioactive photolabile analogue of LPA. [32P]-[(10-azido-stearoyl)]-LPA. When a high-speed supernatant obtained from bacteria transformed with vector containing the insert encoding Hs-endothelin I was subjected to UV-irradiation in the presence of the photolabile LPA, a [32P]-labelled 40K band was observed (Fig. 4c, left) that coincided with recombinant endothelin I detected by protein staining (Fig. 4c, right). Labelling of this band was dependent on UV-irradiation (Fig. 4c) and was blocked by excess unlabelled LPA (data not shown). The 40K band was not detected when a high-speed supernatant from bacteria transformed with vector lacking insert was used, in contrast to an endogenous bacterial 45K protein that was equally labelled in both supernatants (Fig. 4c). Similar photoaffinity labelling was observed for the GST-endothelin I fusion protein (data not shown). We conclude that endothelin I binds LPA.

Effects of added LPA and fatty acyl-CoA

Addition of 1 μM LPA to the perforated-cell system did not significantly affect the basal SLMV formation observed with low-protein cytosol, but completed blocked the ability of exogenously added recombinant endothelin I to mediate SLMV formation (Fig. 5a). This is consistent with the interpretation that exogenously added LPA competitively prevents endothelin I from acting on LPA in the donor membrane.

To investigate the requirement for fatty acyl-CoA in SLMV formation mediated by endothelin I, we used the unsaturated
fatty acid arachidonoyl-CoA rather than the saturated fatty acid palmitoyl-CoA because, upon stimulation of secretion, the increase in incorporation of arachidonate into phosphatidic acid is greater than that of palmitate. In the standard perforated-cell system using gel-filtered low-protein cytosol, addition of 10 μM exogenous arachidonoyl-CoA in the presence of recombinant endothelin I did not increase SLMV formation above the level obtained by addition of endothelin I alone (Fig. 5b, left). Likewise, arachidonoyl-CoA did not increase the basal SLMV formation observed without addition of endothelin I (data not shown). This indicated that, under these conditions, endogenous arachidonoyl-CoA was not rate-limiting. Presumably, in the presence of gel-filtered cytosol, the source of fatty acyl-CoA was fatty acid derived from membrane phospholipids through endogenous phospholipase A2 (PLA2) followed by activation by cytosolic fatty acyl-CoA synthase.

To remove free fatty acids and fatty acyl-CoA from the membranes, the perforated cells were pretreated with fatty acid-free bovine serum albumin (BSA). Including PLA2 under non-lytic conditions (see Methods) during the BSA pretreatment reduced basal SLMV formation in the subsequent perforated-cell reaction to about half of that observed after BSA pretreatment in the absence of added PLA2; addition of 10 μM arachidonoyl-CoA during the perforated-cell reaction following BSA/PLA2 pretreatment restored basal SLMV formation to the original level (data not shown). We therefore used this pretreatment to show that arachidonoyl-CoA is required for endothelin-I-mediated SLMV formation. Compared to the marked stimulation of SLMV formation by endothelin I seen without BSA/PLA2 pretreatment (Fig. 5b left), BSA/PLA2 pretreatment greatly reduced endothelin-I-mediated SLMV formation in the absence of exogenously added arachidonoyl-CoA (Fig. 5b right); addition of 10 μM arachidonoyl-CoA doubled the endothelin-I-mediated SLMV formation (Fig. 5b right). We conclude that endothelin-I-mediated SLMV formation requires fatty acyl-CoA.

We next investigated whether palmitoyl-CoA, which, like arachidonoyl-CoA, can be used as substrate by endothelin I to generate phosphatidic acid (data not shown), also supported endothelin-I-mediated SLMV formation. In the standard perforated-cell system using low-protein cytosol, addition of 2.5 μM (data not shown) or 10 μM (Fig. 5c) of palmitoyl-CoA completely blocked endothelin-I-mediated SLMV formation, in contrast to arachidonoyl-CoA (Fig. 5b). Addition of 2 μM LPC, which was a much poorer substrate for endothelin I than LPA (Table 2), also inhibited endothelin-I-mediated SLMV formation (Fig. 5d).

A PLA2 inhibitor potentiates SLMV formation

If SLMV formation mediated by endothelin I is due to the conversion of LPA to phosphatidic acid, one might expect that a PLA2 inhibitor acts synergistically with endothelin I in SLMV formation because the reverse reaction, the hydrolysis of phosphatidic acid to LPA and free fatty acid, which presumably occurs during the perforated-cell reaction owing to the activity of cytosolic PLA2, would be prevented. Indeed, the specific inhibitor of cytosolic calcium-dependent and -independent PLA2, methyl arachidonoyl fluorocarbonic acid (MAF), strongly potentiated endothelin-I-mediated SLMV formation (Fig. 6a). This observation indicates that endogenous cytosolic PLA2 may be able to antagonize the action of endothelin I in SLMV formation.

In most of the experiments described above, the effects of recombinant endothelin I on SLMV formation were investigated in the presence of low-protein cytosol, which by itself supported a basal level of SLMV formation. Given the results of Fig. 4c, this basal SLMV formation can largely be attributed to the endothelin I endogenously present in low-protein cytosol (6 μg ml⁻¹). Like the SLMV formation mediated by addition of exogenous endothelin I (Fig. 4a), the basal SLMV formation should therefore be sensitive to agents affecting hydrolysis of phosphatidic acid.

Indeed, addition of MAFP moderately stimulated basal SLMV formation (Fig. 5b). Conversely, addition of exogenous PLA2 derived from snake venom markedly reduced basal SLMV formation (Fig. 5c), the residual level being only slightly above that observed without addition of any cytosol (data not shown).

The role of the SH3 domain of endothelin I

Dynamin, which is an essential component of the machinery that mediates SLMV formation in our perforated-cell system, is recruited to, and forms rings at, the necks of synaptic vesicles budding from their donor membrane. Endothelin I binds, by its SH3 domain, to the proline-rich region of dynamin. We therefore investigated whether SLMV formation mediated by endothelin I involved protein–protein interaction through its SH3 domain. A ~34K deletion mutant of endothelin I lacking the SH3 domain (referred to as del-endothelin I) still exhibited LPAAT activity (Fig. 7a) but no longer mediated SLMV formation, at least not at the concentration tested (~10 μg ml⁻¹). This shows that protein–protein interaction of endothelin I through its SH3 domain is essential for its ability to mediate SLMV formation. Although endothelin I binds not only to dynamin but also synaptojanin, we interpreted the need for its SH3 domain in terms of an interaction with dynamin rather than synaptojanin because, in perforated PC12 cells supplemented with cytosol depleted of both dynamin and synaptojanin, addition of dynamin alone is sufficient to restore SLMV formation. Specifically, we propose that the interaction with dynamin results in the recruitment of endothelin I, and hence its LPAAT activity, to the membrane of budding synaptic vesicles.

Discussion

We have provided several lines of evidence that endothelin I has LPAAT activity. First, endothelin I isolated from brain using ERSpace as the final purification step appears as a single 40K protein on analytical SDS– and 2D PAGE and exhibits LPAAT activity after

Figure 5 Effects of PLA2 and the PLA2 inhibitor MAFP on endothelin-I-mediated SLMV formation. SLMV formation in perforated PC12 cells using complete brain cytosol (0.75 mg protein per ml) in the absence (·, Con) and presence (+) of the indicated additions: a, 10 μg ml⁻¹ purified (metal-chelate) recombinant b-HIV—endothelin I (End I); 10 μM MAFP, b, 10 μM MAFP; c, 10 μg ml⁻¹ PLA2 from C. adamantium. Note that to quantify the reduction by PLA2 below the control, the immunoblot was exposed longer than in the case of a stimulation above the control (such as a, b), resulting in higher than usual arbitrary units for the control value. a–c, Data are the mean of three independent perforated-cell reactions; bars indicate s.d.
Figure 7  Endophilin I lacking its SH3 domain (Δ-endophilin I) still possesses LPAAI activity but does not promote SLMV biogenesis. a, Duplicate LPAAI assays using 5 μM [1,2-3H]arachidonoyl-CoA and 10 μM LPA in the absence (Con) and presence of 10 μM purified (metal-chelate) recombinant Hsa-Δ-endophilin I (Δ-end I) and Δ-end-Hsa-Δ-endophilin I (Δ-end I). PA, phosphatidic acid; Orl, origin. b, SLMV formation in perforated FC12 cells using complete brain cytosol (0.75 mg protein per ml) in the absence (Con) and presence of 10 μM purified (metal-chelate) recombinant Hsa-Δ-endophilin I and Δ-end-Hsa-Δ-endophilin I. Data are the mean of three independent perforated-cell reactions; bars indicate s.d.

renaturation. The protein clearly elutes with a higher apparent molecular mass than the transmembrane LPAAIs described so far54;64; all of the latter migrate with apparent relative molecular masses of ~29–31K, and no such band was detected in the fractions containing the purified endophilin I and exhibiting LPAAI activity. Second, recombinant endophilin I exhibited LPAAI activity after various types of purification (GSH-column for GST–endophilin-I, metal-chelate column for HSA–endophilin-I, poly(lysine) column for either fusion protein). Moreover, recombinant endophilin I was solely responsible for the LPAAI activity observed in the soluble fraction obtained from transformed bacteria lysed by freezing/thawing. Third, endophilin I binds to palmitoyl-CoA and was photoaffinity-labelled by a photoactivatable LPA analogue, showing that it physically interacts with its two substrates.

A phosphorylation-induced increase in LPAAI activity, apparently reflecting the stimulation of LPAAI activity in response to secretagogue originally observed in parotid acinar cells55, has been observed in a postsynaptic membrane fraction56. This led us to anticipate that this LPAAI activity would reside in a membrane-associated protein. We therefore used a crude membrane fraction as a source for LPAAI purification. How, then, can it be explained that this purification yielded endophilin I, which upon hypo-osmotic lysis of synaptosomes is largely recovered in the soluble fraction57, consistent with it being a cytosolic protein? Presumably, the crude membrane fraction used as starting material for the solubilization of LPAAI, which was obtained from a relatively low-speed iso-osmotic supernatant, still contains synaptosomes (sealed nerve terminals) which, in line with the presynaptic localization of endophilin I (ref. 30), are enriched in this protein.

The observations that low-protein cytosol depleted of endophilin I barely supported SLMV formation in the perforated-cell system and that addition of recombinant endophilin I to this cytosol restored SLMV formation to the level obtained with high-protein cytosol indicate an essential role for endophilin I in this process. The function of endophilin I in SLMV formation appears to be mediated by its LPAAI activity. First, under conditions designed to lower the endogenous fatty acyl-CoA content of the perforated cells, endophilin-I-mediated SLMV formation was largely dependent on exogenous arachidonoyl-CoA. Second, exogenous LPA blocked endophilin-I-mediated SLMV formation, presumably by preventing the action of endophilin I on the endogenous LPA in the SLMV donor membrane. Third, endophilin-I-mediated SLMV formation was strongly promoted by the PLA2 inhibitor MAFP, which would stabilize the effect of the LPAAI reaction catalysed by endophilin I, that is, an increase in the phosphatidic acid/LPA ratio.

There are several potential mechanisms by which the LPAAI activity of endophilin I could mediate SLMV formation. One is that the phosphatidic acid generated by endophilin I in the donor membrane either stimulates, by activating phosphatidylinositol-4-phosphate (PtdIns(4)P) 5-kinase58, the formation of PtdIns(4,5)P2, or increases phosphoinositide levels by serving as a precursor in their synthesis. A local increase in the PtdIns(4,5)P2 level in the donor membrane may promote the recruitment of dynamin via its pleckstrin-homology domain, and thereby stimulate SLMV formation14,64. Phosphatidic acid may also directly promote dynamin-mediated changes in membrane shape, given that an increase in the amount of phosphatidic acid in liposomes favours the formation of dynamin-coated tubules15. However, we have observed that addition of phospholipase D (PLD), which increases phosphatidic acid by hydrolysing phosphatidylycholine, does not increase SLMV formation in the perforated-cell system although, in parallel experiments using another cell-free system, it did increase secretory vesicle formation from the trans-Golgi network (data not shown), confirming previous observations34. This may indicate that endophilin I mediates SLMV formation not simply because it increases phosphatidic acid, but also because it concomitantly consumes LPA.

This leads us to consider changes in membrane curvature as a central feature of the mechanism by which endophilin I mediates the formation of SLMVs and, by extrapolation, the recycling of synaptic vesicles. Synaptic vesicles are the smallest physiologically occurring vesicles of biological membranes known. The neck of a budding synaptic vesicle, especially when this budding occurs from the planar plasma membrane, is therefore characterized by greater membrane curvature than that of other membrane vesicles. Speci-
ically, the positive membrane curvature that exists in the cytoplasmic leaflet of a coated pit and bud changes to negative membrane curvature in one of the two spatial dimensions: at the edge of the coated pit as it invaginates from the planar plasma membrane to form a bud, and at the constricting neck of a bud proceeding to fission from either the planar plasma membrane or a tubular plasma membrane invagination. Inverted-cone-shaped membrane lipids and cone-shaped membrane lipids induce positive and negative membrane curvature, respectively\(^\text{25,26}\). LPA is thought to be an inverted-cone-shaped lipid, and phosphatidic acid (especially with arachidonate in position 2 of the glycerol backbone) a cone-shaped lipid\(^\text{27}\). Moreover, inverted-cone-shaped lipids and cone-shaped lipids have been proposed to affect membrane fusion in a differential manner\(^\text{28,29}\). It is therefore conceivable that endophilin I mediates synaptic vesicle formation because the conversion of LPA to an appropriate phosphatidic acid, catalyzed by the LPAT activity of endophilin I, induces the positive-to-negative membrane curvature change in the cytoplasmic leaflet that is required for coated pit invagination and, as shown in Fig. 8, the constriction of the neck of a budding synaptic vesicle during fission.

Such a mechanism would explain why the unsaturated fatty acid arachidonoyl-CoA supported endophilin-I-mediated SLIMV formation, whereas the saturated fatty acid palmitoyl-CoA blocked this process. In line with its ability to use palmitoyl-CoA as substrate (data not shown), endophilin I may have generated a phosphatidic acid with palmitate in position 2. Such a phosphatidic acid may be non-productive for SLIMV formation because its generation from LPA would result in a much less pronounced change in shape of the lipid from inverted cone to cone that than upon addition of arachidonate.

Alternatively, palmitoyl-CoA, which, in contrast to arachidonoyl-CoA, is an inverted-cone-shaped lipid, may have blocked endophilin-I-mediated SLIMV formation as a substrate but by acting directly on the lipid bilayer, which would imply a profound sensitivity of SLIMV formation to lipid shape. Direct evidence for an inhibition of endophilin-I-mediated SLIMV formation by inverted-cone-shaped lipids was obtained using LPC (C10:0 in position 1). LPC is not a substrate for endophilin I and hence would presumably not interfere with endophilin-I-catalyzed conversion of LPA to phosphatidic acid.

Previous models of dynamin function have suggested that this protein itself may operate as a 'pinchase' at the neck of synaptic and other endocytic vesicles\(^\text{30}\). However, evidence has been reported\(^\text{31}\) that dynamin does not function as a force-generating GTPase but rather, like other members of the GTPase superfamily, activates a downstream effector. Our observations raise the possibility that endophilin I constitutes this effector, which would be recruited to the membrane, and perhaps activated, by dynamin to mediate fission. Consistent with this concept, the deletion mutant of endophilin I lacking the SH3 domain, which still exhibited LPAT activity but presumably no longer bound to dynamin, did not mediate SLIMV formation. Activation of endophilin I upon its dynamin-mediated recruitment to the membrane would also explain why the rate of acyl transfer of soluble endophilin I was relatively low (≤17 mU mg⁻¹ protein).

In mammalian tissues, endophilin I belongs to a protein family whose other members are endophilin II and endophilin III (originally called SH3p8 and SH3p13, respectively), which also interact with dynamin\(^\text{32}\). Like endophilin I, whose tissue distribution is indistinguishable from that of dynamin I, endophili ns II and III co-distribute with dynamin II and III, respectively\(^\text{33}\), consistent with the existence of endophilin I–dynamin I–endophilin II–dynamin II and endophilin III–dynamin III complexes. This raises the possibility that the mechanism of positive-to-negative membrane curvature change in vesicle formation that we propose (the conversion of an inverted-cone-shaped lipid to a cone-shaped lipid by addition of a fatty acyl chain to a lysyglycerol–lipid) operates not only in SLIMV/synaptic vesicle formation mediated by endophilin I–dynamin I but, perhaps also, in the formation of other vesicles by endophilin II–dynamin II and endophilin III–dynamin III. Furthermore, this mechanism (operating not necessarily only with arachidonate) may not be confined to dynamin-dependent pinch-off of vesicles from donor membranes, but may be a common feature of vesicle formation and particularly membrane fission. Palmitoyl transfer to an unidentified acceptor is required in the pinching-off of Golgi-derived vesicles\(^\text{34-40}\). Perhaps this acceptor, too, is LPA, given the observation that BARS-50, a protein substrate of brefeldin A-dependent ADP-ribosylation, induces the fission of Golgi tubules by palmitoyl transfer to a membrane lipid (R. Weigert et al., unpublished observation).

Methods

SLMV formation

SLMV formation in perforated PC12 cells, using cell-surface biotinylated synaptophysin as marker (abbreviated as 'Biot. synaptophysin' in the figures) and the single 50% glycerol step centration to isolate SLIMVs, was carried out and quantified as described\(^\text{41}\). Differences in arbitrary units between Figure panels reflect differences in the exposure time of the synaptophysin immunoblot rather than in the efficiency of SLIMV formation. In the glucose control condition (cytosol control, 0.78 mg protein per ml, 0.18 uM LPA), the total biotinylated synaptophysin was recovered in SLIMVs\(^\text{15}\). To address the role of fatty acyl-CoA we used gel-filtered cytosol (PD-10 column, Pharmacia G2000 gel) supplemented with 1 mM GTP. LPA (1-La-hexadecanoyl-phosphate, acid, C16:0 (1:1), Sigma) was added in the form of 10 nmol 100 mM solution stock in LPA buffer (250 mM Hepes-KOH pH 7.4, 10 mM sucrose, 1 mM EDTA, 2.5 mg ml⁻¹ fatty acid-free BSA (Boehringer) sonicated (Sonorex super RK 1021); 2 min; reactions without LPA contained the corresponding amount of LPA buffer. LPC (1-e-hexadecanoyl-choline, decasoy, C16:0, Sigma), palmitoyl-CoA (Sigma) and arachidonoyl-CoA (Fluka) were added in 10 nmol, 10 nmol and 10 nmol respectively, MAP (Biomol Research Laboratories) was added from a 135 mM stock in DMSO; reactions without MAPA contained the corresponding amount of DMSO. PIP₂, from Crotalus adamanteus venom (CellSystems) was added from a 1 mg ml⁻¹ stock in 10 mM Hepes-KOH pH 7.4 to a final concentration of 0.01–0.1 µM. When investigating the role of arachidonoyl-CoA (Fig. 5b right), the pellet of perforated PC12 cells (~15 mg protein) was resuspended in 2 ml of the GGA/glutathione buffer containing 10 mg ml⁻¹ fatty-acid-free BSA, followed by addition of 50 µl PIP₂ from C. adamanteus and incubation at 37°C for 15 min. All subsequent steps were performed at 4°C. The BSA/PPLA₂-treated perforated cells were washed and resuspended in GGA/ glutathione buffer and used for perforated-cell reactions.

Purification of LPAT from bovine brain

All steps were carried out at 4°C. Bovine brains were homogenized with 3 volumes (v/v) of buffer A (50 mM Tris-Cl pH 74, 250 mM sucrose; 2 mM EDTA; 50 mM HEPES, 0.1% (v/v) 2-mercaptoethanol) in a Waring Blender-type homogenizer. A particulate fraction containing most of the LPAT activity present in the homogenate was obtained by differential centrifugation as follows: the homogenate for 10 min at 1,600×g, supernatant for 39 min at 5,000×g, supernatant for 39 min at 50,000×g, supernatant for 39 min at 100,000×g. The particulate fraction was resuspended in buffer A to 15 mg protein per ml and diluted five-fold with buffer B (25 mM Hepes-NaOH pH 7.4, 2 mM EDTA, 4 mM CHAPS, 0.1% (v/v) 2-mercaptoethanol). Insoluble material was removed by centrifugation for 1 h at 100,000×g (S.80Ti, Beckman). The supernatant, referred to as Extract (Table I), was loaded onto a Q-Sepharose column and LPAT-activity was eluted with a 0–1.5 M NaCl-gradient in buffer C (50 mM Tris-HCl pH 7.4; 250 mM sucrose; 2 mM EDTA; 2 mM CHAPS, 0.1% (v/v) 2-mercaptoethanol). The fractions of peak II were combined, dialysed against buffer D (50 mM potassium phosphate, pH 6.2, 2 mM EDTA; 0.1% (v/v) Nonidet P-40; 0.1% (v/v) 2-mercaptoethanol) and loaded onto a Mono Q HR 5/10 column, and LPAT was eluted with a 0–1 M NaCl gradient in buffer D. LPAT-containing fractions were combined, dialysed against buffer E (20 mM Tris-HCl pH 7.4; 10% (v/v) glycerol; 2 mM EDTA; 0.1% (v/v) Nonidet P-40; 0.1% (v/v) 2-mercaptoethanol) and loaded onto a Superdex 75 HiLoad 16/60 column in buffer F. LPAT-containing fractions were combined, dialysed against buffer F (20 mM Tris-HCl pH 7.4; 1 mM EDTA; 10% (v/v) glycerol; 100 mM NaCl 0.1% (v/v) Nonidet P-40; 10 mM HEPES, 0.1% (v/v) 2-mercaptoethanol) and chromatographed on a Superdex 75 HiLoad 16/60 column by analytical SDS-PAGE (Fig. 2a). Fractions containing the purified protein ~40K were diluted with buffer E (2 ml buffer E per 0.1 ml fraction) and concentrated to 0.1 ml by Centricon-30 centrifugation. This was repeated twice and, after this renaturation protocol, aliquots of the fractions were used for LPAT activity (Fig. 2b, Table II). Fractions containing the ~40K protein as the only detectable band and possessing LPAT activity were combined and used for 2D-PAGE, sequence analysis and determination of substrate specificity.
Poly-L-proline) Sepharose adsorption and elution

All steps were performed at 4°C. Rat brain cytoplasm (1.3-5 ml, 7.5 mg protein per ml) was subjected to a single passage over a column containing ~1 ml of packed poly-L-proline)-coated Sepharose beads and the flow-through was collected (depleted cytoplasm). The column was washed with 20 ml of GGA buffer and eluted either directly with 2 ml of 8 M urea containing 10 mM Hepes-KOH, pH 7.4 or with 2 ml of 30% (w/v) DMSO diluted in GGA buffer followed by 2 ml of 8 M urea containing 10 mM Hepes-KOH, pH 7.4. The DMSO eluate (and, as control, 30% DMSO in GGA buffer) was extensively dialysed against 10 mM Tris-Cl pH 7.4 containing 5 mM reduced glutathione, followed by concentration using Centricon-10 to ~200 ml, which contained ~30 μg of endophilin I as judged by SDS-PAGE using recombinant endophilin I as standard.

Protein identification

For MALDI, protein bands were excised from the gel and in-gel digested with trypsin as described12. Peptide-mass mapping was performed on a Bruker Reflex mass spectrometer, and the list of peptide masses searched against a non-redundant protein sequence database with an accuracy better than 50 ppm.

For protein sequencing of tryptic peptides, ~10 μg of the 40K protein purified from bovine brain through the ES/AGE step was digested twice for 2 h at 37°C with 0.1 μg TPCK-treated trypsin. Peptides were separated by RP-HPLC (Vydac 218 TP) using a 0.1% TFA/acetonitrile gradient and sequenced by the Edman method using an ABI sequencer.

Palmitoyl-CoA agarose adsorption and elution

All steps were performed at 4°C. Rat brain cytoplasm (0.8 ml, 7.5 mg protein per ml) was subjected, with or without 40 mM added free palmitoyl-CoA, to a single passage over a column containing 1.5 ml of packed palmitoyl-CoA-agarose beads (Sigma, equilibrated in GGA buffer), and the flow-through was collected. The column was washed with 20 ml of GGA buffer and eluted with 0.3 ml of GGA buffer containing 4 mM free palmitoyl-CoA (kept in the column overnight) followed by 3.5 ml of GGA buffer (total eluate 3.5 ml).

Recombinant endophilin I

A full-length endophilin I cDNA was generated by PCR (Pwo) using a mouse brain cDNA library and primers (sense primer 5′-GGATGAGCCGACTTGGGTTGGGCTGTGCCGATGG-3′, antisense primer 5′-GCTTACAGGAATCTTAAATGGCCGCACCAACCAG-3′) designed according to the sequence of mouse endophilin I (ref. 18). The cDNA was cloned into pCAGGS (Pharmacia) and pQE30 (Qiagen) to obtain GST- and His-tagged fusion proteins, respectively.

A cDNA coding for a truncated endophilin I (amino acids 1-299, referred to as del-endophilin I), which lacks the SH3 domain, was generated by PCR using the full-length endophilin I cDNA as template, and the above sense primer and the antisense primer 5′-TCGGCAAGCTGCTTGGGTTGGGCTGTGCCGATGG-3′, which encodes a stop codon after amino acid 293 and contains a Kpel restriction site. The PCR product was digested with BamHI and Kpel and ligated into pQE30 to obtain His-Δdel-endophilin I.

The fusion proteins were expressed in BL21 or BL21-D (inducible for lac promoter (Stratagene). Bacteria were lysed by two cycles of rapid freezing/thawing in PBS (25 ml per 1 L culture pellet). All subsequent steps were performed at 4°C. After addition of aprogin (5 μg ml⁻¹), leupeptin (10 μg ml⁻¹), benzamidine (1 mM), PMSF (0.5 mM) and DTT (0.5 mM), cells were sonicated and centrifuged for 15 min, the lysate was centrifuged for 10 min at 10000g and the resulting supernatant for 75 min at 20000g. The high-speed supernatant (~6 mg protein per ml) was used for LPA assay (Fig. 3a).

LPA-photofluorography (Fig. 4c), and the purification of recombinant endophilin I by either poly-L-proline) affinity chromatography (used to obtain the data shown in Figs 1c, 3a, 3b, 4b and 5a). The column chromatography in each experiment was run as follows: 2.5 ml of 10 mM NaH₂PO₄, pH 8.0, 300 mM NaCl, 10 mM imidazole, 2 mM Pefabloc (Roche) or Brönsted ion exchange followed by cation exchange. The purified proteins were eluted with elution buffer (40 mM Hepes-NaOH, pH 7.4, 5 mM MgCl₂, 0.23 mM ATP and 7.5 μM catalytic subunit of PKA). After the photofluorography step, samples were subjected to LPA assay. The LPA activity observed after a photofluorophorization reaction in the presence of added PKA was similar to that observed without performing the photofluorophorization reaction.

For the results shown in Figs 3 and 7, aliquots of 1°C (carboxyl)CoA (75-750 nM, 50 nM per nmol, Merck Biochemicals) in ethanol/water (1:1) were dried in a SpeedVac and resuspended in 150 μl of GGA buffer containing 5 mM reduced glutathione followed by sonication for 2 min. The tubes were then transferred to ice and brought to a final reaction volume of 300 μl by sequential additions as follows: (1) 137 μl or 122 μl of GGA buffer depending on the source of endorphin I to be added; (2) 1 μl of either the 10 mM ATP stock (see above in Materials and Methods) or 10 mM MgCl₂ stock by 10-fold dilution of the 10 mM stock with GGA buffer followed by sonication for 4 min); or LPA buffer (3) 10 μl of GGA buffer without or with ~3 μg of recombinant endorphin I, or 25 μl of the dialysed, concentrated and glycerol-diluted DMSO eluate without or with ~5 μg of endorphin I. Reactions were carried out for 15 min at 37°C and terminated by addition of 300 μl ice-cold 0.8 M KCl, 0.2 M H₃PO₄. Samples were mixed with 900 μl chloroform/methanol (2:1, vol:vol), subjected (along with a 1°C (carboxyl)phosphonic acid standard, NEN) to TLC using silica gel 60 and chloroform/methanol/formic acid (50:30:7) as solvent, and analysed by photofluorography.

Miscellaneous

Recombinant human profilin I and mouse profilin II were obtained as described12. Two-dimensional PAGE was performed using ampholines pH 3-10 for isoelectric focusing. A rabbit antiserum specifically recognizing endophilin I was obtained from Eurogentec, using the synthetic peptide (AcNH₂-KRPMRFLEAPGDTG-DKH), corresponding to residues 258-271 of endophilin I (ref. 30), coupled to KLH-Glu as antigen. Anti-Gerb antibody was obtained from Transduction Laboratories.

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