LETTERS

Endophilin and CtBP/BARS are not acyl transferases in endocytosis or Golgi fission

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Endophilins have been proposed to have an enzymatic activity (a lysophosphatidic acid acyl transferase or LPAAT activity) that can make phosphatidic acid in membranes[1–3]. This activity is thought to change the bilayer asymmetry in such a way that negative membrane curvature at the neck of a budding vesicle will be stabilized. An LPAAT activity has also been proposed for CtBP/BARS (carboxy-terminal binding protein/brefeldin A-ribosylated substrate), a transcription co-repressor that is implicated in dynamin-independent endocytosis and fission of the Golgi in mitosis[4–6]. Here we show that the LPAAT activity associated with endophilin is a contaminant of the purification procedure and can be also found associated with the pleckstrin homology domain of dynamin. Likewise, the LPAAT activity associated with CtBP/BARS is also a co-purification artefact. The proposed locus of activity in endophilins includes the BAR domain, which has no catalytic site but instead senses positive membrane curvature. These data will prompt a re-evaluation of the molecular details of membrane budding.

The lipid composition of a membrane helps to define the identity of an organelle, the flexibility and permeability of the bilayer, and its interaction partners. In phospholipid biosynthesis, glycerol-3-phosphate is acylated to form lysophosphatidic acid (LPA), which is further acylated (on the *sn*-2 position) by LPAAT activity to form phosphatidic acid. Phosphatidic acid is a precursor for glycerophospholipids (including phosphatidylethanolamine and phosphatidylinositols), some of which are increasingly recognized to have important roles in membrane traffic. The highly active LPAATs that are involved in major lipid biosynthetic pathways are transmembrane proteins: LPAAT-α has ubiquitous tissue distribution and is localized to the ER; LPAAT-β has more limited tissue distribution, is highly expressed in various tumour cells and is a drug target[7,8].

Low LPAAT activities have been also described for endophilin and CtBP/BARS, cytosolic protein families that are implicated in membrane trafficking[1,2,4,5], and have generated great interest. Endophilin A proteins are enriched presynaptically and are required for efficient synaptic vesicle retrieval[3,9]. These endophilins can tubulate membranes *in vitro* by their N-BAR domain[10] (and our unpublished data) and their intrinsic LPAAT activity is proposed to change membrane curvature by altering the lipid composition[1,3,11]. Similarly, endophilin B1 is proposed to have LPAAT activity and to be involved in maintaining mitochondrial morphology[2,12]. Another protein with proposed intrinsic LPAAT activity is CtBP3 (also termed BARS50)[4], a minor splice variant of CtBP1. There is no sequence homology between either of these protein families and transmembrane LPAATs, and there is no structural homology between CtBP proteins and endophilins[11,13,14]. CtBP3 is found at the Golgi and in the nucleus and is postulated to be involved in Golgi fission[5], in fission of vesicles involved in basolateral transport from the Golgi to the plasma membrane[6], in dynamin-independent endocytosis[6] and in transcriptional regulation[15]. Thus, its precise function is debated. CtBP proteins have a dehydrogenase domain that binds differentially to NAD^+/NADH and in transcription its co-repressor activity is proposed to be sensitive to the redox potential[16]. The proteins bind to transcription repressors such as E1A and RIP140 and to the tumour suppressor adenomatous polyposis coli (APC) through PxDLS motifs[13,17]. Mouse knockouts of CtBP1 and CtBP2 show gross developmental defects[18]. CtBP2 has an alternative promoter that gives rise to Ribeye, a protein that is enriched in dense bodies at ribbon synapses and is likely to function in synaptic vesicle exocytosis through interactions with active zone proteins[19,20]. CtBP1 is also present in synaptic terminals[19].

The involvement of endophilin in endocytosis and CtBP/BARS in Golgi remodelling has led to a model in which negative curvature at the neck and indeed membrane fission may be aided by a local change in lipid composition from LPA, an inverted-cone-shaped lipid, to phosphatidic acid, a cone-shaped lipid[1,4,21,22] (Fig. 1a). The similar mechanisms proposed for these non-homologous proteins led us to investigate further the basis of these reported activities. Doubt has previously been cast on the LPAAT activity of endophilin because it tubulates liposomes in the absence of LPAAT substrates[10]; however, the LPAAT activity implicates the protein in negative curvature generation and/or stabilization (Fig. 1b) and the *in vitro* tubulation assays do not address this type of curvature. Thus, a different approach is needed to test whether endophilin or CtBP/BARS do indeed have LPAAT activity.

In a previous study[1], the LPAAT activity of endophilin was localized to a region just larger than the N-BAR domain (amino acids 1–293). The activity was low but this might have been due to the non-optimal presentation of substrate or the absence of activators in the assay. We confirmed the observation of LPAAT activity using full-length rat endophilin A1 (Fig. 1c). We also found that the N-BAR domain (residues 1–247) has this activity (Fig. 1d, e).

We solved the structure of the endophilin A1 BAR domain and found, by comparison with other BAR domains that do not have LPAAT activity, that the only region that is different (and thus could be responsible for the activity) is an insertion in the BAR domain of 22 residues on the membrane interaction face that is not present in the structure (our own unpublished data). One might speculate that this region folds on substrate binding; however, we found that a deletion mutant of the region has LPAAT activity identical to that of the wild-type N-BAR domain (Fig. 1d).

Similarly to previous findings[1], we found LPAAT activity in a supernatant obtained after freeze–thaw cycles and high-speed centrifugation of endophilin-transformed bacteria, and none in that obtained after the same treatment of vector-transformed bacteria (Fig. 1e). Transformation with a construct encoding a control lipid-binding domain, namely the dynamin double pleckstrin homology (PH) domain, also had LPAAT activity in its supernatant (Fig. 1e). LPAAT activity co-purified with the PH domain on glutathione *S*-transferase (GST) purification (Fig. 1f), but was separated away

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from the protein by gel filtration (Fig. 1f and Supplementary Fig. 1e, f). Notably, purification of low LPAAT activity was more reproducible with the PH domain than with endophilin. The activity was not always observed with endophilin and seemed to correlate inversely with protein expression (that is, when we had much less protein expression, the GST purification was less effective but there was more LPAAT activity). As endophilin is a membrane-binding and tubulating protein, it may well fragment bacterial membranes or bind to small vesicles, resulting in co-purification of LPAAT activity. We consistently found no LPAAT activity in our endophilin BAR domain purified for crystallography.

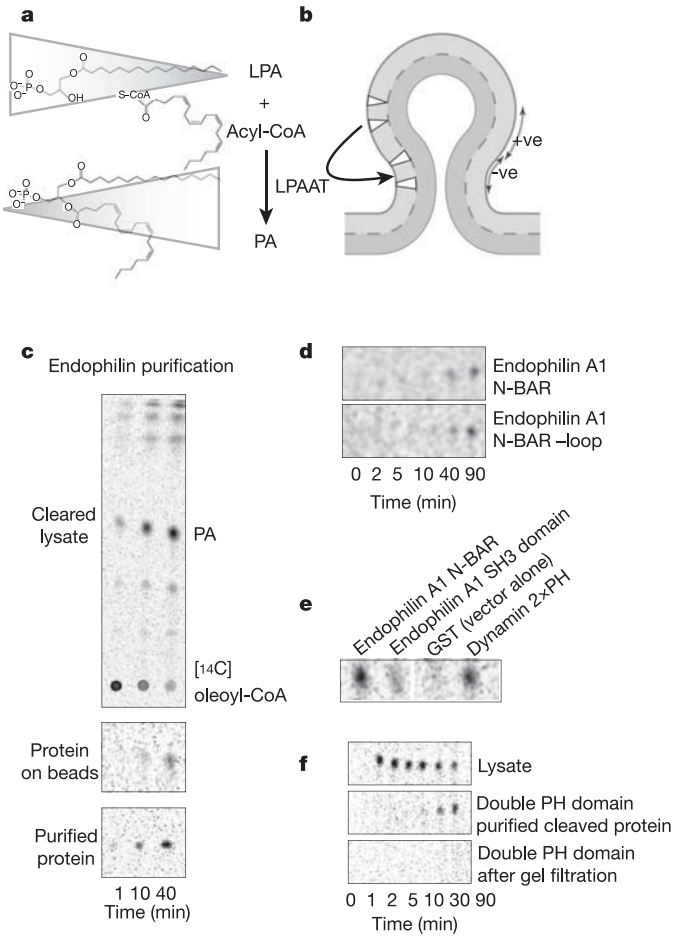

Figure 1 | LPAAT activity may be a co-purification artefact. **a**, LPAAT adds an acyl chain onto LPA to make phosphatidic acid (PA). **b**, LPA and phosphatidic acid should favour different curvatures during vesicle budding. **c**, Endophilin has LPAAT activity, as measured by the production of phosphatidic acid from oleoyl-CoA. Phosphatidic acid was separated by thin layer chromatography and identified by comparison with a standard (see Supplementary Fig. 1). GST-tagged full-length endophilin protein was expressed in BL21 *E. coli* and after affinity purification the GST tag was removed with PreScission. The LPAAT activity of the BL21 lysate was more robust and less variable than that of the purified protein (which was always freshly prepared before measurements). Only the region corresponding to phosphatidic acid is shown. **d**, The N-BAR domain (used at half the protein concentration) also has LPAAT activity, which is not affected by excision of the N-BAR loop. The methods were the same as in **c**. **e**, Although the freeze–thaw/high-speed supernatant from bacteria transformed with vector only does not have LPAAT activity, that from bacteria transformed with the control lipid-binding double PH domain of dynamin does (visible after 90 min of reaction time). **f**, LPAAT assays for purification of the dynamin double PH domain. Activity is present in the lysates (as expected) and also in the purified cleaved protein. No activity is observed after gel filtration. See Supplementary Fig. 1e for purification.

To test the possibility that LPAAT activity is not intrinsic to endophilin, we used a complementation assay in bacteria that has been previously used to identify LPAATs[24]. A JC201 strain of *Escherichia coli* lacking endogenous LPAAT activity grows at 30 °C but not at 42 °C (ref. 25). This defect at 42 °C is complemented by transformation of an *Escherichia coli* native LPAAT[25] (Fig. 2a). Transformation of JC201 with endophilin A1, other BAR domain constructs and control lipid-binding proteins showed that these constructs did not rescue temperature sensitivity (Fig. 2a) or growth kinetics

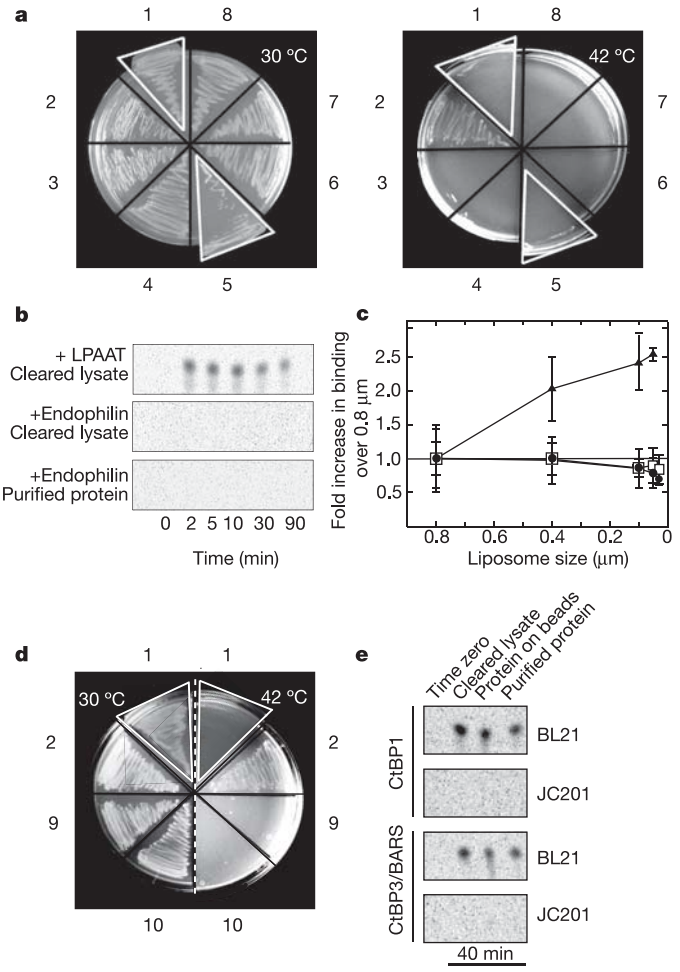

Figure 2 | Endophilin and CtBP/BARS do not have LPAAT activity and endophilin effects positive, rather than negative membrane curvature. **a**, *E. coli* that has no endogenous LPAAT activity (JC201 strain) cannot grow at 42 °C (sector 1). This growth defect can be rescued by other LPAATs (sector 2) but not by endophilin (sectors 3–5). LPAAT-negative *E. coli* were transformed with nothing (1); *pPlsC* (the *E. coli* gene encoding LPAAT), which rescues temperature sensitivity (2); GST-tagged full-length endophilin A1 (3); GST-tagged endophilin A1 N-BAR (4); C-terminally His-tagged endophilin A1 N-BAR (5); amphiphysin 1 N-BAR+ (6); epsin 1 ENTH domain (7); and arfaptin 2 (8). **b**, No LPAAT activity was observed for full-length endophilin in purifications from LPAAT-negative JC201 *E. coli*. **c**, The endophilin BAR domain binds better to smaller liposomes (liposomes of increased positive curvature). Filled triangles, endophilin A1 BAR domain; open squares, epsin 1 ENTH domain; filled circles, endophilin A1 N-BAR domain. The N-BAR actively tubulates liposomes and shows no preference in this assay. (See also Supplementary Methods and Supplementary Fig. 4.) Results are the mean ± s.d. of three experiments. **d**, CtBP1 or CtBP3/BARS does not rescue the temperature-sensitive growth of LPAAT-negative bacteria. JC201 were transformed with nothing (1) as in **a**; *pPlsC* (2) as in **a**; GST–CtBP1 (9); and GST–CtBP3/BARS (10). **e**, LPAAT assays on CtBP/BARS purified from BL21 and JC201 at time zero or after 40 min of incubation with the indicated fractions. CtBP/BARS has no LPAAT activity when purified from JC201.

(Supplementary Fig. 2). Thus, endophilin does not complement LPAAT-defective cells. Endophilin purified from this JC201 strain does not have LPAAT activity (Fig. 2b). In support of the LPAAT activity of endophilin, a previous study showed binding of this protein to the LPAAT substrates palmitoyl-CoA (linked to agarose) and LPA[1]. We cast doubt on the significance of these interactions in Supplementary Figs 3 and 4.

Functionally, LPAAT activity was proposed to generate or to aid the formation of negative curvature found at the neck of a budding vesicle. We found that the endophilin BAR domain, as expected from its homology to other BAR domains, actually binds preferentially to smaller liposomes, which have increased positive curvature rather than the negative curvature suggested by the LPAAT hypothesis (Fig. 2c). This indicates that if endophilin does function in formation of the vesicle neck then the curvature generated is positive rather than negative. Notably, the curvature inherent in the BAR domain corresponds more closely to that of the neck than the vesicle dome. The membrane-binding characteristics of the proposed locus of LPAAT activity are therefore at odds with the LPAAT hypothesis and suggest that endophilin has an alternative role in endocytosis.

CtBP/BARS has also been reported to have LPAAT activity[4]. We tested CtBP/BARS in the JC201 complementation assay and again found no rescue of growth at 42 °C (Fig. 2d). CtBP1 and its splice variant CtBP3 were expressed in BL21 and JC201 *E. coli*, and LPAAT activity was tested under the maximum activity conditions reported previously[4] (Fig. 2e). Although we observed the activity in protein purified from BL21, we did not observe it in protein purified from JC201, despite correct protein folding and the binding of either NADH to the nucleotide binding domain or PxDLS motifs from APC to the substrate binding domain (Supplementary Fig. 5). We further tested the specificity of LPAAT activity of CtBP/BARS using the proposed competitive inhibitor NADH[15]; the lack of inhibition observed again supports the idea that the activity is a co-purification artefact (Supplementary Figs 5 and 6).

The dynamic modulation of membrane curvature by localized LPAAT activity is an attractive proposition; however, we have presented evidence that this activity is not contained in endophilin or CtBP/BARS. Indeed, the reported activity of endophilin was less than 1 mol min^{-1} per mol of endophilin[1], which is much too slow to effect curvature during vesicle invagination unless an activator is used. An involvement of CtBP in membrane trafficking is strongly supported by its localization in dense bodies of synaptic terminals[19], where we propose that it might function in vesicle tethering rather than lipid modification. As in transcriptional regulation, this function is likely to be responsive to the redox balance, which would be a particularly useful readout of cellular energy status given the energy required for neurotransmission.

This study should act as a caution in interpreting enzymatic assays, even those done with purified recombinant protein. Although affinity tags make crude purifications very easy, our results indicate that this approach is not a replacement for rigorous purification. In the absence of evidence for direct and timely conversion of LPA to phosphatidic acid at the vesicle neck, the contribution of fission proteins such as dynamin takes on renewed importance, and the mechanism of negative curvature generation at the neck may well be a relaxation of the bilayer inner and outer leaflet lipid imbalance caused by vesicle neck formation rather than active generation.

METHODS

LPAAT assay. *E. coli* were lysed by French pressing and clarified by ultracentrifugation (186,000*g*, 40 min) except in Fig. 1e when they were lysed by freeze–thaw and centrifuged (280,000*g*, 75 min) according to the method of ref. 1. GST protein was bound to glutathione Sepharose (following the instructions of Amersham) and the tag cleaved using thrombin (Serva) or PreScission (Amersham) for 1 h at room temperature in 150 mM NaCl, 20 mM HEPES pH 7.4, 2 mM DTT. The purification was performed rapidly to avoid inactivation of a possible activity. LPAAT assays were performed in both the above buffer and in 100 mM KCl, 25 mM sucrose, 10 mM Tris pH 8, plus 1 mg ml^{-1} fatty acid-free BSA and 0.01% Triton X-100 with substrate concentrations (20–200 μM LPA) at 25 °C and 37 °C with ^{14}C-labelled oleoyl-CoA (Amersham) at a specific activity of 50 or 0.67 μCi ml^{-1} depending on the final concentration (10–100 μM). Three microlitres of the 100-μl reactions (using 5 and/or 10 μg protein) were spotted onto K60 silica gel chromatography plates (Merck) and run in chloroform:methanol:acetone:acetic acid:water, 50:20:10:10:5 followed by autoradiography.

Protein constructs. The following proteins were expressed in bacteria from either pET or pGex vectors: rat endophilin A1 N-BAR domain (residues 1–247), rat endophilin A1 N-BAR-loop domain (deletion of 59–87 and insertion of two glycines), full-length human arfaptin 2, rat epsin 1 ENTH domain (1–164), bovine dynamin 1 double PH domain (510–633), rat amphiphysin 1 N-BAR+ (1–377), full-length mouse CtBP1 and full-length mouse CtBP3 (where the first 13 residues of CtBP1 are replaced with Met,Ser), and GST–(Dros)E-APC (488–597). Proteins were affinity purified by GSH-Sepharose or Ni-NTA agarose (see Supplementary Fig. 1). If a tag is not present then the protein has been previously N-terminally GST tagged and the tag has been cleaved with thrombin.

Curvature sensing. Liposomes made from Folch brain lipid extract at 0.2 mg ml^{-1} in 20 mM HEPES pH 7.4, 150 mM NaCl, 2 mM DTT were extruded 11 times through cyclopore filters with given pore sizes[26]. Endophilin N-BAR (2 μM), endophilin BAR (2 μM) and epsin 1 ENTH domain (15 mg) were incubated in a volume of 100 μl for 10 min and sedimented at 100,000*g*. Sample buffer was added to the pellet and run on SDS–PAGE and stained with Coomassie brilliant blue. The amount of protein bound to the 0.8 μm liposomes was defined as 1 and the fold increase in binding was calculated relative to this value.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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