Importance of the pleckstrin homology domain of dynamin in clathrin-mediated endocytosis

Yvonne Vallis, Patrick Wigge*, Bruno Marks*, Philip R. Evans and Harvey T. McMahon

The GTPase dynamin plays an essential role in clathrin-mediated endocytosis [1–3]. Substantial evidence suggests that dynamin oligomerisation around the necks of endocytosing vesicles and subsequent dynamin-catalysed GTP hydrolysis is responsible for membrane fission [4,5]. The pleckstrin homology (PH) domain of dynamin has previously been shown to interact with phosphoinositides, but it has not been determined whether this interaction is essential for dynamin's function in endocytosis [6–9]. In this study, we address the *in vivo* function of the PH domain of dynamin by assaying the effects of deletions and point mutations in this region on transferrin uptake in COS-7 fibroblasts. Overexpression of a dynamin construct lacking its entire PH domain potently blocked transferrin uptake, as did overexpression of a dynamin construct containing a mutation in the first variable loop of the PH domain. Structural modelling of this latter mutant suggested that the lysine residue at position 535 (Lys535) may be critical in the coordination of phosphoinositides, and indeed, the purified mutant no longer interacted with lipid nanotubes. Interestingly, the inhibitory phenotype of cells expressing this dynamin mutant was partially relieved by a second mutation in the carboxy-terminal proline-rich domain (PRD), one that prevents dynamin from binding to the Src homology 3 (SH3) domain of amphiphysin. These data demonstrate that dynamin's interaction with phosphoinositides through its PH domain is essential for endocytosis. These findings also support our hypothesis that PRD–SH3 domain interactions are important in the recruitment of dynamin to sites of endocytosis.

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Results and discussion

Overexpression of dynamin-1 lacking its PH domain blocks endocytosis in COS cells

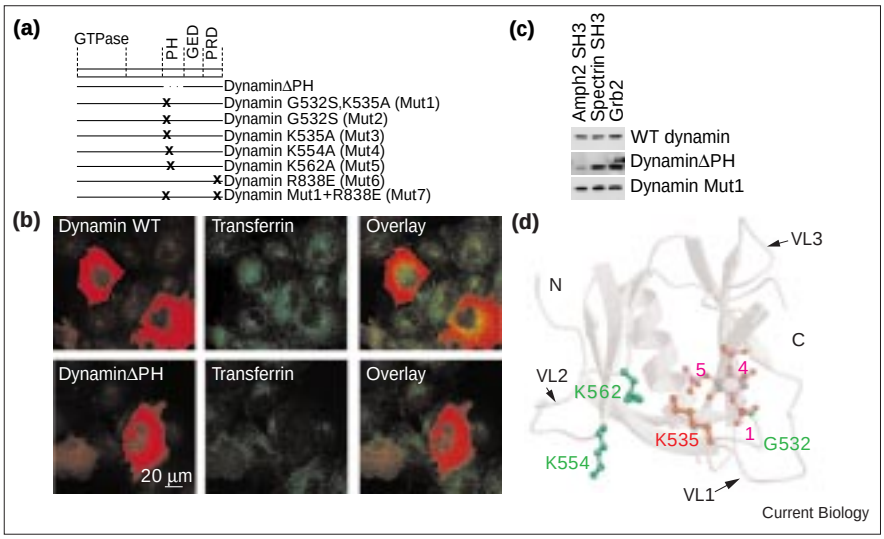
The structure of the PH domain of dynamin has been reported in four separate studies [10–13]. There is general agreement that the PH domain can interact with phosphoinositides including phosphatidylinositol (4,5)-bisphosphate ($PI(4,5)P_2$) and phosphatidylinositol (3,4,5)-trisphosphate ($PI(3,4,5)P_3$) [6,8,9,14]. But, there is a lack of correlation between the phosphoinositide binding of PH-domain mutants and their ability to inhibit rapid endocytosis when injected into chromaffin cells [14,15]. Dynamin mutants (Figure 1a) were screened for their ability to inhibit transferrin uptake in a well established endocytosis assay [16–18]. Expression of a dynamin mutant containing a deletion of the entire PH domain (dynaminΔPH) blocked transferrin uptake in 88 ± 5% of cells (Figure 1b, lower panels). It is striking that even weakly expressing cells, such as the one on the left-hand side of the lower panels in Figure 1b, showed reduced endocytosis. Cells overexpressing wild-type dynamin did not show any reduction in transferrin uptake (Figure 1b, upper panels).

Lys535 is a crucial residue for the *in vivo* function of dynamin

The deletion of the entire PH domain from its internal position in dynamin has multiple effects on dynamin function (see Figure 1c and Supplementary material published with this paper on the internet). More specific PH-domain mutations that would affect only phosphoinositide binding were therefore designed on the basis of structure. The dynamin PH-domain structure (Figure 1d) consists of an electrostatically polarised, seven-stranded β sandwich, with three variable loops opposite a carboxy-terminal α helix [12]. By superimposing this structure upon the structure of the phospholipase Cδ (PLCδ) PH domain complexed with its inositol-1,4,5-trisphosphate (IP_3) ligand [19] — and making the assumption that the IP_3 ligand would adopt the same binding conformation in the dynamin PH domain as it does in PLCδ — we were able to identify candidate residues for point mutagenesis.

As shown in Figure 1d, Lys535 comes into close proximity to the 5′ phosphate of the inositol ring. An adjacent residue, Gly532, previously implicated in dynamin-1 PH-domain function [15], was mutated to serine, and the Lys535 residue mutated to alanine, creating a double mutant (G532S, K535A) called Mut1. These residues were also mutated singly and the mutants called Mut2 and Mut3, respectively (Figure 1a). Two additional lysines in

Figure 1



PH-domain mutants. (a) Scheme of the constructs of bovine dynamin Ia designed in this study. Domains are abbreviated as follows: GED, GTPase effector domain; PH, pleckstrin homology; PRD, proline-rich domain. The dynamin mutants are shown underneath. (b) Dynamin lacking its PH domain (lower panels), but not wild-type (WT) dynamin (upper panels), blocks transferrin uptake in COS cells. Dynamin is stained red; transferrin is stained green. (c) Interaction of dynamin mutants with SH3 domains. GST fusion proteins containing the SH3 domains of amphiphysin2 (amph2), spectrin or Grb2 were immobilised on glutathione–agarose beads and incubated with extracts of COS cells transfected with each of the dynamin constructs shown (all three of which are expressed at approximately equivalent levels, as determined by immunoblotting). (d) Crystal structure of the dynamin-1 PH domain, showing the position of each of the four residues mutated. The structure (PDB code 1DYN [12]) is shown with the mutated residues highlighted (K535, mutation of which blocks endocytosis, in red and other residues, mutations of which do not affect endocytosis, in green). The IP_3 ligand is shown in a position taken from the structure of its complex with the PH domain of PLCδ-1 (PDB code 1MAI [19]), after superimposing the β-sheet strands of the two structures. Note that the position is very approximate, because the binding loops have very different conformations in the two structures. The first, second and third variable loops are abbreviated as VL1, 2, and 3, respectively.

the second variable loop were also mutated singly (Mut4 and Mut5); these residues are situated further away from the modelled IP_3 ligand (Figure 1d). Mut1, unlike dynaminΔPH, interacted normally with the SH3 domain of amphiphysin (Figure 1c).

Each of these five point mutants was tested for its ability to inhibit transferrin uptake (Figure 2a,b). Mut1 potently inhibited endocytosis with 78 ± 3% of cells showing undetectable levels of transferrin staining (Figure 2b). The Lys535 single mutant, Mut3, produced a similar block, suggesting that the Gly532 residue that was also mutated in Mut1 is not important for endocytosis. This was confirmed by the finding that cells expressing Mut2 showed normal transferrin uptake (Figure 2b). The lysine mutations in Mut4 and Mut5, and also two tyrosine mutations (Y596F and Y599F) in the third variable loop, had no effect on transferrin uptake (Figure 2a and Supplementary material).

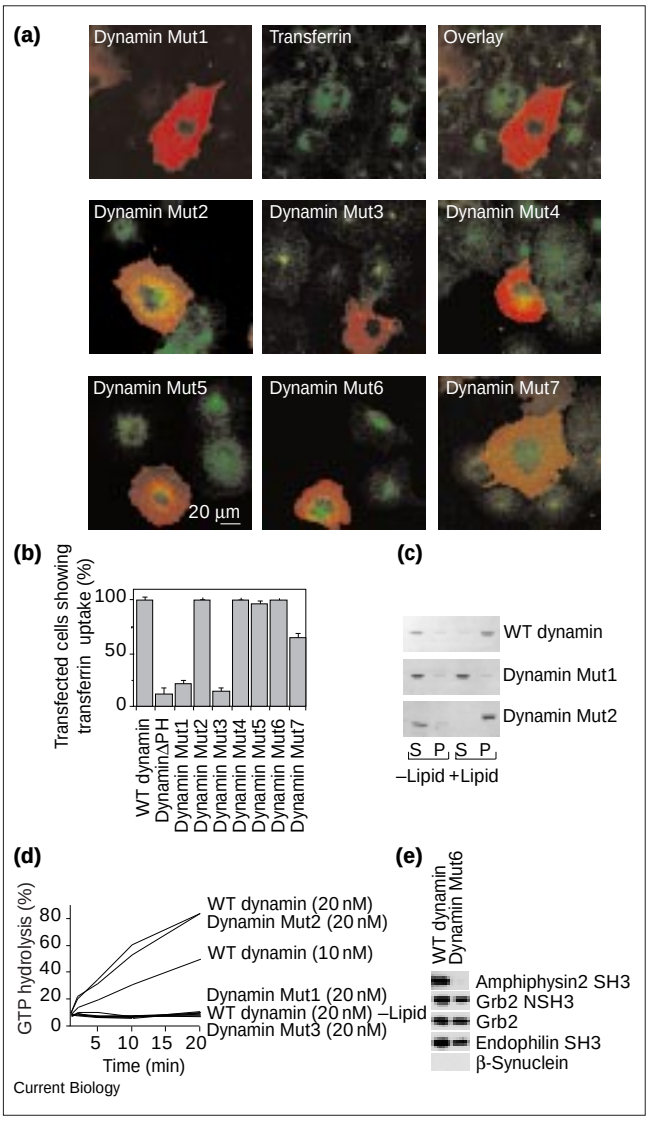
Mut1 dynamin cannot interact with PI(4,5)P_2 lipid nanotubes and has no lipid-activated GTPase activity

To verify whether the inhibitory effect of Mut1 is due to an alteration in phospholipid binding, wild-type and Mut1 dynamins were expressed in COS cells and purified from COS cell lysates using the amphiphysin-2 SH3 domain as an affinity ligand [20]. Each dynamin was then incubated with lipid nanotubes containing 10% PI(4,5)P_2 [21]. The ability of dynamin to cosediment with lipid nanotubes was then assayed by ultracentrifugation followed by SDS–PAGE and Coomassie staining (Figure 2c), and, in parallel, its ability to hydrolyse GTP, an activity that is strongly activated by lipid binding [6,21,22], was measured by thin-layer chromatography (Figure 2d). These results show that Mut1 dynamin, in contrast to wild type, did not bind to PI(4,5)P_2 and, consistent with this, its GTPase activity (measured under conditions of physiological salt concentration) was inhibited to background levels (Figure 2d). Mutating Lys535 alone (Mut3) produced the same effect, whereas the single Gly532 substitution (Mut2) showed normal lipid binding and GTPase activity (Figures 2c,d). Sedimentation experiments in which dynamin multimerisation was assayed in low ionic strength buffer demonstrated normal oligomerisation of Mut1 dynamin (data not shown); therefore, the inability of this mutant to support activated GTP hydrolysis reflects its greatly reduced phospholipid-binding properties.

The inability of Mut1 dynamin to interact with PI(4,5)P_2 confirms the modelling predictions that key basic residues in the first variable loop (notably Lys535) are essential for binding to the phosphates of the inositol head group. This is consistent with previous mutagenesis studies on the isolated PH domain [9]. Although the mutagenesis experiments argue that phosphoinositides constitute important ligands for dynamin *in vivo*, the data do not exclude the possibility that additional ligands for this PH domain (such as G protein βγ subunits [23–25]) may exist.

Figure 2

Inhibition of endocytosis by mutation of Lys535 and rescue by mutation of the PRD. (a) Dynamin Mut1 and Mut3, but not other point mutants, block endocytosis in COS cells. Note the weakly expressing cell in the upper left-hand corner (upper panels) that is still blocked in transferrin uptake. Cells overexpressing the dynamin double mutant Mut7 show increased transferrin uptake compared to cells expressing Mut1. The PRD mutation on its own, Mut6, has no effect on endocytosis. Dynamin is stained red; transferrin is stained green. For the upper panels, dynamin and transferrin staining are shown separately and then as an overlay; for all other panels, only the overlay is shown. (b) Bar graph showing the quantitation of the effect on transferrin uptake from at least three separate experiments, where between 10–20 fields are examined per experiment. (c) Mut1 dynamin, in contrast to wild type, cannot bind to phospholipid nanotubes. Purified dynamins were incubated with lipid nanotubes and cosedimentation was assayed by ultracentrifugation followed by Coomassie-stained gel analysis. P, pellets; S, supernatants. (d) The ability of nanotubes to activate GTP hydrolysis by Mut1 dynamin is abolished. Time points were taken at 1, 2, 5 and 20 min. GTPase assays were carried out on WT, Mut1, Mut2 and Mut3 dynamins (at the indicated concentration), each of which was purified on two separate occasions. (e) The PRD mutation Mut6 abolishes amphiphysin binding and, to a lesser extent, the binding of other SH3 domains. The binding of dynamin to recombinant proteins was detected by immunoblotting using the D632 polyclonal antibody. The β-synuclein protein was used as a negative control. Grb2 NSH3 represents the amino-terminal SH3 domain of Grb2 alone.



Mutagenesis of the amphiphysin-binding site partially rescues the dominant-inhibitory effect of Mut1 dynamin

We and others have postulated that dynamin is recruited to clathrin-coated pits through interactions between its PRD and the SH3 domain of amphiphysin [18,26,27]. To further test this hypothesis, the amphiphysin-binding site in dynamin, PSRPNR [20,28] (in the single-letter amino-acid code) was mutated to PSRPNE. The resulting point mutant, Mut6, was expressed in COS cells, and binding studies with various SH3 domains revealed that the interaction with amphiphysin is selectively abolished (Figure 2e). No detectable reduction in transferrin uptake was observed in cells expressing Mut6, however (Figure 2a). This lack of a dominant-negative effect is consistent with previous experiments in which truncation of the dynamin carboxyl terminus had no effect on endocytosis [16,29]. We speculate that Mut6 dynamin either is recruited by virtue of its dimerisation with endogenous dynamin and functions normally or is not recruited to endocytosis zones, remaining inactive in the cytosol.

A further mutant of dynamin, Mut7, that contains the PH-domain mutations of Mut1 and the amphiphysin-binding-site mutation of Mut6, was made. In contrast to Mut1, transferrin uptake occurred in the majority of cells (65 ± 4%) expressing Mut7 (Figure 2b), although uptake per cell was reduced and the perinuclear staining observed was less pronounced than in control cells (Figure 2a). The rescue of the Mut1 phenotype by the addition of the PRD mutation may be due to prevention of targeting of mutant dynamin to sites where it exerts its dominant-negative effect. This proposal is in agreement with the hypothesis that amphiphysin recruits dynamin to sites of endocytosis *in vivo*.

To summarise, mutagenesis of Lys535 in the first variable loop of dynamin is sufficient to abolish phosphoinositide binding and to exert a dominant-negative effect on endocytosis. Our data provide direct evidence for the physiological role of the dynamin PH domain in binding to membrane lipids. We suggest that amphiphysin targets dynamin to the clathrin-coated pit through SH3 domain–PRD interactions; dynamin then binds to phosphoinositides through Lys535, an interaction that is essential for its subsequent collaring of nascent clathrin-coated vesicles.

While this manuscript was under review, another paper describing the importance of dynamin's PH domain in receptor-mediated endocytosis was published by Achiriloaie *et al.* [30].

Materials and methods

Preparation of lipid nanotubes

Lipid nanotubes were prepared by a modification of the procedure described by Goldstein *et al.* [31], in which the dimethyl formamide (DMF) has been omitted and instead the lipids have been extruded through a filter of pore size 0.2 μm. The lipid composition was 40% phosphatidylcholine, 40% non-hydroxy fatty acid galactocerebrosides (NFA-GalCer), 10% cholesterol, 10% $PI(4,5)P_2$. These gave nanotubes of average diameter 28 nm and average length 500 nm.

Purification of dynamin mutants

Five dishes (90 mm) of COS cells transfected with plasmids expressing each of the mutant versions of dynamin were harvested after 3 days by scraping into buffer A (150 mM NaCl, 20 mM HEPES pH 7.4, 1 mM DTT and a protease inhibitor cocktail) containing 0.1% Triton X-100. The resulting lysate was centrifuged at 60,000 rpm for 20 min. A glutathione-S-transferase (GST) fusion protein of the amphiphysin2 SH3 domain, purified on glutathione–agarose beads, was incubated for 1 h with the clarified extract. After washing the beads extensively three times in buffer A, purified dynamin was eluted with elution buffer (1.2 M NaCl, 20 mM PIPES pH 6.2, 1 mM DTT). For sedimentation and GTPase assays, this eluate was diluted eightfold into 20 mM HEPES pH 7.4 and prespun at 60,000 rpm, to give a final dynamin concentration in the range of 0.5–1 μM.

COS cell transfections and staining methods

These have been described previously [18] and further details are given in the Supplementary material.

Dynamin GTPase assay and sedimentation assay

These assays have been described previously [20,27] and further details are given in the Supplementary material.

Supplementary material

Further methodological details, the effects of the dynaminΔPH mutant, and a structural alignment of the PH domains of dynamin and PLCδ showing the point mutations used are published with this paper on the internet.

References

1. Schmid SL, McNiven MA, De Camilli P: Dynamin and its partners: a progress report. *Curr Opin Cell Biol* 1998, 10:504-512.
2. McNiven MA: Dynamin: a molecular motor with pinchase action. *Cell* 1998, 94:151-154.
3. McClure SJ, Robinson PJ: Dynamin, endocytosis and intracellular signalling. *Mol Memb Biol* 1996, 13:189-215.
4. Takei K, Haucke V, Slepnev V, Farsad K, Salazar M, Chen H, *et al.*: Generation of coated intermediates of clathrin-mediated endocytosis on protein-free liposomes. *Cell* 1998, 94:131-141.
5. Sweitzer SM, Hinshaw JE: Dynamin undergoes a GTP-dependent conformational change causing vesiculation. *Cell* 1998, 93:1021-1029.
6. Barylko B, Binns D, Lin K-M, Atkinson MAL, Jameson DM, Yin HL, *et al.*: Synergistic activation of dynamin GTPase by Grb2 and phosphoinositides. *J Biol Chem* 1998, 273:3791-3797.
7. Scaife RM, Margolis RL: The role of the PH domain and SH3 binding domains in dynamin function. *Cell Signal* 1997, 9:395-401.
8. Zheng J, Cahill SM, Lemmon MA, Fushman D, Schlessinger J, Cowburn D: Identification of the binding site for acidic phospholipids on the PH domain of dynamin: implications for stimulation of GTPase activity. *J Mol Biol* 1996, 255:14-21.
9. Salim K, Bottomley MJ, Querfurth E, Zvelebil MJ, Gout I, Scaife R: Distinct specificity in the recognition of phosphoinositides by the pleckstrin homology domains of dynamin and Bruton's tyrosine kinase. *EMBO J* 1996, 15:6241-6250.
10. Fushman D, Cahill S, Lemmon MA, Schlessinger J, Cowburn D: Solution structure of pleckstrin homology domain of dynamin by heteronuclear NMR spectroscopy. *Proc Natl Acad Sci USA* 1995, 92:816-820.
11. Timm D, Salim K, Gout I, Guruprasad L, Waterfield M, Blundell T: Crystal structure of the pleckstrin homology domain from dynamin. *Nat Struct Biol* 1994, 1:782-788.
12. Ferguson KM, Lemmon MA, Schlessinger J, Sigler PB: Crystal structure at 2.2 A resolution of the pleckstrin homology domain from human dynamin. *Cell* 1994, 79:199-209.
13. Downing AK, Driscoll PC, Gout I, Salim K, Zvelebil MJ, Waterfield MD: Three-dimensional solution structure of the pleckstrin homology domain from dynamin. *Curr Biol* 1994, 4:884-891.
14. Klein DE, Lee A, Frank DW, Marks MS, Lemmon MA: The pleckstrin homology domains of dynamin isoforms require oligomerization for high affinity phosphoinositide binding. *J Biol Chem* 1998, 273:27725-27733.
15. Artalejo CR, Lemmon MA, Schlessinger J, Palfrey HC: Specific role for the PH domain of dynamin-1 in the regulation of rapid endocytosis in adrenal chromaffin cells. *EMBO J* 1997, 16:1565-1574.
16. Herskovits JS, Burgess CC, Obar RA, Vallee RB: Effects of mutant rat dynamin on endocytosis. *J Cell Biol* 1993, 122:565-578.
17. Okamoto PM, Herskovits JS, Vallee RB: Role of the basic, proline-rich region of dynamin in Src homology 3 domain binding and endocytosis. *J Biol Chem* 1997, 272:11629-11635.
18. Wigge P, Vallis Y, McMahon HT: Inhibition of receptor-mediated endocytosis by the amphiphysin SH3 domain. *Curr Biol* 1997, 7:554-560.
19. Ferguson KM, Lemmon MA, Schlessinger J, Sigler PB: Structure of the high affinity complex of inositol trisphosphate with a phospholipase C pleckstrin homology domain. *Cell* 1995, 83:1037-1046.
20. Owen DJ, Wigge P, Vallis Y, Moore JDA, Evans PR, McMahon HT: Crystal structure of the Amphiphysin-2 SH3 domain and its role in prevention of dynamin ring formation. *EMBO J* 1998, 17:5273-5285.
21. Stowell MHB, Wigge P, Marks B, McMahon HT: Nucleotide dependent structural changes in dynamin: evidence for a mechanochemical molecular spring. *Mol Biol Cell* 1998, 9:195a.
22. Tuma PL, Stachniak MC, Collins CA: Activation of dynamin GTPase by acidic phospholipids and endogenous rat brain vesicles. *J Biol Chem* 1993, 268:17240-17246.
23. Lin HC, Gilman AG: Regulation of Dynamin-I GTPase activity by G-protein beta-gamma subunits. *J Biol Chem* 1996, 271:27979-27982.
24. Liu JP, Yajima Y, Li H, Ackland S, Akita Y, Stewart J, *et al.*: Molecular interactions between dynamin and G-protein beta gamma-subunits in neuroendocrine cells. *Mol Cell Endocrinol* 1997, 132:61-71.
25. Lin HC, Duncan JA, Kozasa T, Gilman AG: Sequestration of the G protein beta gamma subunit complex inhibits receptor-mediated endocytosis. *Proc Natl Acad Sci USA* 1998, 95:5057-5060.
26. Shupliakov O, Low P, Grabs D, Gad H, Chen H, David C, *et al.*: Synaptic vesicle endocytosis impaired by disruption of dynamin–SH3 domain interactions. *Science* 1997, 276:259-263.
27. Wigge P, Kohler K, Vallis Y, Doyle CA, Owen D, Hunt SP, *et al.*: Amphiphysin heterodimers: potential role in clathrin-mediated endocytosis. *Mol Biol Cell* 1997, 8:2003-2015.
28. Grabs D, Slepnev VI, Songyang Z, David C, Lynch M, Cantley LC, *et al.*: The SH3 domain of amphiphysin binds the proline-rich domain of dynamin at a single site that defines a new SH3 binding consensus sequence. *J Biol Chem* 1997, 272:13419-13425.
29. Shpetner HS, Herskovits JS, Vallee RB: A binding site for SH3 domains targets dynamin to coated pits. *J Biol Chem* 1996, 271:13-16.
30. Achiriloaie M, Barylko B, Albanesi JP: Essential role of the dynamin pleckstrin homology domain in receptor-mediated endocytosis. *Mol Cell Biol* 1999, 19:1410-1415.
31. Goldstein AS, Lukyanov AN, Carlson PA, Yager P, Gelb MH: Formation of high-axial-ratio-microstructures from natural and synthetic sphingolipids. *Chem Phys Lipids* 1997, 88:21-36.

Supplementary material

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Supplementary results

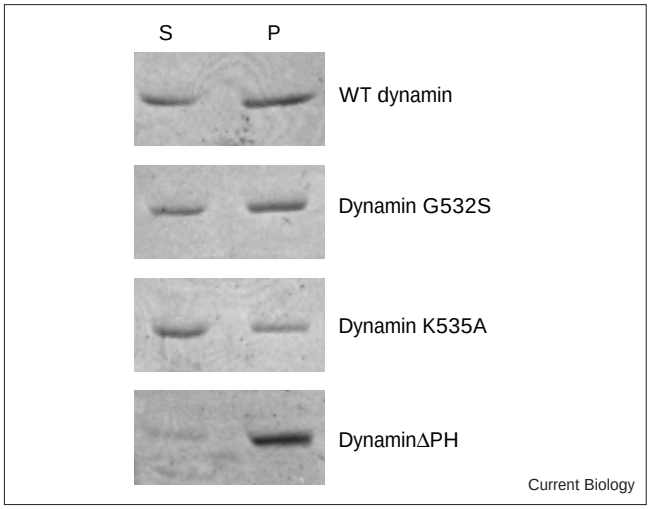
Studies of the dynamin PH-domain deletion construct

The dominant-negative effect of relatively low levels of dynaminΔPH expression on endocytosis is probably due to its increased tendency to participate in dynamin–dynamin interactions (thus effectively sequestering endogenous dynamin more efficiently than any of the point mutants). It is clear that this protein cannot bind lipid nanotubes, an interaction that requires the PH domain. But two lines of evidence indicate that it has an increased ability to form multimeric complexes with other dynamin molecules: first, sedimentation assays of this mutant in the absence of a lipid template (that is, in a necessarily low ionic strength) show an increased proportion of dynaminΔPH appearing in the pellet fraction (Figure S1); and second, GTPase assays under identical conditions revealed this greater sedimentation to be due to the formation of at least a proportion of functional multimers that can hydrolyse GTP (data not shown). Although dynamin's GTPase activity is much lower in the absence of lipid nanotubes, this experiment showed a higher rate of hydrolysis for dynaminΔPH than for wild-type, Mut2 or Mut3 constructs.

These data raise the possibility that the deletion of dynamin's PH domain in some way improves the accessibility of its GED region (and/or other domains responsible for dynamin–dynamin interactions) such that dynamin's oligomerisation state is shifted in favour of multimeric forms, be they higher-order ring structures, or possibly tetramers.

This hypothesis would explain why amphiphysin binding to dynaminΔPH is selectively reduced (Figure 1c). Although SH3 domains of spectrin and Grb2 do not affect dynamin's monomer–multimer equilibrium (that is, they bind equally well to either form of the dynamin molecule), there is good evidence that SH3 domains of amphiphysin cannot bind to the multimeric form [S1]. The data also predict that transfection of such a dynaminΔPH construct into COS cells will be even better at sequestering endogenous dynamin into inactive multimers than any of the point mutants. Thus, dynaminΔPH potently inhibits endocytosis, more so than the double-point mutant Mut1. Whatever the exact reason for the potent dominant-negative effect of dynaminΔPH, we pursued a more refined analysis of the function of dynamin's PH domain by point mutagenesis.

Figure S1



Oligomerisation assay for mutant dynamins. Wild-type and mutant versions of dynamin were purified from COS cells and assembly was tested in low ionic strength buffer in the absence of a lipid template. Multimerised dynamin was pelleted and the amount of dynamin contained in either the supernatant (S) or pellet (P) fraction was determined by SDS–PAGE followed by Coomassie staining.

Morphology of cells expressing dynaminΔPH

One feature of the cells transfected with constructs that inhibit binding to phosphoinositides was that they tended to form clusters on the coverslip, reminiscent of clonal colonies, and displayed a loss of contact inhibition compared with control cells. We speculate that this may be due to failure to endocytose activated growth factor receptors, leading to constituitive signalling and a loss of control of proliferation.

Studies of tyrosine mutants

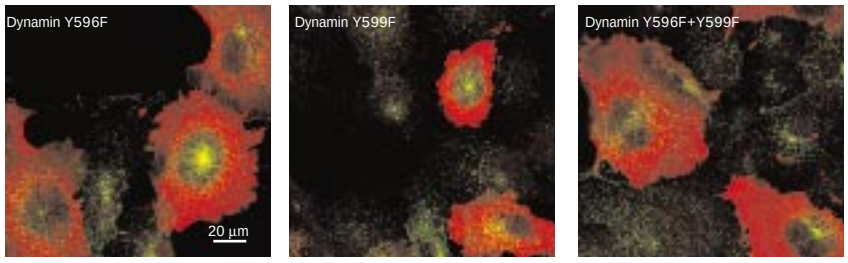
Immunofluorescence images of COS cells transfected with point mutants Y596F and Y599F are shown in Figure S2. These mutants serve as negative controls and are potentially important residues in the third variable loop; the lack of an inhibitory effect (Figure S2) argues that the tyrosines mutated are not crucial for endocytosis *in vivo*.

Supplementary materials and methods

Site-directed mutagenesis

Bovine dynamin I (pCMV96-1a, from T.C. Südhof, Dallas) was used as a template for reverse and forward 20mer PCR primers incorporating

Figure S2



Tyrosine mutations in the third variable loop of the PH domain of dynamin do not affect transferrin uptake. Y596 and Y599 were mutated singly and in combination. Dynamin is stained red; transferrin is stained green. The absence of an effect on transferrin uptake argues against an important role for these mutants in lipid binding.

altered bases. After 10 cycles the product was digested for 1 h with *Dpn*I and transformed into bacteria for plasmid preparation. Plasmids were checked by sequencing using the Big Dye terminator kit (ABI). The various mutants made are illustrated in Figure 1a.

Transient transfection of COS-7 cells

COS-7 cells plated onto 10 cm dishes were transfected using DEAE–dextran and chloroquine, followed by glycerol shock, according to the following brief description. Cells were plated 24 h prior to transfection (day 0) at a density of 1.4×10^5 cells/ml (10^6 cells in 7 ml normal DMEM medium). On day 1, plasmid DNA to be transfected (2.2 μg per dish) was mixed gently for 5 min with DEAE–dextran (80 μl of a 5 mg/ml stock freshly prepared in sterile water). The DNA–dextran complexes formed were then diluted by the addition of 550 μl 2 × Tris-buffered saline (TBS) and 418 μl water per dish to be transfected. All 1 ml was then applied to the dish of cells seeded the previous day, washed twice beforehand in 1 × TBS. Dishes were incubated for 30 min in a 37°C incubator containing 5% CO_2. DNA-containing solution was aspirated, and 10 ml DMEM containing 0.1 mM chloroquine added (100 × stock freshly made up from 7 mg solid and 1.5 ml medium). After incubation for 3 h at 37°C, cells were washed twice in 1 × TBS and glycerol-shocked by incubating with 20% glycerol in DMEM for 4 min at room temperature. After gently aspirating and gently washing twice in 1 × TBS, cells were left to recover in 7 ml DMEM at 37°C. In experiments involving biochemical analysis, cells were left for 48–60 h before harvesting in lysis buffer (buffer A; 150 mM NaCl, 20 mM HEPES pH 7.4, 1 mM DTT and a protease inhibitor cocktail plus 0.1% Triton X-100). For immunofluorescence analysis of transfected cells, however, they were left for only 18 h before replating onto poly-L-lysine-coated coverslips.

Transferrin uptake assays and immunofluorescence microscopy

Cells were incubated 18 h after replating onto poly-L-lysine-coated coverslips (36 h post-transfection) at 37°C in serum-free DMEM for 1 h, then at 37°C in DMEM containing 25 μg/ml biotinylated human transferrin (Sigma). After washing three times in phosphate-buffered saline at room temperature (to reduce surface and non-specific labelling), cells were fixed in 4% paraformaldehyde, permeabilised with 0.2% saponin, and blocked in 10% goat serum. Transferrin uptake was visualised with FITC-conjugated streptavidin and dynamin with the polyclonal antibody D632. Secondary antibodies were Texas Red-conjugated goat anti-rabbit for dynamin. Coverslips were mounted under AIRWOL plus 1% DABCO and cells imaged on an MRC 1024 scanning confocal microscope. For bar chart quantitation, 'blocked cells' were defined as all transfected cells in which transferrin uptake was < 20% of normal. These cells were counted and divided by the total number of transfected cells (in multiple fields), and extent of inhibition thus expressed as a percentage. Similarly, cells 'rescued' in endocytosis were defined as all those cells in which transferrin was internalised to levels of at least 30% of normal. SEMs were calculated from the results of at least three independent experiments.

Dynamin GTPase assays

All GTPase assays were performed at room temperature. Nanotubes (0.1 mM lipid) and dynamin (typically 0.02 μM) were incubated for 10 min at room temperature in buffer B (135 mM NaCl, 5 mM KCl, 20 mM HEPES, 1 mM $MgCl_2$) and then reactions (in 20 μl) were initiated by the addition of GTP (to 25 μM) and 10 μCi/ml [α-^{32}P]GTP. Hydrolysis of GTP was analysed by thin-layer chromatography on PEI cellulose (Sigma) and the results were quantitated using a phosphoimager and ImageQuant software (Molecular Dynamics).

Dynamin sedimentation assays

Purified dynamin (0.02 μM) was self-assembled *in vitro* by incubation for 20 min in either low ionic strength buffer (for the formation of ring structures in the absence of a lipid template) or in 135 mM NaCl, 5 mM KCl, 20 mM HEPES and 1 mM $MgCl_2$; to each mixture 1 mM lipid nanotubes plus 0.1 mM nucleotide (for the formation of ring structures in more physiological conditions, around a membrane template) was added. Multimerised dynamin was then sedimented by ultracentrifugation at 80,000 × *g* for 20 min. The extent of self-assembly was assayed

Figure S3

Structural alignment of dynamin and PLCδ PH domains. Mutated resides used in this manuscript are shown by arrows on top of the sequence. Regions of structural similarity are indicated by dots; β sheets and α helices are also indicated by blue and red lettering, respectively. The interactions of PLCδ with IP_3 phosphates (1, 4 and 5) through amino-acid side chains or backbone (B) as deduced by Ferguson *et al.* [S2] are shown.

by running pellet (P) or supernatant (S) fractions on 12% SDS–PAGE, followed by Coomassie staining.

References

S1. Owen DJ, Wigge P, Vallis Y, Moore JDA, Evans PR, McMahon HT: Crystal structure of the Amphiphysin-2 SH3 domain and its role in prevention of dynamin ring formation. *EMBO J* 1998, 17:5273-5285.

S2. Ferguson KM, Lemmon MA, Schlessinger J, Sigler PB: Structure of the high affinity complex of inositol trisphosphate with a phospholipase C pleckstrin homology domain. *Cell* 1995, 83:1037-1046.