Inhibition of receptor-mediated endocytosis by the amphiphysin SH3 domain

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Background: Receptor-mediated endocytosis appears to require the GTP-binding protein dynamin, but the process by which dynamin is recruited to clathrin-coated pits remains unclear. Dynamin contains several proline-rich clusters that bind to Src homology 3 (SH3) domains, which are short modules found in many signalling proteins and which mediate protein–protein interactions. Amphiphysin, a protein that is highly expressed in the brain, interacts with dynamin *in vitro*, as do Grb2 and many other SH3 domain-containing proteins. In this study, we examined the role of amphiphysin in receptor-mediated endocytosis *in vivo*.

Results: To address the importance of the amphiphysin SH3 domain in dynamin recruitment, we used a transferrin and epidermal growth factor (EGF) uptake assay in COS-7 fibroblasts. Amphiphysin is present in these cells at a low level and indeed in other peripheral tissues. Confocal immunofluorescence revealed that cells transfected with the amphiphysin SH3 domain showed a potent blockade in receptor-mediated endocytosis. To test whether the cellular target of amphiphysin is dynamin, COS-7 cells were cotransfected with both dynamin and the amphiphysin SH3 domain; here, transferrin uptake was efficiently rescued. Importantly, the SH3 domains of Grb2, phospholipase Cγ and spectrin all failed to exert any effect on endocytosis. The mechanism of amphiphysin action in recruiting dynamin was additionally tested *in vitro*: amphiphysin could associate with both dynamin and α-adaptin simultaneously, further supporting a role for amphiphysin in endocytosis.

Conclusions: Our results suggest that the SH3 domain of amphiphysin recruits dynamin to coated pits *in vivo*, probably via plasma membrane adaptor complexes. We propose that amphiphysin is not only required for synaptic-vesicle endocytosis, but might also be a key player in dynamin recruitment in all cells undergoing receptor-mediated endocytosis.

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Background

The uptake of membrane at the cell surface by clathrin-mediated endocytosis plays a crucial role in synaptic-vesicle recycling in neurons as well as in the acquisition of nutrients and the downregulation of receptor–ligand complexes by all cells [1]. The uptake process is thought to begin with the recruitment of adaptor complexes (AP-2) to the plasma membrane where they bind to the cytoplasmic tails of receptors that are destined to be internalised, and concentrate them in patches. Clathrin, a triskelial protein, also binds to the adaptor complexes and drives the formation of a clathrin lattice at the endocytosing region of the membrane. Although the clathrin coat provides the structural scaffold for the early budding vesicle, completion of the process of vesicles 'pinching off' from the plasma membrane requires dynamin, a large GTPase protein. The *shibire* mutation in *Drosophila* corresponds to a mutation in dynamin that renders it incapable of GDP–GTP cycling; *shibire* mutants accumulate tubular invaginations that are collared with ring-like structures, and display a complete block to all receptor-mediated endocytosis [2–4]. The same invaginated vesicle intermediates that have not completely pinched off from the plasma membrane are observed using electron microscopy when isolated nerve terminals are treated with GTPγS [5]. Immunogold labelling demonstrates that the rings coating these structures are composed of dynamin. The current model predicts that dynamin is recruited to the coated pit and subsequently oligomerises into rings around the neck of the pit. GTP hydrolysis is triggered, driving a conformational change in the molecule that pinches off the vesicle (for review, see [6]). Although the role of dynamin in receptor-mediated endocytosis is now well documented, pinning down its protein partners has been more elusive [7]. Recently, De Camilli and colleagues [8] have shown that the major dynamin-binding partner in brain extracts is the Src homology 3 (SH3)-domain-containing protein amphiphysin, which was originally identified as a synaptic-vesicle-associated protein

expressed in the brain [9]. Amphiphysin has also been reported to interact via a distinct domain with the appendage (ear) domain of the α subunit of adaptin in the AP-2 adaptors [8,10]. This interaction has led to the proposal that amphiphysin could potentially recruit dynamin to clathrin-coated pits, perhaps by binding to plasma membrane adaptor proteins [8], despite a previous report of a direct interaction between dynamin and α-adaptin [10]. In this study, we tested whether amphiphysin could link dynamin to adaptin ear domains *in vitro* and *in vivo*. We used a transferrin uptake assay to demonstrate that, although many SH3-domain-containing proteins interact with dynamin *in vitro*, only the amphiphysin SH3 domain appeared to affect transferrin uptake when expressed in fibroblasts. These results underline the importance of amphiphysin in all clathrin-mediated endocytosis as well as in the specialised pathway of synaptic-vesicle retrieval.

Results

The interaction of different domains of amphiphysin with dynamin and adaptor proteins was initially tested in brain extracts (Figure 1a). The carboxy-terminal domain of amphiphysin (Amph-SH3) was found to bind to dynamin (in agreement with [10]), whereas the amino-terminal 300 amino acids of amphiphysin (AmphAB) interacted with AP-2 complexes (see scheme in Figure 1a). We therefore tested whether full-length amphiphysin could interact with both molecules simultaneously, and thus act as a potential linker, recruiting dynamin to adaptor complexes clustered at the coated pit. We incubated the ear domain of α-adaptin (previously reported to be sufficient for interaction with amphiphysin [10]) with extracts of COS cells overexpressing dynamin and amphiphysin. A direct interaction between dynamin and the ear domain was barely detectable; however, in the presence of amphiphysin, the amount of bound dynamin was greatly enhanced (Figure 1b). This suggests that the three proteins can form a complex, with amphiphysin serving a bifunctional role in interacting through separate domains with dynamin and adaptor proteins.

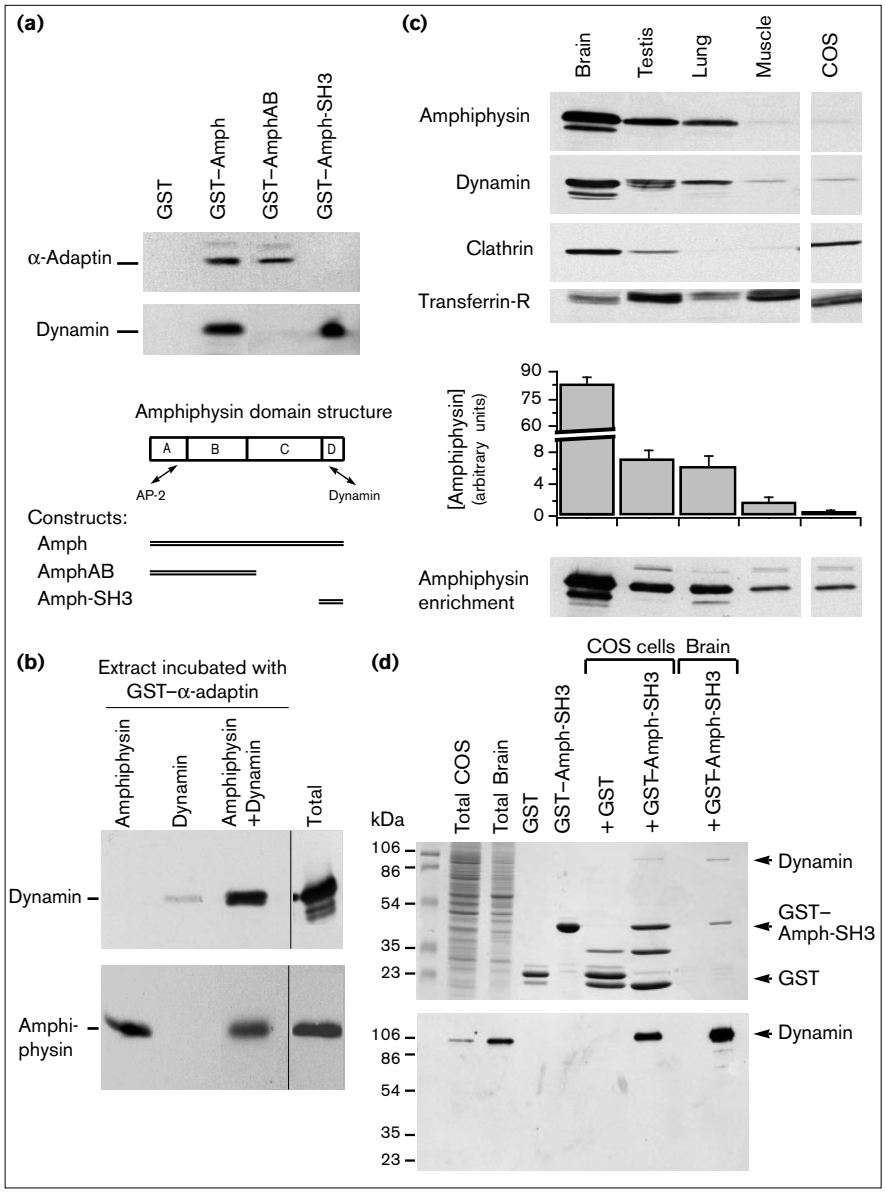
Because the expression of amphiphysin has previously been shown to be restricted to brain and endocrine tissues [9,11], we first established whether amphiphysin is expressed in COS cells and in peripheral tissues. Figure 1c shows that the expected 125 kDa amphiphysin protein found in brain was also present, albeit at a lower level, in testis, lung, muscle and fibroblasts. The levels of expression are seen more clearly upon enrichment of the extracts with a glutathione S-transferase (GST)-tagged ear domain of α-adaptin. Indeed, the levels of amphiphysin correlate closely with those of dynamin, both being at least 10-fold higher in the brain (see bar chart in Figure 1c). To verify that amphiphysin can interact with dynamin in COS cells, we used the GST-tagged SH3 domain of amphiphysin and affinity-purified a single protein of about 100 kDa from COS cell extracts (Figure 1d); this 100 kDa protein migrated at the same size as dynamin purified from rat brain, and, indeed, reacted with a dynamin monoclonal antibody.

To test the hypothesis that the SH3 domain of amphiphysin is essential for dynamin recruitment *in vivo*, we followed the uptake of transferrin in COS cells with biotinylated-transferrin as an assay for receptor-mediated endocytosis. Under confocal microscopy, untransfected COS cells gave the classic punctate, perinuclear transferrin staining, characteristic of the endosomal transferrin-internalisation pathway. Cells transfected with a Myc-tagged amphiphysin SH3 domain showed an ~80% block in transferrin uptake (Figure 2a, see also Figure 4c for quantitation) and an abolition of the classic punctate, perinuclear staining. The most likely explanation for this is that the interaction of dynamin with endogenous amphiphysin was disrupted in these cells. The disruption of this interaction would prevent targeting of dynamin to the coated pit, blocking endocytosis at the late stage of vesicle budding. Thus these data indicate that the amphiphysin–dynamin interaction observed in Figure 1a,b,d is likely to have physiological importance.

The SH3 domain of amphiphysin has also been reported to interact with synaptojanin (p145) [12]. A tagged version of the amphiphysin SH3 domain bound to both dynamin and a 145 kDa protein from brain extracts, although dynamin bound approximately 10-fold more amphiphysin than the 145 kDa protein (data not shown). It is therefore possible that the SH3 domain of amphiphysin acts in a dominant-negative manner by sequestering not dynamin but synaptojanin, or perhaps some other, unidentified SH3-binding protein present in COS cells. To address this possibility, COS cells were cotransfected with the amphiphysin SH3 domain and full-length rat dynamin. In over 80% of cotransfected cells, transferrin uptake was rescued (Figure 2b, see also Figure 4b for quantitation). One cell in the overlay panel, marked with an arrow, shows that the rescue was dependent on dynamin levels: in this cell, transferrin uptake was still blocked, presumably because dynamin was not expressed at a sufficiently high level. Expression of dynamin alone did not inhibit transferrin uptake (Figure 2c, and in agreement with Herskovits *et al.* [13]).

Amphiphysin is, however, not the only SH3-domain-containing protein that can interact with dynamin. Grb2, phospholipase Cγ (PLCγ) and phosphatidylinositol-3-kinase (PI(3)K), among others, have all been reported to bind to the polyproline domain of dynamin *in vitro* [14–16]. The physiological significance of all of these interactions is not understood, but it was important to test the effect of overexpression of these other SH3 domains on transferrin uptake. COS cells transfected with either

Figure 1

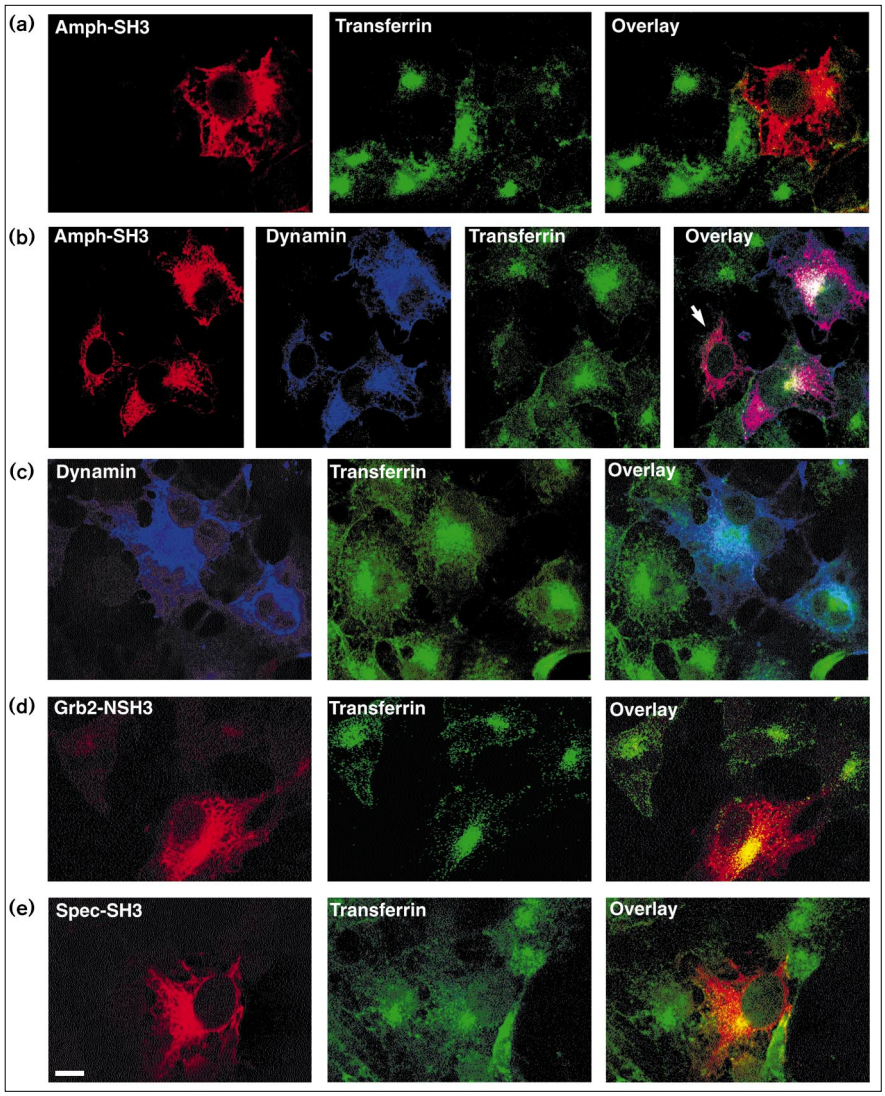


(a) Amphiphysin interacts with α-adaptin and dynamin via separable, non-overlapping domains. Full-length amphiphysin (Amph), the amino-terminal domain (AmphAB) or the carboxy-terminal domain (Amph-SH3) were expressed as glutathione S-transferase (GST) fusion proteins, and incubated with 10 mg rat brain extract. Bound proteins were subjected to 7% SDS–PAGE and detected by immunoblotting. A schematic view of amphiphysin (1–683 amino acids) and the constructs used in these studies is also shown. The domains are as follows: A, predicted α-helical region (amino acids 1–131); B, proline-rich region (132–342); C, acidic domain (343–605); D, SH3 domain (606–683). **(b)** Amphiphysin can efficiently recruit dynamin to the ear domain of α-adaptin *in vitro*. GST–α-adaptin ear domain was incubated with extracts of COS cells overexpressing either amphiphysin or dynamin, or both, as shown, and bound proteins were analysed by immunoblotting with polyclonal antibodies to amphiphysin (Ra3 antibody) and dynamin. The lane labelled 'Total' contains extracts from COS-7 cells overexpressing either amphiphysin or dynamin. **(c)** Amphiphysin is expressed in COS cells and other peripheral tissues. Extracts were prepared from each tissue in buffer A (see Materials and methods) containing 1% TX-100 and assayed by Bradford protein detection. Equal amounts of protein (50 μg, except 150 μg COS extract) were subjected to SDS–PAGE followed by immunoblotting for amphiphysin (Ra4.2 antibody), clathrin, dynamin (monoclonal antibody) or the transferrin receptor (Transferrin-R) as a measure of protein loading). Note that the Ra4.2 and the dynamin monoclonal antibodies (Transduction Laboratories) detect low levels of expression of amphiphysin and dynamin, respectively, in other tissues compared with expression levels in the brain. The bar chart shows the quantitation of the tissue distribution of amphiphysin from three experiments normalised to protein concentration (± standard deviation). The enrichment of amphiphysin in all five tissues by the GST–α-adaptin ear domain is shown below the bar chart. **(d)** The amphiphysin SH3 domain binds specifically to dynamin in COS cells. GST-tagged SH3 domain, or GST (both immobilised on glutathione–agarose) were incubated with extract from either brain or COS cells (both at 10 mg/ml), in buffer A containing 1% TX-100. The bound proteins were analysed by Coomassie staining (upper panel) or immunoblotting with a DynI/II monoclonal antibody (lower panel). Several major proteins are bound nonspecifically by GST, but the 96 kDa dynamin band is precipitated only by GST–Amph-SH3. This comigrates with dynamin present in total COS or brain extracts. Also shown are total COS cell and brain extracts, and total recombinant GST and GST–Amph-SH3 proteins (10 μg of each) before incubation with dynamin extracts.

the Grb2 amino-terminal SH3 domain, the SH3 domain from spectrin (Figure 2d,e) or PLCγ (data not shown) exhibited completely normal transferrin uptake. These SH3 domains, like the amphiphysin SH3 domain or full-length Grb2, bound to purified dynamin *in vitro* (Figure 3). The blockade of endocytosis thus appears to be specific to the SH3 domain of amphiphysin.

It was important to exclude the possibility that the blockade by the amphiphysin SH3 domain was an artefact specific to the transferrin receptor. We therefore examined the effect of the amphiphysin SH3 domain on other markers of receptor-mediated endocytosis, for example, the uptake of epidermal growth factor (EGF). Uptake of labelled EGF was inhibited in all cells examined, averaging 76% inhibition

Figure 2



The amphiphysin SH3 domain inhibits transferrin uptake in COS cells. Cells were transfected by the DEAE-dextran method as described in Materials and methods with pCMV constructs of **(a)** the SH3 domain of amphiphysin (Amph-SH3), **(b)** Amph-SH3 and dynamin, **(c)** dynamin alone, **(d)** the amino-terminal SH3 domain of Grb2 (Grb2-NSH3) and **(e)** the SH3 domain of spectrin (Spec-SH3). Cells were subsequently visualised for the uptake of biotin-labelled transferrin by confocal microscopy. The bar in (e) represents 10 μM.

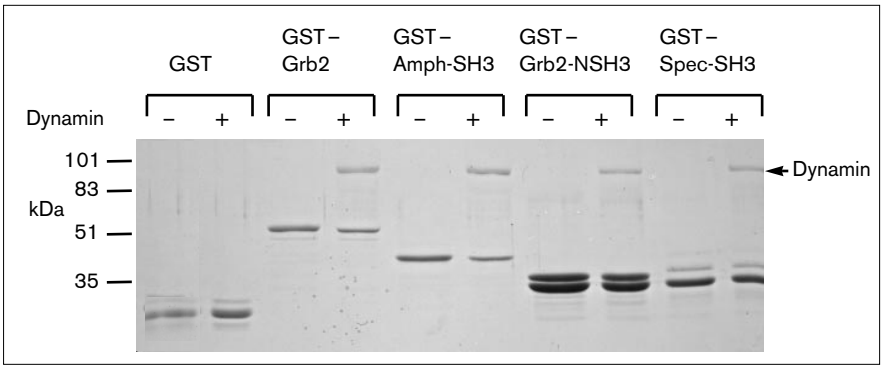
over 20 cells quantitated (Figure 4a–c) and the blockade was rescued by coexpression of dynamin. In a few cells, the rescue was incomplete but this correlated with a low level of dynamin overexpression, and thus is similar to the dynamin-mediated rescue of transferrin uptake (cell marked by arrow in Figure 2b). The bar graph (Figure 4c) shows the statistical analysis of 20 cells randomly selected from three experiments for each of the conditions analysed. This analysis demonstrates the extent of the inhibition and of the dynamin rescue for both EGF and transferrin.

In contrast to receptor-mediated endocytosis, fluid-phase uptake of dextran was not affected in COS cells expressing the amphiphysin SH3 domain (Figure 4d), eliminating the possibility that the endocytic blockade is due to a defect in COS cell integrity or cytoskeletal changes. Dominant-negative dynamin mutants similarly are selective in their inhibition of receptor-mediated, but not fluid-phase, uptake [13]. This finding is consistent with evidence suggesting that fluid-phase endocytosis occurs by a different pathway that does not involve clathrin coats or dynamin [17].

Discussion

To date, it has not been clear exactly how dynamin is recruited to budding clathrin-coated pits where it acts in vesicle fission. As well as a polyproline and a GTPase domain, dynamin has a pleckstrin homology domain, which has been reported to bind to acidic phospholipids [18]. Expression of dynamin deletion mutants in COS cells indicates that although the pleckstrin homology domain is dispensable for colocalisation of dynamin with coated pits, the carboxy-terminal polyproline cluster is not required for this colocalisation [19], suggesting that dynamin recruitment

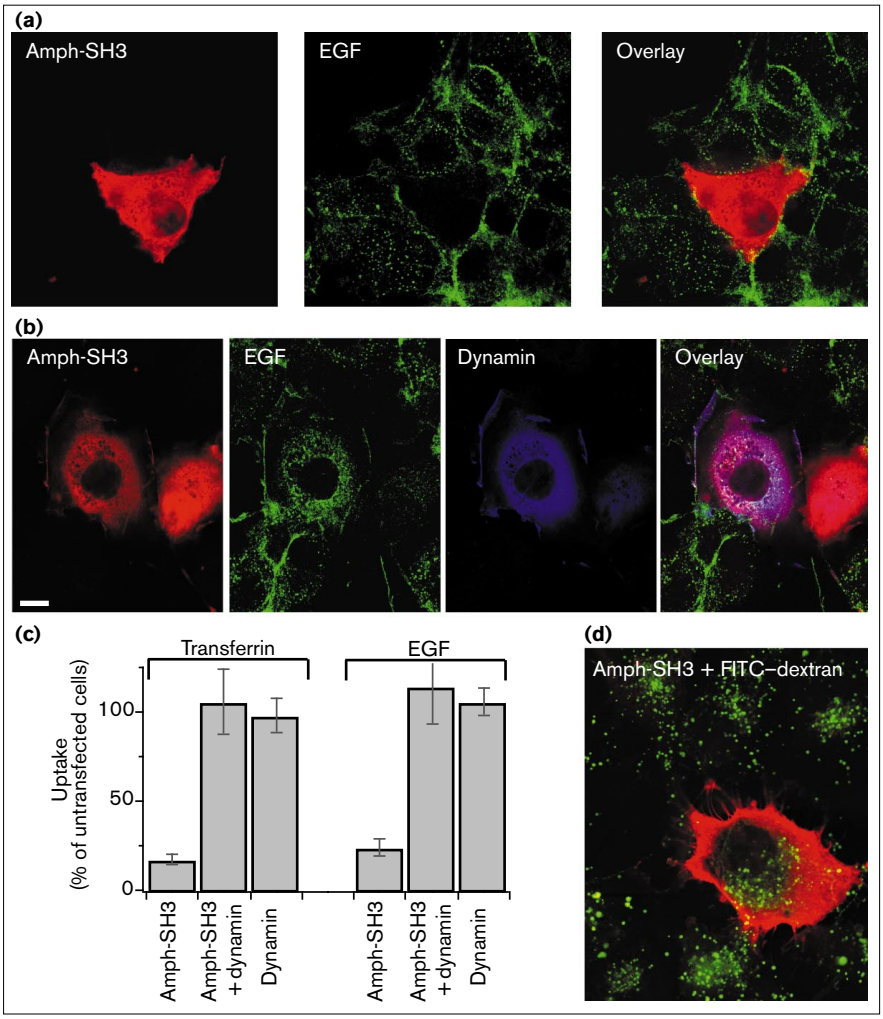
Figure 3



Binding of dynamin to SH3 domains. Recombinant SH3 domains were purified on glutathione–agarose beads. After incubation with 3 µg purified dynamin, bound dynamin was visualised by Coomassie staining following SDS–PAGE.

occurs via an SH3-domain-containing protein. This mechanism of recruitment is supported in our experiments which show that transfection of COS cells with the SH3 domain of amphiphysin leads to a potent block in the uptake of transferrin and EGF. Two observations indicate that the SH3 domain is acting to disrupt the recruitment of dynamin to

Figure 4



Specificity and quantitation of the endocytic blockade by the amphiphysin SH3 domain. **(a)** The effect of the amphiphysin SH3 domain on EGF uptake (see Materials and methods). **(b)** Rescue of the amphiphysin SH3-domain-mediated endocytic blockade by dynamin cotransfection. For (a,b), labelled EGF was incubated with the transfected cells for 5 min before washing and fixing as described in Materials and methods. The bar represents 10 µM. **(c)** Quantitation of the inhibition of transferrin and EGF uptake, and their rescue by dynamin. For each transfection experiment, polygons were drawn around 20 transfected cells and 20 untransfected control cells and the number of green pixels counted in each, normalised for area and expressed as a percentage of the untransfected control. Error bars represent standard error of the mean. **(d)** Disruption of the amphiphysin SH3 domain–dynamin interaction does not affect fluid-phase uptake. COS cells transfected with the Myc-tagged amphiphysin SH3 domain (red) were incubated with FITC-labelled lysine-fixable dextran (green) for 60 min before fixing the cells. Transfected cells were identified by labelling with the anti-Myc antibody. Uptake of FITC–dextran into a number of untransfected cells is also visible in this same panel.

clathrin-coated pits in these cells: firstly, the isolated SH3 domain binds tightly to dynamin *in vitro*; and secondly, coexpression of both dynamin and the amphiphysin SH3 domain rescues the transferrin uptake. Preliminary analysis of amphiphysin SH3-domain-transfected cells indicates that there is less dynamin in the particulate fraction compared to control cells, a finding in agreement with this hypothesis (data not shown).

Although we have not demonstrated a lack of dynamin recruitment to coated pits *per se*, this has been shown in a study by De Camilli and colleagues [20], albeit using a different system. Their paper describes the blockade of synaptic-vesicle recycling in the lamprey reticulospinal synapse by microinjection of recombinant amphiphysin SH3 domain. Electron microscopy has elegantly demonstrated the accumulation of clathrin-coated invaginations lacking dynamin rings; these invaginations are exaggerated under conditions of synaptic stimulation. The central conclusion, that dynamin recruitment to synaptic clathrin-coated pits is blocked by dominant-negative-acting SH3 domains, therefore agrees with our results. However, the lamprey experiments shed no light on the recruitment of dynamin to coated pits in cells other than neurons.

Clathrin-mediated synaptic-vesicle endocytosis is likely to be a specialisation of the 'slow' receptor-mediated pathway that operates in all cells [1], because both pathways require dynamin, adaptor proteins and clathrin, but the extent of similarity of their uptake mechanisms is unclear. Could dynamin be recruited by Grb2, amphiphysin, or other SH3-domain-containing proteins in these cells? Our findings discriminate between these candidate SH3-domain proteins by showing that the SH3 domains of Grb2, spectrin and PLCγ do not affect transferrin uptake when overexpressed in COS cells. For amphiphysin to act in dynamin recruitment, however, it has to be localised to the endocytosing membrane, which probably occurs via binding of amphiphysin to the AP-2 adaptor complex. Our observation that dynamin recruitment to adaptor proteins *in vitro* is greatly enhanced in the presence of amphiphysin suggests that the three molecules are sufficient to form a targeting complex that could direct the formation of coated-pit structures at the endocytosing membrane.

Conclusions

We have shown that amphiphysin can recruit dynamin to AP-2 adaptors *in vitro*, and that overexpression of the amphiphysin SH3 domain in fibroblasts specifically blocks receptor-mediated endocytosis. Amphiphysin therefore appears to be a key component of the endocytic machinery *in vivo*, not only in synaptic-vesicle recycling in neurons where it is highly expressed, but also in general clathrin-mediated endocytic pathways.

Materials and methods

Cloning

Rat amphiphysin was generated by degenerate PCR from a rat brain cDNA library using the degenerate primers ATGGCCGACA TSAAGACGGG CATCTTCGCC and CTAWTCYARR TGKCGKGTGA AGTTCTCTGG (where W indicates A/T, Y indicates C/T and R indicates A/G) from comparison of the chick and human sequences: for binding studies, amphiphysin was subcloned into the expression vector pGEX4T1. The amino-terminal domain of amphiphysin (AmphAB; residues 1–376) and the SH3 domain of amphiphysin (Amph-SH3; residues 595–683) were cloned into a Myc-tagged version of the pCMV5 expression vector (nucleotides ATGGAGCAGA AGCTGATCAG CGAGGAGGAC CTGAAC inserted in the *Eco*RI site) and into pGEX4T1. The dynamin I expression construct was a kind gift of T.C. Südhof, Dallas, Texas (pCMV-96-1a). Rat Grb2 full-length protein, Grb2 amino-terminal SH3 domain, and the rat α-spectrin SH3 domain were all cloned by PCR into pCMV-MYC from a rat brain cDNA library using primers as described in Gout *et al.* [14].

Protein–protein interactions

Amphiphysin (individual domains or the full-length protein) and the ear domain of α-adaptin were expressed in bacteria as GST fusion proteins. Protein was purified from bacterial lysates by incubation with glutathione–agarose beads in buffer A (20 mM HEPES pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 10 μg/ml leupeptin, 100 μg/ml Pefabloc, 10 μg/ml aprotinin, and 1 μg/ml pepstatin) for 1 h at 4°C. The purified protein (10 μg attached to beads) was then incubated on a rotator at 4°C with either brain extract (rat brains homogenised in buffer A containing 1% Triton X-100 and pelleted at 100 000 × *g* in a Beckman ultracentrifuge to remove debris) or extract from COS cells expressing amphiphysin or dynamin. After washing beads three times in buffer A containing 0.1% TX-100, the bound protein was analysed by boiling in SDS–PAGE sample buffer, and electrophoresing through 7% SDS polyacrylamide gels. The antibodies used were anti-dynamin I/II polyclonal antibody (D632, gift of T.C. Südhof, Dallas, Texas), anti-dynamin I/II monoclonal, clathrin monoclonal antibody (Transduction Labs), anti-α-adaptin antibody (Sigma), anti-β-adaptin antibody (gift of M.A. Robinson, Cambridge, UK), anti-γ-adaptin antibody (Sigma), anti-Myc antibody (gift of S. Moss, London, UK), anti-p62 antibody (gift of M. Stewart, Cambridge, UK), or anti-amphiphysin antibody (Ra3 and Ra4.2 raised in rabbits to purified GST–AmphAB and His–Amph-SH3, respectively).

Immunocytochemistry

COS-7 cells plated onto 60 mm dishes were transfected using DEAE-dextran/chloroquine, followed by glycerol shock, with either $pCMV_{Myc}$-Amph1-SH3, $pCMV_{Myc}$-Grb2-NSH3, $pCMV_{Myc}$-Spectrin-SH3, or pCMV-DynI. Cells were replated 18 h after transfection onto poly-L-lysine-coated coverslips for subsequent processing. After a further 18 h, cells were incubated at 37°C in serum-free DMEM for 1 h, then at 37°C in DMEM containing either 25 μg/ml biotinylated human transferrin (Sigma) or 1.25 mg/ml FITC-conjugated lysine-fixable dextran (MW10,000; Molecular Probes) for 1 h, or with 100 ng/ml biotinylated EGF for 5 min (the short incubation time was used to reduce background uptake as seen by other investigators [21]). After washing three times in PBS 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, SH3 domains with the anti-Myc monoclonal antibody 9E10 and dynamin with the polyclonal antibody D632. Secondary antibodies were Cy5-conjugated goat anti-rabbit antibody for dynamin, and horseradish-peroxidase-conjugated goat anti-mouse antibody followed by indirect tyramide signal amplification (Dupont NEN) for the other antigens, using the fluorophore Texas red conjugated to streptavidin. Coverslips were mounted under Vectashield (Vector labs) and cells were observed using an MRC 1024 scanning confocal microscope.

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