Clathrin interacts specifically with amphiphysin and is displaced by dynamin

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Abstract Amphiphysin is an SH3 domain protein that has been implicated in synaptic vesicle endocytosis. We have recently cloned a second amphiphysin isoform, Amph2 (sequence submitted to GenBank, Y13380). Proteins capable of forming a complex with amphiphysin were isolated from rat brain by using recombinant GST-Amph2 for binding experiments. As well as interacting with dynamin I, the full-length protein bound to a weaker 180-kDa band. Immunoblotting demonstrated this protein to be clathrin. To address whether this is a direct interaction, the clathrin binding to amphiphysin was reconstituted in vitro with purified proteins. The N-terminal domain of Amph2 is sufficient for clathrin binding. Dynamin, which interacts with the SH3 domain of Amph2, displaces clathrin from the N-terminus. We propose a model that may explain how clathrin and dynamin are recruited to non-overlapping sites of the coated pit.

Key words: Amphiphysin; Clathrin; Coated pit; Dynamin; Synaptic vesicle Endocytosis

1. Introduction

During receptor-mediated endocytosis, clathrin is recruited to adaptor complexes (AP-2) at the plasma membrane. Clathrin is a triskelial protein that assembles into a lattice-like structure of hexagons and pentagons, providing the structural scaffold for the early coated pit. The next stage is catalysed by dynamin, a large GTP-binding protein first identified in *Drosophila* [1]. Mutations in dynamin demonstrate it to be crucial in the 'pinching off' stage of clathrin-coated vesicle endocytosis [2–4]. The current model [5] proposes that it is recruited to the coated pit, probably via its proline-rich C-terminus, and subsequently redistributes to form ring-like structures collaring the neck of the invagination. Upon stimulation of GTP hydrolysis, a conformational change in the molecule is thought to cause closure of the ring and release of the newly formed vesicle.

While much evidence supports dynamin's role in endocytosis, it is less clear how it becomes recruited to clathrin-coated pits and is targeted to the site where ring formation occurs. Recently it has become evident that amphiphysin may play a role in this process. Through its SH3 domain, it interacts with dynamin's polyproline region in vitro, and the two proteins colocalise at the nerve terminal [6]. Experiments in living cells have demonstrated that the amphiphysin SH3 domain may have an important role in the dynamin recruitment process. When this domain is microinjected into the lamprey synapse [7], or transfected into fibroblasts [8], a potent block in clathrin-mediated endocytosis is observed. Mutations in the Rvs yeast homologues [9–13] and studies of the neurological side effects of autoimmunity against amphiphysin in Stiff Man's syndrome [11] also support a role for amphiphysin in endocytosis.

We recently identified a novel amphiphysin isoform, amphiphysin 2, the sequence of which we have submitted to GenBank. In this paper we have examined the interactions of this protein with other partners in the brain. Amph2 interacts with dynamin and with α-adaptin, as has been reported previously for Amph1 [6,8]. In this paper, we report that it also binds, via a distinct domain, with clathrin. Furthermore, this interaction is effectively inhibited by dynamin binding to amphiphysin's SH3 domain. We speculate that this competition of the two amphiphysin partners may be physiologically important in ensuring the correct targeting of dynamin and clathrin to non-overlapping sites at the clathrin-coated pit.

2. Materials and methods

2.1. Plasmid construction

The vectors pGEX-4T2 (Pharmacia) and pET-15b (Novagen) were used to make fusion proteins with glutathione-*S*-transferase (GST) and a hexahistidine tags respectively. The following constructs, cloned by PCR, were used:

- GST- and His-Amph2-1: full length rat Amph2-1
- GST-Amph2-1AB: residues 1-422
- GST-Amph2-SH3: residues 494–588
- GST-α-Adap$_E$: α-adaptin$_C$ ear domain, residues 701–938
- GST-β-Adap$_E$: β-adaptin ear domain, residues 701–937.

2.2. In vitro binding experiments

Brain extract was prepared by homogenising one rat brain in 20 ml of buffer A [150 mM NaCl, 20 mM HEPES pH 7.4, 1 mM $MgCl_2$, 1 mM EGTA, and a protease inhibitor cocktail (10 μg/ml leupeptin, 100 μg/ml Pefabloc, 10 μg/ml aprotinin, and 1 μg/ml pepstatin)] in a glass-Teflon blender at 200 rpm. To complete lysis, Triton X-100 was added to the homogenate to a final concentration of 1%, and residual debris pelleted by centrifugation at 100000×*g* for 20 min in a Beckman ultracentrifuge.

GST fusion proteins and His-tagged fusion proteins were expressed in *E coli* and purified on glutathione-agarose and Ni-NTA-agarose resins respectively. Purified protein in this form was incubated with the brain extract prepared above. After 2 h of gentle mixing at 4°C, the beads were pelleted at 5000×*g* for 2 min and washed extensively (3×5 min washes) in the same buffer. Bound protein was eluted by boiling in an equal volume of 2×SDS-PAGE sample buffer and run

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Abbreviations: AP-2, adaptor protein complex 2; Amph, amphiphysin; GST, glutathione-*S*-transferase; GTP, guanosine triphosphate; PAGE, polyacrylamide gel electrophoresis; SH3, src-homology 3

Accession number information. The sequence data for rat Amph2 have been submitted to the GenBank database under accession numbers Y13380.

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on SDS-PAGE (7% or 13%) for immunoblotting or Coomassie staining.

2.3. Purification of Clathrin and dynamin

Coated vesicles were prepared from fresh pig brain as described [14]. Clathrin was purified from coated vesicles following the method of Crowther and Pearse (1981) [15], with the substitution of triethanolamine for Tris buffer in buffer C, and using Sepharose CL-4B gel filtration media.

Dynamin was purified from rat brain as described [16].

2.4. Antibodies

Polyclonal antiserum against Amph2 was raised by injecting His-Amph2 protein into rabbits (3×100 μg injections). The Amph2 antiserum reacts predominantly to the expected 92-kDa band in brain extracts. Other antibodies used are the following (with their suppliers and generous donors shown in brackets): Dynamin (D632, Tom Südhof), α-adaptin (AC1-M11 from Margaret Robinson), AP180 (Transduction Laboratories) and clathrin heavy chain (Transduction Laboratories).

3. Results

3.1. Interaction of amphiphysin 2 with Brain proteins

To investigate whether Amph2 complexes with novel proteins in the brain, the full-length Amph2 cDNA was cloned into pGEX and expressed as a GST fusion protein in bacteria. After purification on glutathione agarose, the protein was incubated with a crude extract of rat brain. Bound proteins were analysed by SDS-PAGE and immunoblotting. In addition to a ~100-kDa dynamin band (see Fig. 1), a weak 180-kDa band was specifically precipitated (as visualised by coomassie). Several brain-enriched proteins migrate around this size, including AP180 and clathrin. Immunoblotting revealed the identity of the 180-kDa band to be clathrin (Fig. 1). As a control, the band was not immunoreactive to a monoclonal antibody against AP180. A similar interaction of clathrin with Amph1 has also been observed (results not shown).

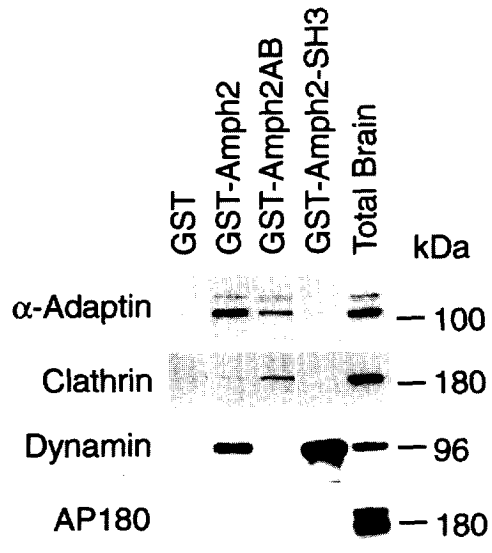

Fig. 1. Immunoblot showing interaction of clathrin with the N-terminal domain of amphiphysin. GST fusion proteins of amphiphysin 2, or GST as a control, were incubated with 10 mg/ml rat brain extract for 2 h and bound proteins were separated on SDS-PAGE followed by immunoblotting with each of the indicated antibodies. Ten μg of each fusion protein was used. The last lane (positive control) demonstrates that dynamin, clathrin, α-adaptin and AP180 are all present in total brain.

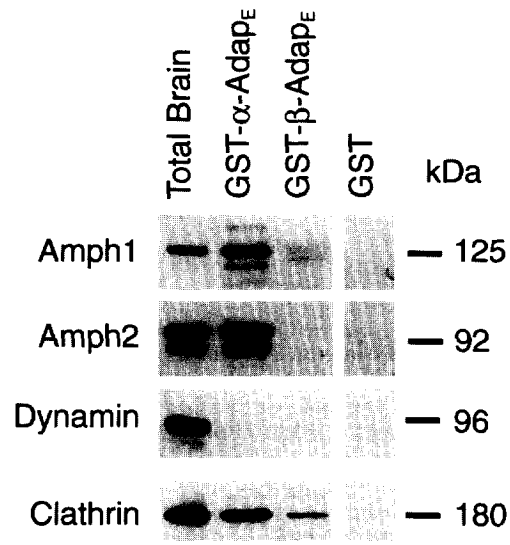

Fig. 2. Enrichment of amphiphysin complexes from the brain with a GST-α-adaptin matrix. A GST fusion protein of the ear domain of alpha-adaptin (or as a control, beta-adaptin) were used to enrich for amphiphysin-interacting proteins in 10 mg/ml rat brain extract. Ten μg of each fusion protein was used. Bound proteins were separated on SDS-PAGE followed by immunoblotting with each of the indicated antibodies.

To examine what domain of amphiphysin is important in clathrin binding, the full-length Amph2 was divided into two domains, the AB domain (N-terminal 422 amino acids) and the SH3 domain (C-terminal 94 amino acids). Fig. 1A reveals that clathrin binds most strongly to the AB domain, a region predicted to be largely α-helical. By contrast, the dynamin-binding C-terminal SH3 domain does not bind clathrin at all (Fig. 1).

In addition to precipitating clathrin and dynamin, Amph2 binds the α subunit of the AP-2 complex (Fig. 1). This interaction still occurs with the AB domain, showing that truncating the protein does not eliminate α-adaptin binding. A similar interaction of this adaptin [via its ear (appendage) domain] with Amph1 has been reported previously [6,17]. The ear domain of the α subunit was therefore expressed as a GST fusion protein and used to enrich for native, amphiphysin complexes from the brain. Fig. 2 reveals that Amph1 and Amph2 (the latter appearing as a doublet, due to an additional splice form) are precipitated in a form that binds very little dynamin, but instead appears to be associated predominantly with clathrin.

In Fig. 1, we see a form of Amph2 that binds dynamin and α-adaptin but only weakly to clathrin, while in Fig. 2, the α-adaptin ear enriches for a complex containing mostly clathrin, and little dynamin. This may be due to the presence of a 23-kDa GST tag at the N-terminus of the recombinant fusion proteins used in Fig. 1, which may reduce the accessibility of this domain for clathrin.

3.2. Dynamin and clathrin binding to amphiphysin are mutually exclusive

The result in Fig. 2, and other experiments indicating that levels of clathrin and dynamin binding to amphiphysin are inversely related (data not shown), raised the possibility that the two molecules cannot bind to amphiphysin at the same

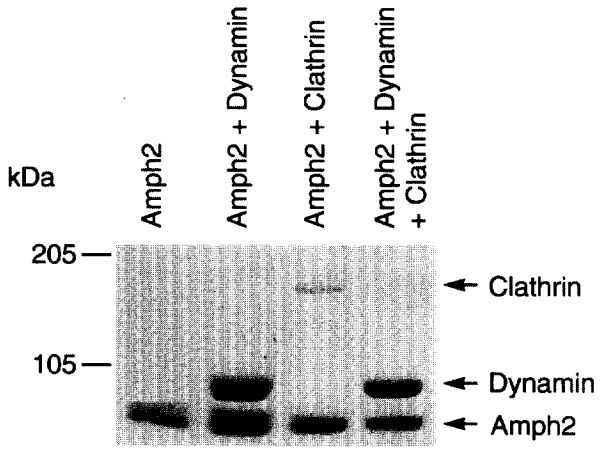

Fig. 3. Dynamin displaces clathrin from amphiphysin 2. Twenty μg His-tagged amphiphysin 2 protein, immobilised on Ni^{2+}-NTA agarose beads, was incubated for 30 min with 20 μg of purified dynamin and/or clathrin in buffer A. Beads were washed three times in buffer A containing 0.1% TX-100, and resuspended in sample buffer. Bound proteins were separated on 7% SDS-PAGE followed by coomassie staining.

time. Fig. 1 further illustrates this idea, where clathrin is more weakly bound to full-length Amph2 (where dynamin is bound), than to the N-terminal domain (to which dynamin cannot associate). It appears that deletion of the dynamin-binding site of the amphiphysin molecule may 'liberate' it to interact more strongly with clathrin. To answer the question of whether clathrin and dynamin binding to amphiphysin are mutually exclusive, and to rule out the possibility that the clathrin-amphiphysin interaction might be an *indirect* one, we reconstituted the interactions in vitro. Clathrin was purified from pig brain coated vesicles (see the Methods in Section 2). Dynamin was purified from rat brain extract by two sequential chromatography steps [16]. Full-length His-tagged Amph2 interacts strongly with dynamin (Fig. 3, lane 2), and weakly with clathrin (lane 3). In the presence of dynamin, the amount of clathrin bound is significantly reduced (lane 4). We therefore conclude that clathrin associates directly with Amph2 and is displaced upon addition of dynamin.

4. Discussion

In this paper we have investigated those proteins which may complex with amphiphysin 2 in the brain. As has been previously shown for Amph1 [6], Amph2 interacts with dynamin and with the α subunit of the AP-2 adaptor complex. In addition, we show that amphiphysin can also interact with clathrin in brain extracts. Clathrin is known to be recruited via the AP-2 adaptor complex, via the hinge domain of the β subunit; however, this interaction is a weak one [18]. The presence of amphiphysin at the membrane (perhaps via AP-2 localisation) could thus provide additional binding sites for clathrin recruitment.

One striking result emerging from our experiments is the observation that clathrin is displaced via dynamin's interaction with the C-terminal SH3 domain of amphiphysin. Because the two proteins interact with distinct, separate domains of the amphiphysin molecule (clathrin at the N-terminus and dynamin at the C-terminus), this is unlikely to be due to direct competition for binding to the same site; more probably it is an indirect effect, possibly mediated through a steric hindrance or a conformational change in the amphiphysin molecule.

Together, the data presented in this paper suggest that, in addition to recruiting dynamin [7,8], amphiphysin could aid the formation of a clathrin lattice at the endocytosing membrane. This may have important implications for our understanding of the sequence of events in clathrin-coated vesicle formation. Immunogold electron microscopy of nerve terminals treated with GTPγS, which results in the accumulation of many constricted clathrin-coated invaginations, shows that dynamin predominantly forms rings at the collar or 'neck' of the invaginated vesicles [19]. Clathrin is not seen where dynamin forms these rings. How is this precise localisation brought about? We speculate that the reasons for the non-overlapping distribution of clathrin and dynamin at the coated pit are due our observations presented in this paper, that the two molecules cannot bind to amphiphysin at the same time. In the following model, we suggest how these early events in coated vesicle formation could be explained at the molecular level. Initially, clathrin is recruited throughout the coated pit area, via the β subunit of the AP-2 complex, and also via membrane-localised amphiphysin. Dynamin recruited to the collar of the pit, via amphiphysin's SH3 domain, causes displacement of the clathrin in this vicinity, allowing a free zone around which ring formation can occur, enabling the apposing membranes to come into closer proximity and finally 'pinch off' the vesicle. For this model to be correct, amphiphysin should have sites for interacting with both dynamin and clathrin, and it should be localised to the plasma membrane. It is likely that this localisation occurs through its interaction with AP-2 complexes [6], and indeed, we have shown that Amph2, like Amph1, can interact with the α subunit of this adaptor complex.

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References

[1] Poodry, C.A. and Edgar, L. (1979) J. Cell Biol. 81, 520–527.
[2] Herskovits, J.S., Burgess, C.C., Obar, R.A. and Vallee, R.B. (1993) J. Cell Biol. 122, 565–578.
[3] Damke, H., Baba, T., Warnock, D.E. and Schmid, S.L. (1994) J. Cell Biol. 127, 915–934.
[4] van-der-Bliek, A.M., Redelmeier, T.E., Damke, H., Tisdale, E.J., Meyerowitz, E.M. and Schmid, S.L. (1993) J. Cell Biol. 122, 553–563.
[5] DeCamilli, P., Takei, K. and McPherson, P.S. (1995) Curr. Opin. Neurobiol. 5, 559–565.
[6] David, C., McPherson, P.S., Mundigl, O. and deCamilli, P. (1996) Proc. Natl. Acad. Sci. USA 93, 331–335.
[7] Shupliakov, O., Low, P., Grabs, D., Gad, H., Chen, H., David, C., Takei, K., DeCamilli, P. and Brodin, L. (1997) Science 276, 259–263.
[8] Wigge, P., Vallis, Y. and McMahon, H.T. (1997) Curr. Biol. (in press).
[9] Crouzet, M., Urdaci, M., Dulau, L. and Aigle, M. (1991) Yeast 7, 727–743.
[10] Desfarges, L., Durrens, P., Juguelin, H., Cassagne, C., Bonneu, M. and Aigle, M. (1993) Yeast 9, 267–277.
[11] David, C., Solimena, M. and DeCamilli, P. (1994) FEBS Lett. 351, 73–79.
[12] Sivadon, P., Bauer, F., Aigle, M. and Crouzet, M. (1995) Mol. Gen. Genet. 246, 485–495.
[13] Munn, A.L., Stevenson, B.J., Geli, M.I. and Riezman, H. (1995) Mol. Biol. Cell 6, 1721–1742.

[14] Campbell, C., Squicciarini, J., Shia, M., Pilch, P.F. and Fine, R.E. (1984) Biochemistry 23, 4420–4426.
[15] Crowther, R.A. and Pearse, B.M.F. (1981) J. Cell Biol. 91, 790–797.
[16] Gout, I., Dhand, R., Hiles, I.D., Fry, M.J., Panayotou, G., Dad, P., Truong, O., Totty, N.F., Hsuan, J. and Booker, G.W. et al. (1993) Cell 75, 25–36.
[17] Wang, L.-H., Südhof, T.C. and Anderson, R.G.W. (1995) J. Biol. Chem. 270, 10079–10083.
[18] Shih, W., Gallusser, A. and Kirchhausen, T. (1995) J. Biol. Chem. 270, 31083–31090.
[19] Takei, K., McPherson, P.S., Schmid, S.L. and DeCamilli, P. (1995) Nature 374, 186–190.