14. A. Saito, M. Inui, M. Radermacher, J. Frank, S. Fleischer, *J. Cell Biol.* **107**, 211 (1988).
15. B. A. Block, T. Imagawa, K. P. Campbell, C. Franzini-Armstrong, *ibid.*, p. 2587.
16. High density SR from skeletal muscle was incubated with [^{3}H]ryanodine, solubilized with CHAPS detergent, and centrifuged for 10 hours on a 10 to 32% linear sucrose gradient at 26,000 rpm and 2°C in a Sorvall AH-629 rotor (17-ml tubes) (*2*, *9*).
17. S. O. Marx, K. Ondrias, A. R. Marks, unpublished data.
18. W. Shou *et al.*, *Nature* **391**, 489 (1998).
19. M. Endo, M. Tanaka, Y. Ogawa, *ibid.* **228**, 34 (1970).
20. L. E. Ford and R. J. Podolsky, *Science* **167**, 58 (1970).
21. E. Ríos and G. Pizarro, *News Physiol. Sci.* **3**, 223 (1988).
22. Recombinant rabbit skeletal muscle RyR1 was expressed in Sf9 cells, purified by sucrose density gradient centrifugation, and reconstituted into liposomes, which were then fused to planar lipid bilayers (*2*, *9*). For some experiments, recombinant FKBP12 was expressed with RyR1 in Sf9 cells; in other experiments, 150 nM FKBP12 protein was added to the cis chamber after fusion, as described (*2*). Thirty minutes after addition of FKBP12 (or immediately in the case of RyR1 co-expressed with FKBP12), RyR1 was activated with caffeine (1 mM) or by adjusting the free Ca^{2+} concentration in the cis chamber to 250 nM.
23. SR vesicles were isolated from rabbit skeletal muscle and incorporated into planar lipid bilayers as described [F. A. Lai, H. P. Erickson, E. Rousseau, Q. L. Liu, G. Meissner, *Nature* **331**, 315 (1988)], with the following modifications: (i) Vesicles were isolated in buffer containing 150 mM KCl from the 32 to 40% (w/v) sucrose interface. (ii) Vesicles were incorporated immediately into planar lipid bilayers, such that fusion occurred instantly or within a few minutes, under high hydrostatic pressure. And (iii) solutions in the cis and trans chambers were supplemented with 50 mM KCl. For recordings from RyR1 channels, black lipid membranes were formed across a hole (diameter, 0.05 to 0.3 mm) separating the cis and trans chambers, as described [C. Miller, *Ion Channel Reconstitution* (Plenum, New York, 1986), pp. 3–151]. The voltage across the bilayer membrane was clamped at 0 mV, and the current carrier was Ca^{2+}. The cis solution contained 250 mM Hepes (pH 7.35), 125 mM tris, 50 mM KCl, 1.0 mM EGTA, and 0.7 mM $CaCl_2$, to give a free Ca^{2+} concentration of 250 nM; the trans solution contained 53 mM $Ca(OH)_2$, 250 mM Hepes (pH 7.35), and 50 mM KCl. The trans chamber was connected to the head-stage input of an Axon 200 amplifier (Axon Instruments, Foster City, CA) by a Ag-AgCl electrode and agar-KCl bridges. Single-channel currents were continuously monitored and recorded on a Digital Audio Tape (DAT) and with a chart recorder. Recordings were filtered through a low-pass Bessel device (Frequency Devices, Haverhill, MA) at 1 to 2 kHz and digitized at 4 to 8 kHz. For construction of current amplitude histograms and channel traces, data were filtered at 500 Hz. Channel properties were evaluated with the use of pClamp 6. Open probabilities were determined by analyzing data at 5- and 10-s intervals. The binomial distribution of open probabilities was calculated from the equation

$$P1_{calc} = 2\sqrt{P2}\cdot(1-\sqrt{P2})$$

where $P1_{calc}$ is the calculated open probability of the 4-pA currents and $P2$ is the observed open probability of the 8-pA currents (*10*). The formula is useful when the open probabilities of two channels are approximately equal and pertains to all experimentally observed values for $P2$. Student's t test was used for statistical analysis of the dwell-time distributions and open probabilities.
24. We thank R. Kass, Q. Al-Awqati, S. Siegelbaum, B. Ehrlich, and S. Hamilton for critical reading of the manuscript and for helpful discussions. Supported by NIH grants R01HL56180, R01A139794, and R03TW00949 (A.R.M.), the Muscular Dystrophy Association (A.R.M.), the American Heart Association (A.R.M. and S.O.M.), and the Richard and Lynne Kaiser Family Foundation.

31 December 1997; accepted 24 June 1998

REPORTS

Role of Phosphorylation in Regulation of the Assembly of Endocytic Coat Complexes

Vladimir I. Slepnev, Gian-Carlo Ochoa, Margaret H. Butler, Detlev Grabs, Pietro De Camilli*

Clathrin-mediated endocytosis involves cycles of assembly and disassembly of clathrin coat components and their accessory proteins. Dephosphorylation of rat brain extract was shown to promote the assembly of dynamin 1, synaptojanin 1, and amphiphysin into complexes that also included clathrin and AP-2. Phosphorylation of dynamin 1 and synaptojanin 1 inhibited their binding to amphiphysin, whereas phosphorylation of amphiphysin inhibited its binding to AP-2 and clathrin. Thus, phosphorylation regulates the association and dissociation cycle of the clathrin-based endocytic machinery, and calcium-dependent dephosphorylation of endocytic proteins could prepare nerve terminals for a burst of endocytosis.

Clathrin-mediated endocytosis plays a key role in the recycling of synaptic vesicles in nerve terminals, and several components of the molecular machinery involved in this process have been identified (*1*). These include, in addition to clathrin and the clathrin adaptors, the guanosine triphosphatase dynamin 1, the amphiphysin dimer, and synaptojanin 1. Dynamin 1 oligomerizes into collar structures at the neck of deeply invaginated clathrin-coated pits, and its conformational change is thought to be an essential step leading to vesicle fission (*2*). Synaptojanin 1 is a presynaptic inositol 5-phosphatase enriched on endocytic intermediates (*3*). The amphiphysin dimer (*4*–*6*) binds to both dynamin 1 and synaptojanin 1 through the COOH-terminal SH3 domains of its two subunits (*4*, *7*, *8*). Disruption of SH3-mediated interactions of amphiphysin blocks clathrin-mediated endo-

Howard Hughes Medical Institute and Department of Cell Biology, Yale University School of Medicine, 295 Congress Avenue, New Haven, CT 06510, USA.

*To whom correspondence should be addressed. E-mail: pietro.decamilli@yale.edu

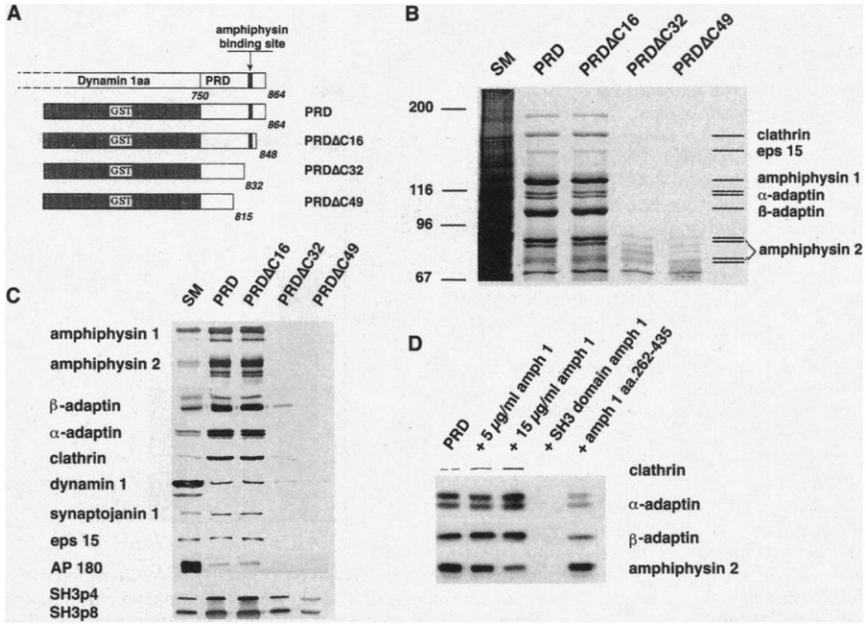

Fig. 1. A macromolecular complex comprising several endocytic proteins can be affinity-purified from a Triton X-100 brain extract by the PRD of dynamin (*13*). (**A**) Schematic drawing of the GST fusion proteins used for affinity chromatography (*8*). (**B**) Coomassie blue staining of the starting rat brain extract and of the material affinity purified by the four constructs shown in (A). (**C**) Immunoblot analysis of the material shown in (B). (**D**) Immunoblot analysis of the material affinity-purified by the PRD in the absence and presence of the indicated amounts of amphiphysin 1, SH3 domain of amphiphysin 1, and central fragment of amphiphysin 1 (amino acids 262 to 435).

cytosis at the step of invaginated coated pits (*9*). The amphiphysin dimer also binds to clathrin (*10, 11*) and to the α-adaptin subunit of the plasma membrane clathrin adaptor AP-2 (*9, 12*) and thus may mediate recruitment of dynamin 1 and synaptojanin 1 to the site of clathrin-mediated endocytosis.

To identify potential major binding partners for the proline-rich domain (PRD) of dynamin other than amphiphysin, a total brain extract was affinity purified on glutathione S-transferase (GST) fusion proteins comprising the entire PRD or COOH-terminal truncations of the PRD (Fig. 1A) (*13*). A set of proteins was specifically bound by full-length PRD and by a deletion construct missing the last 16 amino acids (PRDΔC16) but not by a construct lacking an additional 16 amino acids (PRDΔC32) or a larger portion of the PRD (Fig. 1, B and C). The crucial 16 amino acids contain the amphiphysin binding site (*8*). The major affinity-purified protein bands were identified as amphiphysins 1 and 2, the α and β subunits of the AP-2 clathrin adapter complex (α- and β-adaptin, respectively) (*14*), and clathrin, based on both their electrophoretic mobilities and coenrichment during affinity purification with the corresponding immunoreactivities as determined by immunoblotting. Additional proteins were found by immunoblotting to be specifically retained by the PRD constructs containing the amphiphysin binding site (Fig. 1C) (*15*). These included dynamin 1, synaptojanin 1, and other components of the endocytic machinery, such as eps15 and AP180 (*16*). Two other SH3 domain–containing proteins, SH3p4 (endophilin 1) and SH3p8 (endophilin 2) (*17*), bound to all GST-PRD constructs (Fig. 1C), suggesting a localization of the binding site for these proteins upstream of the amphiphysin binding site.

Other than amphiphysin, none of endocytic proteins that were affinity-purified by using the PRD and PRDΔC16 constructs contain an SH3 domain. Thus these proteins may form multimeric complexes with amphiphysin. In agreement with this possibility, binding of AP-2 and clathrin was increased by the addition of full-length recombinant amphiphysin 1 and decreased by the addition of amphiphysin fragments (Fig. 1D). Furthermore, antibodies to amphiphysin could coprecipitate proteins of the complex from brain lysates (see below). If these complexes occur in vivo, their formation would be likely to undergo regulation because of the cyclic nature of the association and dissociation of the endocytic machinery. Because several proteins of the complex are known to be phosphoproteins [for example, clathrin, AP-2 (*18, 19*), dynamin 1, synaptojanin 1, and amphiphysin (*4, 20, 21*)], we used immunoprecipitation experiments to determine whether protein-protein interactions with the complexes were regulated by phosphorylation.

Rat brain extract (*22*) was depleted of nucleotides and incubated in the presence or absence of adenosine triphosphate (ATP) and either a protein kinase inhibitor or a protein phosphatase inhibitor mixture. This material was subjected to immunoprecipitation with monoclonal antibodies specifically directed against the NH_2-terminal region of amphiphysin 1, which do not recognize amphiphysin 2 (*22*). Amphiphysin 1 had a slower mobility after incubation in the presence of

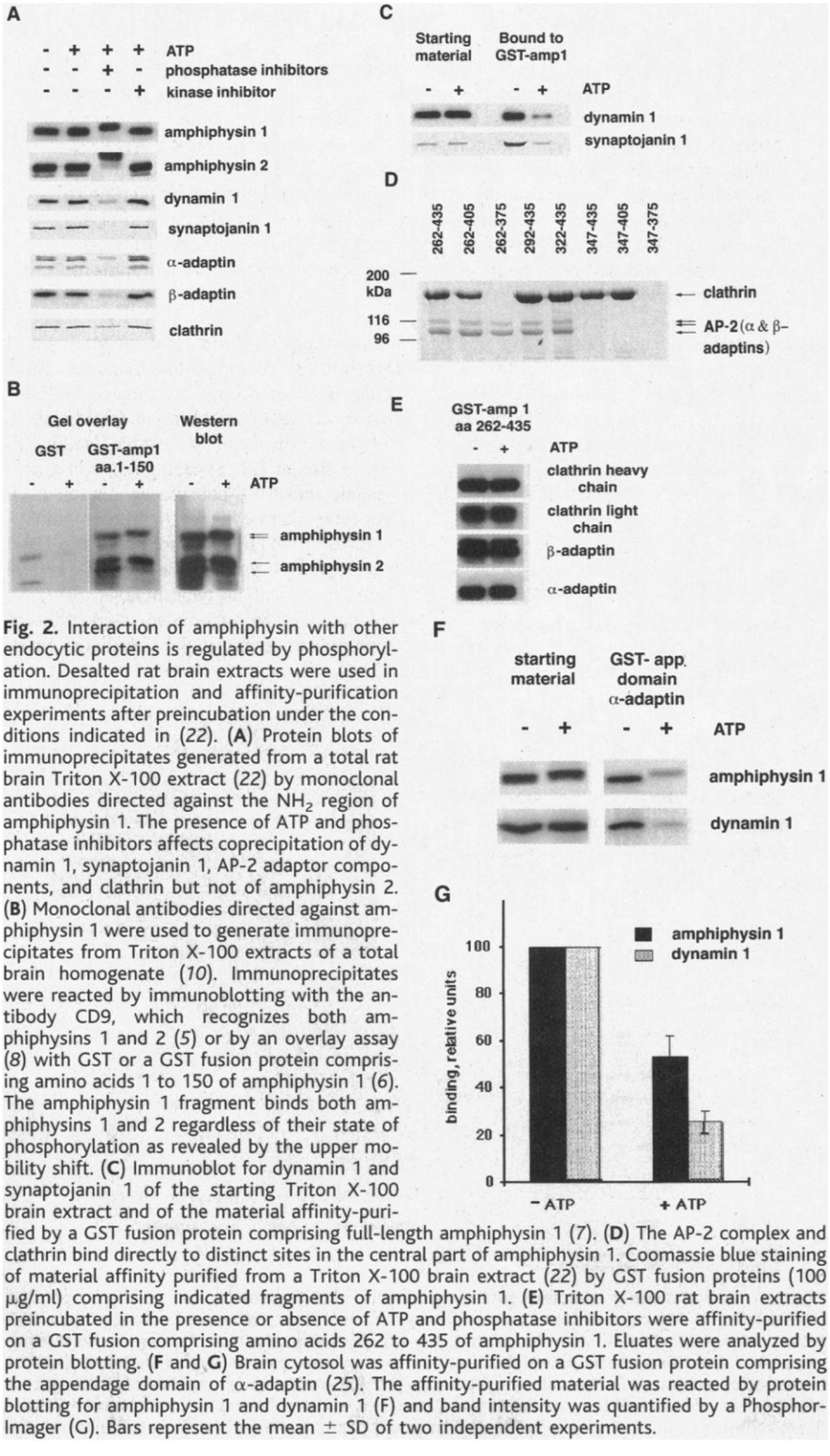

Fig. 2. Interaction of amphiphysin with other endocytic proteins is regulated by phosphorylation. Desalted rat brain extracts were used in immunoprecipitation and affinity-purification experiments after preincubation under the conditions indicated in (*22*). (**A**) Protein blots of immunoprecipitates generated from a total rat brain Triton X-100 extract (*22*) by monoclonal antibodies directed against the NH_2 region of amphiphysin 1. The presence of ATP and phosphatase inhibitors affects coprecipitation of dynamin 1, synaptojanin 1, AP-2 adaptor components, and clathrin but not of amphiphysin 2. (**B**) Monoclonal antibodies directed against amphiphysin 1 were used to generate immunoprecipitates from Triton X-100 extracts of a total brain homogenate (*10*). Immunoprecipitates were reacted by immunoblotting with the antibody CD9, which recognizes both amphiphysins 1 and 2 (*5*) or by an overlay assay (*8*) with GST or a GST fusion protein comprising amino acids 1 to 150 of amphiphysin 1 (*6*). The amphiphysin 1 fragment binds both amphiphysins 1 and 2 regardless of their state of phosphorylation as revealed by the upper mobility shift. (**C**) Immunoblot for dynamin 1 and synaptojanin 1 of the starting Triton X-100 brain extract and of the material affinity-purified by a GST fusion protein comprising full-length amphiphysin 1 (*7*). (**D**) The AP-2 complex and clathrin bind directly to distinct sites in the central part of amphiphysin 1. Coomassie blue staining of material affinity purified from a Triton X-100 brain extract (*22*) by GST fusion proteins (100 μg/ml) comprising indicated fragments of amphiphysin 1. (**E**) Triton X-100 rat brain extracts preincubated in the presence or absence of ATP and phosphatase inhibitors were affinity-purified on a GST fusion comprising amino acids 262 to 435 of amphiphysin 1. Eluates were analyzed by protein blotting. (**F** and **G**) Brain cytosol was affinity-purified on a GST fusion protein comprising the appendage domain of α-adaptin (*25*). The affinity-purified material was reacted by protein blotting for amphiphysin 1 and dynamin 1 (F) and band intensity was quantified by a PhosphorImager (G). Bars represent the mean ± SD of two independent experiments.

both ATP and phosphatase inhibitors (Fig. 2A), which confirms the effectiveness of the phoshorylation reaction under these conditions (*21*). A similar shift (Fig. 2A) was exhibited by amphiphysin 2 (*23*). In addition to amphiphysin 1, the antibodies coprecipitated amphiphysin 2, dynamin 1, synaptojanin 1, AP-2 (α- and β-adaptin), and clathrin. Preincubation of the extract with ATP and phosphatase inhibitors did not affect coprecipitation of amphiphysin 2 but did cause a significant decrease in coprecipitation of other components of the complex, which suggests that phosphorylation affects these interactions (Fig. 2A).

The coprecipitation of amphiphysin 2 from both phosphorylated and dephosphorylated brain extracts supports the presence of amphiphysin heterodimers (*4*) and indicates that amphiphysin dephosphorylation does not affect heterodimer stability. A GST fusion protein comprising the first 150 amino acids of amphiphysin 1 bound to both amphiphysins 1 and 2 in an overlay assay (Fig. 2B), indicating that dimerization is mediated by this coiled-coil region of amphiphysin; this suggests the possibility that both heterodimers and homodimers are present. Binding was not affected by phosphorylation (Fig. 2B).

To determine whether the phosphorylation site or sites that affect binding of dynamin 1 and synaptojanin 1 to amphiphysin were on these proteins or on amphiphysin, we incubated brain extracts in the presence of ATP and the phosphatase inhibitor mixture and then loaded them on a GST-amphiphysin 1 fusion protein column (*7*) after we terminated the kinase reactions by adding EDTA. In both cases, binding of dynamin 1 and synaptojanin 1 to the SH3 domain of amphiphysin 1 was significantly reduced by previous exposure of the cytosol to ATP (Fig. 2C) (*24*). Thus, phosphorylation of dynamin 1 and synaptojanin 1 regulates their interaction with amphiphysin.

We next tested whether clathrin and AP-2 from control and ATP-pretreated cytosol (*21*) bound differently to recombinant amphiphysin. In preliminary affinity-chromatography experiments the AP-2 binding site was localized to a region (amino acids 322 to 375 of human amphiphysin 1) distinct from, but adjacent to, the clathrin binding site (amino acids 347 to 405 of human amphiphysin 1) (*11*) (Fig. 2D). Therefore, we used a GST fusion protein of an amphiphysin 1 fragment comprising both regions (amino acids 262 to 435 of human amphiphysin 1) for these experiments (Fig. 2E). Binding of clathrin and α- and β-adaptin was very similar in the two conditions (Fig. 2F), arguing against an effect of clathrin and AP-2 phosphorylation on their binding to amphiphysin. The phosphorylation of amphiphysin, however, was found to affect its binding to AP-2. When a rat brain cytosolic extract was affinity-purified on a GST fusion protein comprising the appendage domain of α-adaptin (*25*)—that is, the amphiphysin binding portion of AP-2—the phosphorylated forms of both amphiphysin 1 and 2 were retained less efficiently than the corresponding dephosphorylated forms (Fig. 2, F and G).

Thus, complex formation of a multimeric complex between various endocytic proteins is inhibited by phosphorylation (*26*). We explored the effect of protein kinase inhibitors on coprecipitation with amphiphysin 1 of dynamin 1 and AP-2 (*27*). The general protein kinase inhibitor K252a (*28*) strongly inhibited the effect of ATP on both coprecipitation and amphiphysin mobility (Fig. 3, A and B). The kinase or kinases responsible for these effects remain to be identified.

Dynamin 1, synaptojanin 1, and the amphiphysins undergo stimulation-dependent dephosphorylation in nerve terminals, and dephosphorylation is blocked by inhibitors of the Ca^{2+}/calmodulin-dependent phosphatase calcineurin (*4, 20, 21*). In the absence of phosphatase inhibitors, ATP was not sufficient to produce a significant mobility shift of the amphiphysins in SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and a corresponding inhibition of the binding of dynamin to amphiphysin (Fig. 3C). The calcineurin inhibitor cyclosporin A, however, enhanced the shift and decreased the copre-

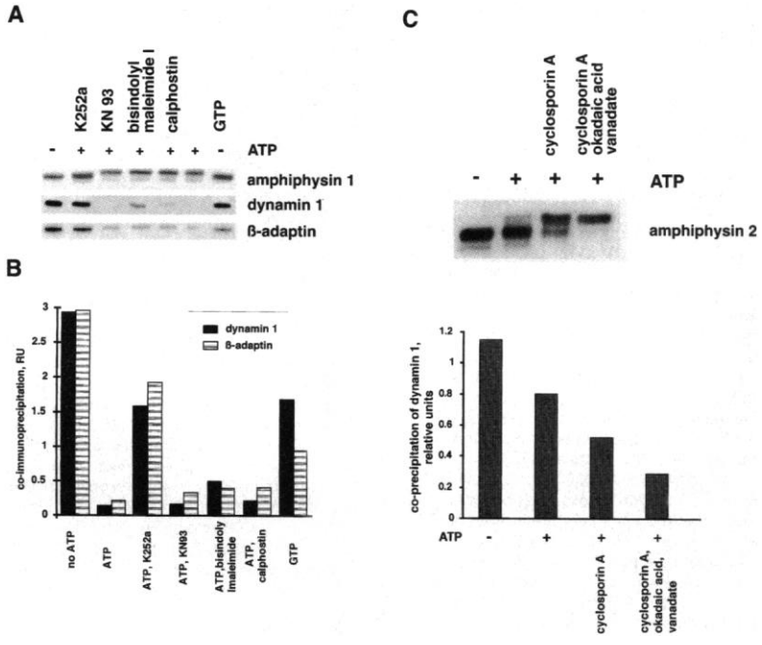

Fig. 3. Effect of protein kinase and protein phosphatase inhibitors on coprecipitation of dynamin 1 and AP-2 (β-adaptin subunit) with amphiphysin 1. Monoclonal antibodies directed against amphiphysin 1 were used to generate immunoprecipitates from rat brain cytosol (*22*), which had been preincubated with ATP, a phosphatase inhibitor mixture, and the compounds indicated. Immunoprecipitates were reacted by protein blotting (**A**) and band intensity was quantified by a PhosphorImager (**B**). (**C**) Monoclonal antibodies directed against amphiphysin 1 were used to generate immunoprecipitates from rat brain cytosol incubated in the presence of ATP and phosphatase inhibitors as indicated. Immunoprecipitates were reacted by protein blotting with antibody to amphiphysin 2 (upper) and the relative amount of coprecipitated dynamin 1 was quantified with a PhosphorImager (lower). Note upper shift of the amphiphysin 2 band correlating with the presence of phosphatase inhibitors. Similar results were obtained for amphiphysin 1.

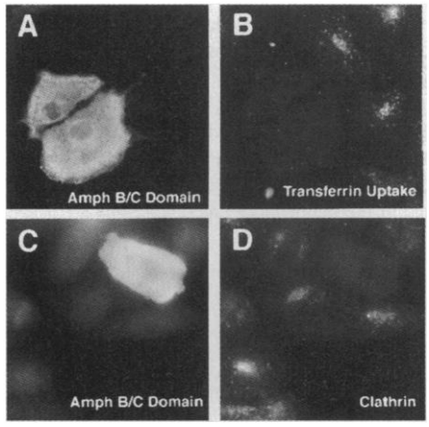

Fig. 4. The amphiphysin 1 region containing the AP-2 and clathrin binding sites has a dominant-negative effect on clathrin-mediated endocytosis. Double immunofluorescence of Chinese hamster ovary cells transfected with an amphiphysin 1 fragment (amino acids 250 to 588) comprising the clathrin and AP-2 binding site (*31*) [B + C region as defined in (*6*)]. Cells were transiently transfected with the B + C region of amphiphysin 1 and then incubated with CY3-labeled transferrin for 20 min. After fixation cells were processed for amphiphysin 1 and clathrin immunofluorescence. Magnification, ×400.

cipitation of dynamin with amphiphysin. An even greater effect on both parameters was observed if two other phosphatase inhibitors, okadaic acid and vanadate, were added to cyclosporin A, which suggests an involvement of other phosphatases in addition to calcineurin (Fig. 3C).

Thus, amphiphysin appears to play a key role as a regulated linker connecting AP-2/clathrin to dynamin 1 and synaptojanin 1. High-level expression of the SH3 domain of amphiphysin, which binds dynamin and synaptojanin 1, has dominant negative effects on clathrin-mediated endocytosis (*9*). A similar effect would be expected for overexpression of the amphiphysin region that contains the AP-2 and clathrin binding sites. Accordingly, transfection of an amphiphysin 1 construct comprising amino acids 250 to 588 (B and C domains) (*31*) in Chinese hamster ovary cells blocked receptor-mediated uptake of transferrin (Fig. 4, A and B). Furthermore, expression of this construct produced a change of the clathrin immunostaining from the typical punctate to a diffuse pattern (Fig. 4, C and D), consistent with a disruption of clathrin assembly.

Phosphorylation and dephosphorylation reactions play an important role in regulation of the endocytic machinery. Ca^{2+}-dependent dephosphorylation of endocytic proteins (*4, 20, 21*) after nerve terminal depolarization may prime the nerve terminal for efficient compensatory endocytosis after a burst of exocytosis. Ca^{2+}-dependent dephosphorylation may underlie some of the reported positive effects of Ca^{2+} on synaptic vesicle endocytosis (*32*) and a dephosphorylation-dependent assembly of cytosolic endocytic coat proteins may explain the increased number of clathrin cages and clathrin-coated pits observed in ATP-depleted cells (*33*). In nonneuronal cells AP-2 assembly into clathrin coats correlates with its dephosphorylation (*19*). It is possible that a general property of proteins involved in endocytosis is to undergo constitutive phosphorylation and to assemble in the dephosphorylated state.

References and Notes

1. J. E. Heuser and T. S. Reese, *J. Cell Biol.* **57**, 315 (1973); O. Cremona and P. De Camilli, *Curr. Opin. Neurobiol.* **7**, 323 (1997).
2. J. H. Koenig and K. Ikeda, *J. Neurosci.* **9**, 3844 (1989); K. Takei, P. S. McPherson, S. L. Schmid, P. De Camilli, *Nature* **374**, 186 (1995); J. E. Hinshaw and S. L. Schmid, *ibid.*, p. 190; R. Urrutia, J. R. Henley, T. Cook, M. A. McNiven, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 377 (1997).
3. P. S. McPherson *et al.*, *Nature* **379**, 353 (1996); C. Haffner *et al.*, *FEBS Lett.* **419**, 175 (1997).
4. P. Wigge *et al.*, *Mol. Biol. Cell* **8**, 2003 (1997).
5. B. Lichte, R. W. Veh, H. E. Meyer, M. W. Kilimann, *EMBO J.* **11**, 2521 (1992); M. H. Butler *et al.*, *J. Cell Biol.* **137**, 1355 (1997); C. Leprince *et al.*, *J. Biol. Chem.* **272**, 15101 (1997); A. R. Ramjaun, K. D. Micheva, I. Bouhelet, P. S. McPherson, *ibid.*, p. 16700.
6. C. David, M. Solimena, P. De Camilli, *FEBS Lett.* **351**, 73 (1994).
7. C. David, P. S. McPherson, O. Mundigl, P. De Camilli, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 331 (1996).
8. D. Grabs *et al.*, *J. Biol. Chem.* **272**, 13419 (1997).
9. O. Shupliakov *et al.*, *Science* **276**, 259 (1997); P. Wigge, Y. Vallis, H. T. McMahon, *Curr. Biol.* **7**, 554 (1997).
10. H. T. McMahon, P. Wigge, C. Smith, *FEBS Lett.* **413**, 319 (1997).
11. A. R. Ramjaun and P. S. McPherson, *Mol. Biol. Cell Suppl.* **8**, 93a (1997).
12. L. H. Wang, T. C. Südhof, R. G. W. Anderson, *J. Biol. Chem.* **270**, 10079 (1995).
13. GST-PRD fusion proteins were prepared as in (*8*); 6 mg of fusion protein was coupled to 0.5 ml of Affi-Gel 10 beads (Bio-Rad) according to the manufacturer's instructions. Triton X-100 detergent extracts of total rat brain (15 mg of protein per milliliter) were prepared as in (*8*); dialyzed overnight against 20 mM Hepes (pH 7.4) containing 100 mM NaCl, 1 mM $MgCl_2$, and 1% Triton X-100; centrifuged at 40,000*g* for 30 min; and incubated with beads for 4 hours at 4°C. Beads were washed five times with 20 mM Hepes (pH 7.4), 150 mM NaCl, and 1% Triton X-100 and eluted with SDS-PAGE sample buffer. Eluted proteins were separated by SDS-PAGE and stained with Coomassie blue or transferred to nitrocellulose filters for immunoblot analysis. Recombinant human amphiphysin 1, its SH3 domain (*6*), and its central region (amino acids 262 to 435) (*6*) were expressed in pGEX6 vector as GST-fused proteins. Their purification and removal of GST by using PreScission protease (Pharmacia) were performed according to the manufacturer's protocols. For competition experiments, 30 μg of PRD presorbed to glutathione-Sepharose was supplemented with 5 or 15 μg of amphiphysin 1, 150 μg of the SH3 domain of amphiphysin, or 150 μg of the central region (amino acids 262 to 435) and incubated with 1 ml of extract for 2 hours at 4°C.
14. B. M. Pearse and M. S. Robinson, *Annu. Rev. Cell Biol.* **6**, 151 (1990).
15. A number of other nerve terminal proteins tested, including the synaptic vesicle proteins synaptobrevin, synaptotagmin, and Rab3, did not bind to the PRD.
16. P. P. Di Fiore, P. G. Pelicci, A. Sorkin, *Trends Biochem. Sci.* **22**, 411 (1997); S. Ahle and E. Ungewickell, *EMBO J.* **5**, 3143 (1986); S. Zhou *et al.*, *J. Biol. Chem.* **268**, 12655 (1993).
17. N. Ringstad, Y. Nemoto, P. De Camilli, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 8569 (1997); E. de Heuvel *et al.*, *J. Biol. Chem.* **272**, 8710 (1997).
18. J. H. Keen and M. M. Black, *J. Cell Biol.* **102**, 1325 (1986); S. A. Morris, A. Mann, E. Ungewickell, *J. Biol. Chem.* **265**, 3354 (1990); W. J. Schook and S. Puszkin, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 8039 (1985).
19. A. Wilde and F. M. Brodsky, *J. Cell Biol.* **135**, 635 (1996).
20. P. J. Robinson *et al.*, *Nature* **365**, 163 (1993); P. S. McPherson, K. Takei, S. L. Schmid, P. De Camilli, *J. Biol. Chem.* **269**, 30132 (1994).
21. R. Bauerfeind, K. Takei, P. De Camilli, *J. Biol. Chem.* **272**, 30984 (1997).
22. For the preparation of cytosol, rat brains were homogenized in 2 volumes of 10 mM Hepes (pH 7.4) containing 1 mM EDTA in the presence of a protease inhibitor cocktail (PIC; aprotinin, antipain, leupeptin, and pepstatin each at 3 μg/ml). The lysate was centrifuged at 100,000*g* for 2 hours and the resulting supernatant (cytosol) was desalted on Sephadex G-25 at room temperature into KH buffer [20 mM Hepes (pH 7.4), 120 mM KCl, and 1 mM $MgCl_2$]. For preparation of Triton X-100 extract of total rat brain the postnuclear supernatant (1000*g*) was solubilized with 2% Triton X-100 and centrifuged at 150,000*g* for 1 hour. The supernatant was desalted on Sephadex G-25 into KH buffer containing 5 mM $MgCl_2$, PIC, and 1% Triton X-100. Brain extracts were incubated at 30°C for 15 to 40 min in the presence of 2 mM ATP, a mixture of phosphatase inhibitors (2 μM cyclosporin A, 0.2 μM okadaic acid, and 1 mM sodium vanadate), or general protein kinase (4 μM K252a), as indicated. Incubations were terminated by addition of 10 mM EDTA and samples precooled in ice were incubated with monoclonal antibody 8 to amphiphysin 1 [S. Floyd *et al.*, *Mol. Med.* **4**, 29 (1998)] coupled to Ultralink beads (Pierce) for 1 hour at 4°C. Beads were washed with KH buffer containing 0.5% Triton X-100, 1 mM EDTA, and 1 mM sodium vanadate; eluted in SDS-containing buffer; and analyzed by SDS-PAGE and immunoblotting with ^{125}I-labeled protein A (Dupont NEN). Band-associated radioactivity was measured with a PhosphorImager (Molecular Dynamics). The presence of Ca^{2+} up to 50 μM or of 0.2 mM EGTA during the phosphorylation reaction did not produce significant differences on the results of coimmunoprecipitations. A nonhydrolyzable analog of ATP, adenosine-5'-(γ-thio)triphosphate, was not effective in preventing complex formation. No protein bands were precipitated by control immunoglobulin G. For experiments in Fig. 2, B to G, extracts were incubated in the presence of 2 to 5 mM ATP and phosphatase inhibitor mixture (phosphorylation conditions are denoted as +ATP) or in the presence of 4 μM K252a (dephosphorylation conditions are denoted as −ATP).
23. Metabolic labeling of intact rat synaptosomes confirmed that the upper band of amphiphysin 2 represents a phosphorylated form of the protein.
24. The reverse experiment, in which ATP-pretreated material was applied to a dynamin 1 PRD column, did not reveal significant differences in binding of amphiphysins 1 and 2.
25. A GST fusion protein of α-adaptin appendage domain (amino acids 701 to 938 of mouse α_C-adaptin cloned into pGEX4T-1) vector was bound to glutathione Sepharose-4B beads (Pharmacia).
26. This hypothesis is corroborated by the observation that antibodies to amphiphysin did not coprecipitate any ^{32}P-labeled dynamin 1, synaptojanin 1, or AP-2 components from metabolically labeled synaptosomes.
27. Desalted brain cytosol was incubated for 15 min at 30°C in the presence of the phosphatase inhibitor mixture (*22*) and in the absence or presence (as indicated) of 2 mM ATP, 2 mM guanosine triphosphate, and the following protein kinase inhibitors: 4 μM K252a, 15 μM KN93, 1 μM bisinolylmaleimide 1, and 0.6 μM calphostin under room light (all reagents from Calbiochem). Reactions were terminated by addition of 10 mM EDTA and mixtures were immunoprecipitated as in (*22*) with monoclonal antibodies directed against the NH_2-terminal region of amphiphysin 1. For the analysis of phosphatase inhibitors, cytosol was incubated for 30 min at 30°C with 5 mM $MgCl_2$, 5 mM ATP, and the following inhibitors: 2 μM cyclosporin A, 0.2 μM okadaic acid, 1 mM sodium vanadate, and a mixture of the three.
28. U. T. Ruegg and G. M. Burgess, *Trends Pharmacol. Sci.* **10**, 218 (1989).
29. D. Toullec *et al.*, *J. Biol. Chem.* **266**, 15771 (1991); E. Kobayashi, H. Nakano, M. Morimoto, T. Tamaoki, *Biochem. Biophys. Res. Commun.* **159**, 548 (1989).
30. M. Sumi *et al.*, *Biochem. Biophys. Res. Commun.* **181**, 968 (1991).
31. A fragment of cDNA encoding amino acids 250 to 588 of amphiphysin 1 [B/C region (*6*)] was subcloned into pcDNA3 vector (Invitrogen) and transiently transfected into CHO cells for 24 hours. CY3-labeled transferrin (Molecular Probes) (25 μg/ml final concentration) was added during the last 25 min before a short wash in phosphate-buffered saline. Cells were fixed with formaldehyde and processed for immunofluorescence with antibody X22 to clathrin (ATCC) and antibody CD6 to amphiphysin (*6*).
32. B. Ceccarelli and W. P. Hurlbut, *J. Cell Biol.* **87**, 297 (1980); O. Shupliakov, H. Gad, P. Low, E. Zotova, L. Brodin, *Soc. Neurosci. Abstr.* **23**, 2274 (1997).
33. E. M. Merisko, M. G. Farquhar, G. E. Palade, *Pancreas* **1**, 110 (1986); K. L. English and M. C. Nowicky, *J. Physiol (London)* **506**, 591 (1998); O. Shupliakov *et al.*, *Soc. Neurosci. Abstr.* **22**, 777 (1996).

34. We thank C. David for help and discussion in the initial experiments and T. Kirchhausen, P. P. Di Fiore, and R. Jahn for the generous gift of antibodies. Supported in part by grants from the NIH (CA46128 and NS36251), by the Human Frontier Science Program and the U.S. Army Medical Research and Development Command to P.D.C., and by a fellowship from the Deutscher Akademischer Austauschdienst to D.G.

23 April 1998; accepted 29 June 1998