Synaptojanin Forms Two Separate Complexes in the Nerve Terminal

INTERACTIONS WITH ENDOPHILIN AND AMPHIPHYSIN*

Kristina D. Micheva‡§, Brian K. Kay¶, and Peter S. McPherson‡∥

From the ‡Department of Neurology and Neurosurgery, Montreal Neurological Institute, McGill University, 3801 Rue University, Montreal, Quebec H3A 2B4, Canada and the ¶Department of Biology, University of North Carolina, Chapel Hill, North Carolina 27599

Endophilin is a recently discovered src homology 3 domain-containing protein that is a major *in vitro* binding partner for synaptojanin. To further characterize endophilin, we generated an antipeptide antibody. Endophilin is enriched in the brain, and immunofluorescence analysis reveals a high concentration of the protein in synaptic terminals, where it colocalizes with synaptojanin. *In vitro* binding assays demonstrate that endophilin binds through its src homology 3 domain to synaptojanin, and immunoprecipitation analysis with the antiendophilin antibody reveals that endophilin is stably associated with synaptojanin in the nerve terminal. Immunoprecipitation with an antibody against amphiphysin I and II, which interact through their src homology 3 domains with dynamin and synaptojanin at sites distinct from those for endophilin, reveals a second stable complex, which includes dynamin and synaptojanin but excludes endophilin. These data demonstrate that synaptojanin is present in two separate complexes in the nerve terminal and support an important role for endophilin in the regulation of synaptojanin function.

The accuracy and speed of information processing in the nervous system is critically dependent on "fast" synaptic transmission. At the presynaptic level, there are two key events underlying this process: the exocytosis of synaptic vesicles from the nerve terminal, resulting in neurotransmitter release, and the endocytosis of synaptic vesicle membranes, which restores the releasable vesicle pool. Although knowledge of the molecular mechanisms underlying exocytosis has advanced considerably in recent years, the process of endocytosis is still poorly understood.

One of the few molecules with an established function in endocytosis is the GTPase dynamin, which participates in the fission of endocytic vesicles (for review, see Ref. 1). The role of dynamin in endocytosis was first predicted by studies on the *Drosophila shibire* mutant in which mutations of *Drosophila* dynamin lead to a block of synaptic vesicle endocytosis (2, 3). Furthermore, mutations in the GTPase domain of mammalian dynamin inhibit receptor-mediated endocytosis when overexpressed in fibroblasts (4–6). The C terminus of dynamin contains a proline-rich region that interacts with proteins containing Src homology 3 (SH3) domains (7). An important SH3 domain-containing binding partner for dynamin is amphiphysin I (8, 9). Amphiphysin I is enriched in nerve terminals and is homologous to the yeast proteins RVS161 and RVS167 (10), mutations in which cause endocytic defects (11). The SH3 domain-mediated interaction between dynamin and amphiphysin I appears to be an essential step in the endocytosis of synaptic vesicles, serving to target dynamin to clathrin-coated pits (12, 13). In the nerve terminal, amphiphysin I is likely targeted to these endocytic sites through SH3 domain-independent interactions with the clathrin adaptor protein 2 (AP2) (8, 14). Recently, we and others have identified a novel amphiphysin isoform, referred to as amphiphysin II, that also binds through its SH3 domain to dynamin (15–18). Both amphiphysin I and II also interact with clathrin, which is likely to contribute to amphiphysin targeting to the endocytic machinery (17).

Several other proteins besides dynamin and the amphiphysins have been implicated in endocytosis, although compelling evidence for their roles is still lacking. Synaptojanin is a nerve terminal-enriched protein, which, like dynamin, binds to the amphiphysin I and II SH3 domains and undergoes dephosphorylation upon synaptic terminal depolarization (17, 19–21). Synaptojanin is an inositol 5-phosphatase with activity against a number of substrates, including inositol 1,4,5-trisphosphate, inositol 1,3,4,5-tetrakisphosphate, phosphatidylinositol 4,5-bisphosphate, and phosphatidylinositol 3,4,5-trisphosphate (21, 22), and inositol phosphate metabolism is implicated in a variety of membrane trafficking events, including endocytosis (23). The N-terminal domain of synaptojanin is similar to the cytosolic domain of the yeast protein SAC1, which has been implicated in vesicular trafficking, likely via alterations in phospholipid metabolism and diacylglycerol production (24). The C terminus of synaptojanin is proline-rich and binds to the SH3 domains of amphiphysin I and II.

Recently, another SH3 domain-containing protein, SH3P4 (25), was identified as a major binding partner for synaptojanin (26). We had independently isolated SH3P4 as a member of a novel SH3 domain-containing family of proteins with unknown function using an expression library screen with an SH3 domain peptide ligand (25). In the current study, we determined that SH3P4 is brain specific and is enriched in the presynaptic nerve terminal. Using *in vitro* binding assays, we determined that SH3P4, amphiphysin I, and amphiphysin II each bind to distinct sites within the proline-rich tail of synaptojanin, and the three proteins can bind to synaptojanin simultaneously. However, immunoprecipitation analysis revealed that, *in situ*, two separate and stable synaptojanin complexes are formed:

This paper is available on line at http://www.jbc.org

synaptojanin with SH3P4, and synaptojanin with amphiphysin I or II and dynamin. These results suggest an important role for SH3P4 in synaptic vesicle endocytosis, possibly through regulating the recruitment of synaptojanin to the site of endocytosis, and we thus renamed SH3P4 as endophilin.

EXPERIMENTAL PROCEDURES

Antibodies—Polyclonal antiendophilin antibodies (1903 and 1904) were made against an 18-residue peptide (residues 256–272, CQPK-PRMSLEFATGDSTQ), synthesized at the W. M. Keck Biotechnology Resource Laboratory at Yale University (New Haven, CT), and conjugated to KLH (Calbiochem) as described (27). The conjugated peptide was injected into rabbits (approximately 600 μg per rabbit) using Titermax adjuvant (CytRx Corp.) with standard protocols. Antibodies were affinity purified from sera against strips of polyvinylidene difluoride membrane (BioRad) containing the peptide conjugated to bovine serum albumin as described (28). Polyclonal antibodies against amphiphysin and synaptojanin were described previously (8, 17, 20, 26). The following antibodies were generous gifts: a monoclonal antibody against synaptojanin (Drs. Amy Hudson and Pietro De Camilli, Yale University); a polyclonal antisynapsin antibody (G246, Dr. Pietro De Camilli, Yale University) (29); a polyclonal antisynaptophysin antibody (Dr. Reinhard Jahn, Yale University) (30). The monoclonal antibody against dynamin (Hudy I) was purchased from Upstate Biotechnology Inc. Western blot analysis was performed as described previously (31).

Analysis of Tissue and Subcellular Distribution—Postnuclear supernatants from different tissues were prepared and processed for Western blots as described (17). The subcellular distribution of endophilin in various regions of the rat brain was studied on frozen sections by immunofluorescence as described (17).

Production of Fusion Proteins—Endophilin in pEXlox(+) vector (25) was used as a template in PCRs[1] with Vent DNA Polymerase (New England Biolabs) to generate the following GST fusion proteins: full-length endophilin (amino acid residues 1–352), the N-terminal domain of endophilin (residues 1–291), and the SH3 domain of endophilin (residues 292–352). The full-length endophilin was prepared with the forward primer 5′-GCGGGATCCATGTCGGTGGCAGGGCTG (Fa) and the reverse primer 5′-GCGGAATTCTCAATGGGGCAGAGCAACCAG (Ra). For the N-terminal domain, the same forward primer was used (Fa) with the reverse primer 5′-GCGGAATTCTCACATTTGGACACCT-GGAGG (Rb). For the SH3 domain, the forward primer 5′-GCGGGATC-CGATCAGCCCTGCTGCCG (Fb) was used with the reverse primer Ra were used. The PCR products were cloned in frame into the *Bam*HI–*Eco*RI sites of pGEX-2TK (Pharmacia) and the GST fusion proteins were expressed and purified as described (19).

Amphiphysin I and amphiphysin II GST fusion proteins were prepared as described (17).

Affinity Chromatography—Rat brain postnuclear supernatants, prepared as described (17), were incubated with 1% Triton X-100 for 1 h at 4 °C, and insoluble material was removed by centrifugation in a Beckman 50Ti rotor at 45,000 rpm for 1 h. The supernatant was diluted to 2 mg/ml of protein in 20 mM HEPES-OH, pH 7.4, containing 0.83 mM benzamidine, 0.23 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml aprotinin, 0.5 μg/ml leupeptin, and 1% Triton X-100 (Buffer A), and 1-ml aliquots were incubated overnight at 4 °C with the GST fusion proteins described above (approximately 25 μg/ml of each protein) prebound to glutathione-Sepharose. After incubation, samples were washed three times in 1 ml of buffer, and bound proteins were eluted with SDS-PAGE sample buffer and prepared for Western blot analysis.

Overlay Assays—Nitrocellulose membranes with protein fractions purified by endophilin affinity chromatography were incubated overnight at 4 °C in Tris-buffered saline (50 mM Tris-Cl, pH 8.3, 150 mM NaCl) containing 3% bovine serum albumin, 0.1% Tween-20, 1 mM dithiothreitol, and 500,000 cpm/ml of phosphorylated full-length endophilin GST fusion protein. The fusion protein was phosphorylated with heart muscle protein kinase (Sigma) following the protocol supplied by the manufacturer of the pGEX-2TK vector (Pharmacia). Transfers were then washed three times for 5 min each in Tris-buffered saline and exposed to film.

For the overlay assays on synaptojanin, full-length synaptojanin and a synaptojanin deletion mutant lacking the 231-amino acid proline-rich C terminus (synaptojanin R-2) were purified from *Spodoptera frugiperda* (Sf9) cells after baculovirus-mediated expression as described

(26). To construct a synaptojanin deletion mutant lacking the last 127 amino acids of the proline-rich C terminus, PCR was performed on a full-length synaptojanin cDNA (clone 9) (21) with Vent DNA polymerase. The forward primer (5′-GGTGATGTCGACGACTAC) corresponds to nucleotides 3178–3195 spanning a *Sal*I site, and the reverse primer, R-1 (5′-GCGGTCGACAAGCTTTTATGCAAGTCCGGCTTGAGG) corresponds to nucleotides 3619–3636 and encodes a *Sal*I site flanking a *Hin*dIII site. The PCR product was digested with *Sal*I and subcloned into *Sal*I-digested clone 9, generating clone Bluescript R-1. This clone was digested with *Hin*dIII, and the liberated fragment was subcloned into *Hin*dIII-digested clone 1B7 (prepared as described in Ref. 26), generating clone synaptojanin R-1. For synaptojanin 170, PCR was performed on clone 9 with Vent DNA polymerase using the forward primer 5′-GCGAAGCTTGGTAAAAATAAATGGAGCTTG, corresponding to nucleotides 4026–4046, and the reverse primer 5′-GCGAAG-CTTTTATCTTTCTGTGAAGTCCAATG, corresponding to nucleotides 4821–4799. The PCR product was digested with *Hin*dIII and subcloned into the second *Hin*dIII site of clone synaptojanin (26) after a partial *Hin*dIII digestion. The clones were packaged into baculovirus and used to infect Sf9 cells, and the expressed proteins were purified as described (26). Approximately 1 μg each of the various synaptojanin proteins were run on SDS-PAGE, transferred to nitrocellulose, and overlaid with GST-endophilin, GST-amphiphysin I, GST-amphiphysin II, or GST alone as described (19).

Binding Assays—A (His)$_6$-endophilin fusion protein was prepared by digesting the pGEX-2TK plasmid containing full-length endophilin, prepared as described above, with *Bam*HI and *Eco*RI and ligating the liberated fragment into the *Bam*HI–*Eco*RI sites of pTrcHisA (Invitrogen). The (His)$_6$ fusion protein was expressed and purified as recommended for the QIA express system using Ni-NTA resin (Qiagen). To examine the interactions of endophilin and amphiphysin with synaptojanin and dynamin, approximately 2 μg of GST fusion proteins of the SH3 domains of amphiphysin I or amphiphysin II, precoupled to glutathione-Sepharose beads, were incubated with 1 μg of purified synaptojanin or dynamin and increasing concentrations of purified (His)$_6$/endophilin. Dynamin was purified from Sf9 cells after baculovirus-induced expression as described (32) and was a generous gift of Dr. Sandra Schmid (Scripps Research Institute). The reactions were carried out in 1 ml of Buffer A for 4 h at 4 °C. For all binding assays, at the end of the incubations, the beads were pelleted by microcentrifugation, washed three times in 1 ml of Buffer A, resuspended in SDS sample buffer, and prepared for Western blot analysis.

Immunoprecipitation—A crude synaptosomal fraction (P2′) was prepared from rat brain as described (33). It was resuspended in a buffer containing 10 mM HEPES-OH, pH 7.4, 150 mM NaCl, 1 mM EGTA, 0.83 mM benzamidine, 0.23 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml aprotinin, and 0.5 μg/ml leupeptin (Buffer B). The samples were then homogenized with a Polytron, Triton X-100 was added to a final concentration of 0.1%, and the samples were incubated for 20 min at 4 °C before removing insoluble material by centrifugation at 75,000 rpm for 15 min in a Beckman TLA 100.2 rotor. The supernatant was precleared with protein A-Sepharose and was then incubated for 2 h at 4 °C with antiendophilin (1903) or antiamphiphysin sera (1874) precoupled to protein A-Sepharose. Finally, samples were washed three times with 1 ml of Buffer B containing 0.1% Triton X-100 and were eluted with SDS sample buffer.

RESULTS

Endophilin Is a Brain-specific Protein, Concentrated in Synaptic Terminals—To further characterize endophilin, we generated polyclonal rabbit antisera (1903 and 1904) against a peptide sequence specific to the protein. After affinity purification, both antibodies recognized a single 40-kDa band on strips of crude brain homogenate (data not shown). Endophilin has a predicted molecular mass of 39,880 Da (25). When tested on immunoblots from a variety of tissues, endophilin immunoreactivity was observed only in brain (Fig. 1).

We next examined the subcellular localization of endophilin on frozen sections from rat brain using immunofluorescence. Endophilin immunoreactivity had a predominantly punctate appearance, indicative of a concentration of the antigen in presynaptic nerve terminals (Fig. 2, *A* and *B*). There was also a relatively high level of immunoreactivity in the cytoplasm of cell bodies. Cell nuclei were immunonegative, as were axons. The pattern of endophilin immunoreactivity looked very simi-

---

[1] The abbreviations used are: PCR, polymerase chain reaction; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis.
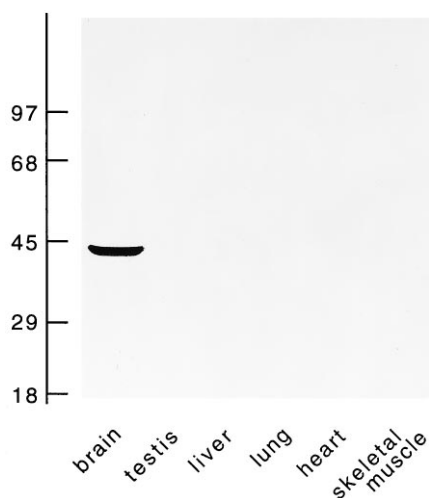
FIG. 1. **Tissue distribution of endophilin.** Proteins from post-nuclear supernatants of various rat tissues (100 μg per lane) were separated by SDS-PAGE, transferred to nitrocellulose, and processed for Western blot with the affinity-purified polyclonal antibody against endophilin. The antibody was revealed with a 4-chloro-1-naphthol-based color development as described previously (31).

lar to that of synaptojanin, as well as the synaptic markers synapsin (Fig. 2*B*) and synaptophysin (data not shown). Interestingly, endophilin, like synaptojanin, appeared to be absent from mossy fiber axon terminals in the CA3 region of the hippocampus (Fig. 2*B*), which was the only brain region examined where endophilin and synaptojanin did not co-localize with known synaptic markers.

Endophilin Binds to Several Brain Proteins, Including Synaptojanin and Dynamin—To identify binding partners of endophilin, we performed affinity chromatography on crude rat brain extracts using a full-length endophilin GST fusion protein. We then overlaid the column elution fraction with radiolabeled endophilin and observed three major bands at 145, 135, and 100 kDa (Fig. 3*A*). Western blots of the elution fractions identified the 145- and 100-kDa bands as synaptojanin and dynamin, respectively (Fig. 3*B*). The identity of the 135-kDa endophilin-binding protein remains unknown. To further delineate the interactions, affinity chromatography was performed using GST fusion proteins encoding the N-terminal and the SH3 domains of endophilin, and both synaptojanin and dynamin were found to specifically bind to the SH3 domain (Fig. 3*B*).

Endophilin, Amphiphysin I, and Amphiphysin II Bind to Different Sites in the Proline-rich C Terminus of Synaptojanin—In addition to endophilin, synaptojanin can also bind to amphiphysin I (8) and amphiphysin II (17). To begin to determine the sites of interaction on synaptojanin for these three proteins, the 170-kDa isoform of synaptojanin (34), as well as the 145-kDa isoform and two deletion constructs lacking the last 127 amino acids of the proline-rich C terminus (synaptojanin R-1) or the entire 231 amino acid proline-rich C terminus (synaptojanin R-2), were purified from Sf9 cells after baculovirus-mediated expression. Western blot analysis confirmed an equal loading of each protein (Fig. 4). These constructs were then overlaid with GST fusion proteins containing either the SH3 domain of endophilin, amphiphysin I, or amphiphysin II. GST alone did not interact with any of the synaptojanin proteins (Fig. 4). In contrast, endophilin and amphiphysin I SH3 domain fusion proteins bound to the 170- and 145-kDa isoforms of synaptojanin and to synaptojanin R-1 but did not bind to synaptojanin R-2. Thus, there is a binding site(s) for the SH3 domains of endophilin and amphiphysin I between amino acids 1061 and 1165 of synaptojanin. Interestingly, the amphiphysin

II SH3 domain did not bind to synaptojanin R-1 or R-2, indicating that the binding site for this protein lies between amino acids 1165 and 1276. These results suggested that endophilin and amphiphysin I bind proline-rich sequence(s) in the same region of synaptojanin that is different from the binding site of amphiphysin II.

To further characterize the interactions between these proteins, we incubated GST fusion proteins encoding the SH3 domains of amphiphysin I or amphiphysin II, prebound to glutathione-Sepharose beads, with constant amounts of synaptojanin in the presence of increasing concentrations of (His)₆/endophilin. In both cases, the formation of a complex of endophilin, synaptojanin, and the amphiphysin isoform was observed (Fig. 5) suggesting that the amphiphysin isoforms bind to proline-rich sequences distinct from the endophilin binding site. Coupled with the results from Fig. 4, these data suggest that the three proteins each interact with a unique site within the proline-rich tail of synaptojanin. Only when endophilin was in large excess to synaptojanin was a slight decrease in the binding of synaptojanin to amphiphysin I or II observed, possibly due to nonspecific binding of endophilin to the amphiphysin I and II binding sites (Fig. 5).

Amphiphysin I or II and Endophilin Can Interact Simultaneously with Dynamin—Like synaptojanin, dynamin can bind to amphiphysin I (8), amphiphysin II (17), and endophilin. To determine whether these interactions can occur simultaneously, we incubated GST fusion proteins of the amphiphysin I or II SH3 domains, precoupled to glutathione-Sepharose, with a constant amount of dynamin and increasing concentrations of (His)₆/endophilin. As was the case for synaptojanin, the formation of a complex of endophilin and dynamin with either amphiphysin isoform was observed (Fig. 5). At high concentrations of endophilin, the amount of bound endophilin reached saturation. At the highest concentration of endophilin (100 μg/ml), a decreased amount of dynamin was detected in the precipitate, probably due, as in the case of synaptojanin, to nonspecific binding of endophilin to the amphiphysin I and II binding sites. These results indicate that, like synaptojanin, dynamin interacts with its SH3-binding partners via distinct proline-rich sequences.

In Situ, Endophilin and the Amphiphysin Isoforms Form Two Separate Complexes with Synaptojanin—To investigate the interactions of endophilin and the amphiphysins with synaptojanin and dynamin *in situ*, we performed immunoprecipitations from rat brain synaptosomes. The antiendophilin antiserum strongly immunoprecipitated endophilin (Fig. 6). Synaptojanin co-immunoprecipitated with endophilin and was similarly enriched in the precipitate compared with the starting material. Neither dynamin nor amphiphysin I or II co-immunoprecipitated with endophilin under these conditions. We then used an antiamphiphysin antiserum that recognizes amphiphysin I and II. The two amphiphysin isoforms were enriched in the immunoprecipitate (Fig. 6). Interestingly, both synaptojanin and dynamin co-immunoprecipitated with the amphiphysins, but no endophilin was detected. It thus appears that endophilin and the two amphiphysin isoforms form two separate complexes with synaptojanin *in situ*: endophilin with synaptojanin; amphiphysin I or II with synaptojanin and dynamin.

DISCUSSION

Endophilin, a member of a novel family of three SH3 domain-containing proteins (endophilin (also known as SH3P4), SH3P8, and SH3P13) (25) was recently identified as a major synaptojanin-binding partner in brain (26). Using an antipeptide antibody, we now demonstrate that endophilin is brain specific and is concentrated in synaptic terminals throughout

A
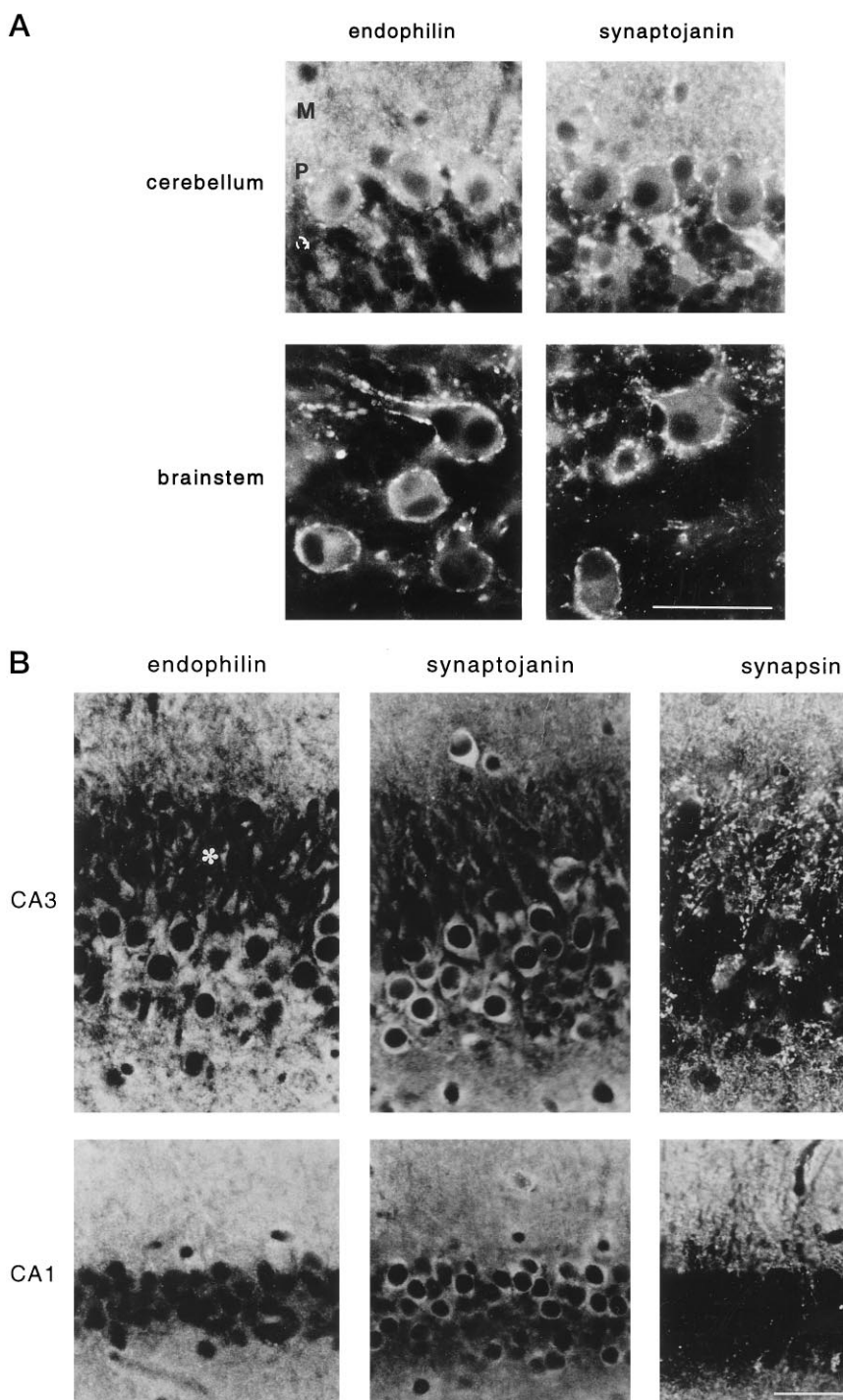
FIG. 2. Localization of endophilin in the rat brain by immunofluorescence. *A*, frozen sections from rat brain cerebellum and brainstem were immunoreacted with antibodies against endophilin and synaptojanin. Note the predominantly punctate appearance of endophilin immunoreactivity, especially surrounding the cell bodies, which is indicative of a concentration of endophilin in presynaptic nerve terminals. The molecular layer of the cerebellum, which is rich in synaptic terminals, is also brightly stained. In contrast, the axonal bundles passing through the brainstem are essentially void of labeling. This pattern closely resembles the synaptojanin immunoreactivity. *M*, molecular layer; *P*, Purkinje cell layer; *G*, granule cell layer. *Scale bar*, 50 μm. *B*, endophilin, synaptojanin, and synapsin immunoreactivity in the CA1 and CA3 regions of the rat brain hippocampus. The mossy fiber terminals (∗), which are brightly stained by the synapsin antibody, do not display endophilin and synaptojanin immunolabeling. Meanwhile, in other regions of the hippocampus, as elsewhere in the brain, the staining patterns for these three proteins were similar and were indicative of a presynaptic enrichment. *Scale bar*, 50 μm.

the brain, where it colocalizes with synaptojanin, dynamin, and amphiphysin I and II (8, 17). One exception to this synaptic localization is in the mossy fiber nerve terminals in the CA3 region of the hippocampus, where neither synaptojanin, endophilin, nor dynamin is detectable, whereas common synaptic markers, such as synapsin and synaptophysin, are present at high levels. This peculiar absence or very low level of endocytic proteins is well correlated with the low firing rates of the granule cells that give rise to these terminals (35). Moreover, the mossy fibers form large terminals, densely packed with synaptic vesicles (36), which also implies a very low demand for endocytic restoration of the vesicle pool. Thus, the tissue and subcellular distribution of endophilin further support a role for endophilin in endocytosis.

The interaction between endophilin and synaptojanin is me-

diated via the endophilin SH3 domain, and synaptojanin can also bind to the SH3 domains of amphiphysin I and II (8, 17). Overlay assays of synaptojanin deletion constructs revealed that endophilin and amphiphysin I interact with synaptojanin between amino acids 1061 and 1165, a region comprising several consensus SH3 domain-binding sites. One such site (PXR-PXR; amino acids 1065–1070) was recently identified as the binding site for amphiphysin I on dynamin (9, 37). Meanwhile, amphiphysin II was found to bind between amino acids 1165 and 1276 of synaptojanin, indicating that amphiphysin II utilizes a distinct binding site. In fact, binding experiments using purified recombinant proteins demonstrated that amphiphysin I, amphiphysin II, and endophilin can interact simultaneously with synaptojanin without competing for binding sites, suggesting that all three proteins interact at distinct sites. These
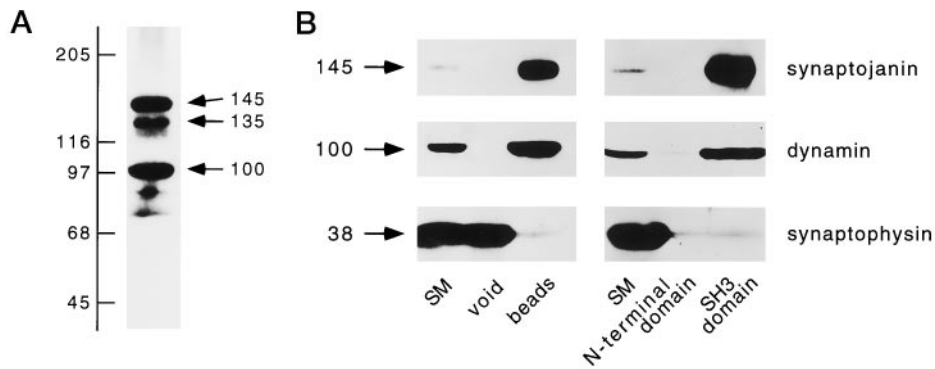
FIG. 3. **Identification of the major binding partners of endophilin.** *A*, nitrocellulose membranes with protein fractions purified by endophilin affinity chromatography were overlaid with radiolabeled endophilin. Three major bands, at 145, 135, and 100 kDa were observed. *B*, *left*, Triton X-100-soluble proteins from rat brain were incubated with a GST fusion protein encoding full-length endophilin conjugated to glutathione-Sepharose. The material bound to the beads was eluted with SDS sample buffer (*beads*), separated on SDS-PAGE along with aliquots of the soluble starting material (*SM*) and unbound material (*void*), transferred to nitrocellulose, and immunoblotted with synaptojanin, dynamin, and synaptophysin antibodies. *B, right*, Triton X-100-soluble proteins were incubated with GST fusion proteins encoding the N-terminal domain or the SH3 domain of endophilin conjugated to glutathione-Sepharose. The material bound to the beads was eluted with SDS sample buffer, separated on SDS-PAGE (*N-terminal domain* and *SH3 domain*) along with aliquots of the soluble starting material (*SM*), and processed for Western blots. The synaptophysin antibody was revealed with a 4-chloro-1-naphthol-based color development as described previously (31), and the dynamin and synaptojanin antibodies were revealed with ECL (Amersham Corp.).
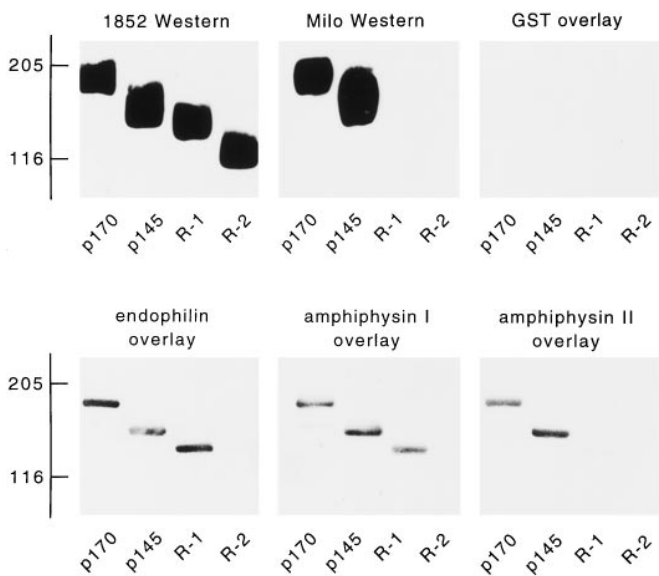

FIG. 4. **Endophilin, amphiphysin I, and amphiphysin II binding to synaptojanin constructs.** The 170-kDa (*p170*) and 145-kDa (*p145*) isoforms of synaptojanin and two deletion constructs lacking the last 127 amino acids of the proline-rich C terminus (synaptojanin R-1) (*R-1*) or the entire 231-amino acid proline-rich C terminus (synaptojanin R-2) (*R-2*) were purified from Sf9 cells after baculovirus-mediated expression. Nitrocellulose membranes with approximately 1 μg of each of the proteins were immunoblotted with a synaptojanin antibody directed against its N terminus (*1852 Western*) and a synaptojanin antibody directed against a region of its C terminus (*Milo Western*), or overlaid with GST-endophilin, GST-amphiphysin I, GST-amphiphysin II, or GST alone. The Western blots were revealed with ECL (Amersham Corp.), and the overlays were revealed with a 4-chloro-1-naphthol-based color development.

observations further confirm the specificity of the SH3 domain-mediated interactions (38) and raise the interesting possibility of the existence in the nerve terminal of a larger protein complex formed through simultaneous interactions of several SH3 proteins with the proline-rich tail of synaptojanin.

In addition to synaptojanin, endophilin was also found to bind in an SH3 domain-dependent manner to dynamin, a nerve terminal-enriched protein whose involvement in endocytosis is supported by a wealth of data (reviewed in Ref. 1). Dynamin has a proline-rich C terminus that mediates its binding to a number of SH3 domain-containing proteins *in vitro*, such as

phopholipase Cγ, Grb2, the regulatory subunit of phosphatidylinositol 3-kinase (p85α) (7) and amphiphysin I and II (8, 9, 17, 37). However, among these potential binding partners, only amphiphysin I and II are concentrated in synaptic terminals. Recent evidence showing that disruption of the interaction between dynamin and amphiphysin I inhibits endocytosis in nerve terminals (13) confirms the importance of the dynamin/amphiphysin I association *in vivo*. We have now identified another SH3 domain-binding partner for dynamin, which, like amphiphysin I and II, is enriched in nerve terminals and may also represent a physiological partner for dynamin. Furthermore, dynamin, like synaptojanin, appears to contain distinct proline-rich binding sites for its different SH3 domain partners and is able to interact simultaneously with several of these proteins.

The present *in vitro* results reveal that the SH3 domain-containing proteins endophilin and amphiphysin I and II have the potential to bind the proline-rich regions of both synaptojanin and dynamin *in vivo*. To investigate the interactions between these proteins occurring *in situ*, we performed immunoprecipitations from crude rat brain synaptosome fractions using antiendophilin and antiamphiphysin antibodies. The results indicate that two separate and stable complexes are formed in the nerve terminal: synaptojanin with endophilin, and synaptojanin with amphiphysin I or II and dynamin. The exact nature of this second complex is still unknown. Regardless, the immunoprecipitation results give an important indication for the stable physiological interactions taking place in the nerve terminal. Meanwhile, these results do not exclude the possibility that other interactions that were observed *in vitro* may also take place, although such interactions might be more transient, possibly limited to certain stages of the synaptic vesicle cycle within the nerve terminal.

Thus, two distinct and stable protein complexes appear to exist in the nerve terminal: amphiphysin I and II with synaptojanin and dynamin, and endophilin with synaptojanin. The amphiphysin-containing complex is well suited for targeting of synaptojanin and dynamin to sites of endocytosis. For example, amphiphysin I binds to the plasmalemmal clathrin adaptor protein AP2 (8, 14), and both amphiphysin I and II bind to clathrin (17). It is, indeed, known that the SH3 domain-mediated interaction between amphiphysin I and dynamin is essential for recruiting dynamin to clathrin-coated pits (12, 13). By a similar mechanism, amphiphysin I and/or II may also partici-

Synaptojanin Protein Complexes
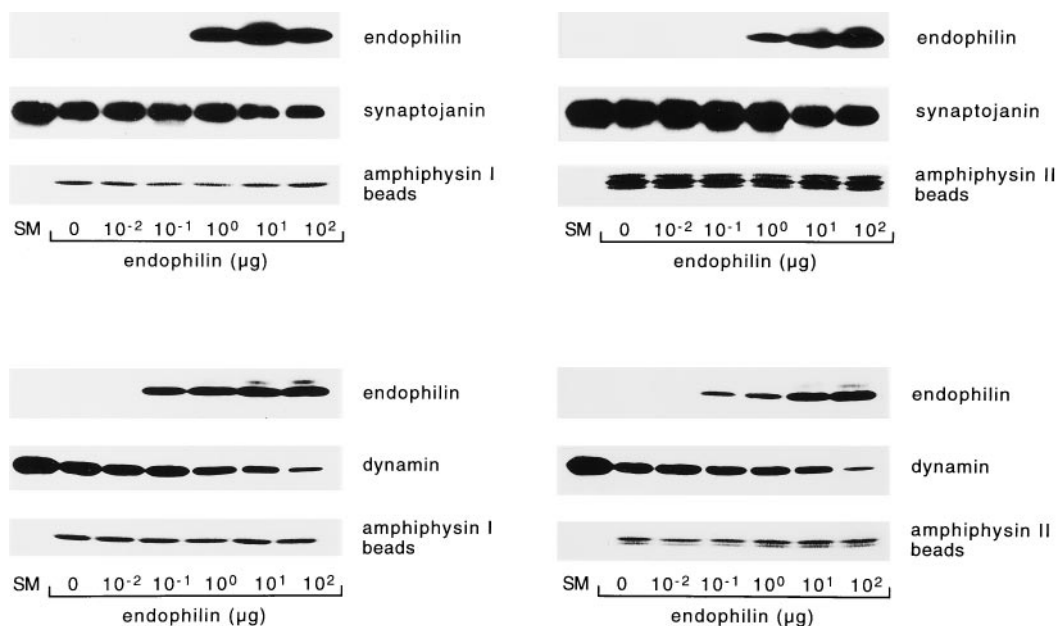
FIG. 5. **Simultaneous binding of endophilin and the amphiphysins to synaptojanin or dynamin.** GST fusion proteins of the SH3 domains of amphiphysin I or amphiphysin II, precoupled to glutathione-Sepharose beads, were incubated with 1 μg of purified synaptojanin (*top panels*) or dynamin (*bottom panels*) and increasing concentrations of purified (His)$_6$/endophilin. Synaptojanin or dynamin alone (1 μg) was used as starting material (*SM*). The material bound to the beads was eluted with SDS-PAGE sample buffer. The panels show immunoblots with endophilin, synaptojanin, and dynamin antibodies and a Ponceau S stain of the amphiphysin I and amphiphysin II GST fusion proteins. The endophilin antibody was revealed with a 4-chloro-1-naphthol-based color development as described previously (31), and the dynamin and synaptojanin antibodies were revealed with ECL (Amersham Corp.).
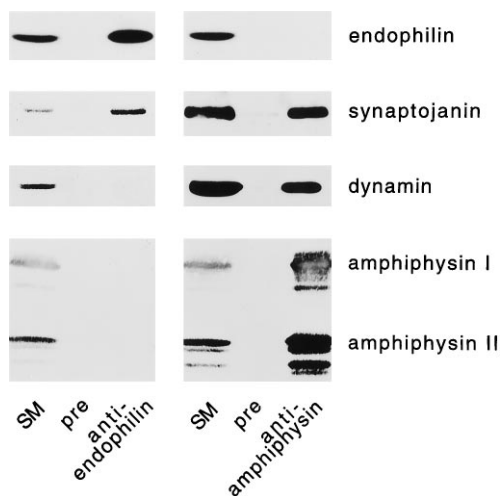
FIG. 6. **Immunoprecipitation analysis of synaptojanin interactions.** Crude synaptosome fractions from rat brain were immunoprecipitated with polyclonal antibodies against endophilin or amphiphysin or with preimmune sera from the rabbits used for the production of these antibodies. Precipitated proteins were separated on SDS-PAGE along with an aliquot of the synaptosome fraction (starting material (*SM*)), transferred to nitrocellulose, and immunoblotted with antibodies against endophilin, synaptojanin, dynamin, and amphiphysin I and II. The endophilin and amphiphysin antibodies were revealed with a 4-chloro-1-naphthol-based color development as described previously (31), and the dynamin and synaptojanin antibodies were revealed with ECL (Amersham Corp.).

pate in the targeting of synaptojanin. Once at clathrin-coated pits, synaptojanin, through its 5-phosphatase activity (21, 22), might function in regulating endocytosis by decreasing the local concentrations of inositol phosphates, which inhibit AP2- and AP3-mediated clathrin assembly (39, 40). Alternatively, synaptojanin could function in the regulation of phospholipase D activity and the formation of phosphatidic acid (41), or it could play a role in endocytosis through rearrangement of actin filaments (42). Less is known about the possible roles of the

endophilin/synaptojanin complex. This complex might serve to regulate the amount of synaptojanin at the sites of endocytosis by limiting the pool of synaptojanin available for interaction with the amphiphysin isoforms. Alternatively, endophilin might not simply buffer excess synaptojanin but rather actively target it to other sites within the neuron, possibly through its central domain, which has a high probability of participating in coiled-coil interactions. Synaptojanin, dynamin, and amphiphysin I are known to undergo dephosphorylation upon nerve terminal depolarization (20, 43–45), and this may account for an activity-dependent regulation of the interactions within and between the two endocytic complexes. Whatever the mechanism, the present results provide strong evidence for the involvement of endophilin in synaptic vesicle endocytosis.

REFERENCES

1. De Camilli, P., Takei, K., and McPherson, P. S. (1995) *Curr. Opin. Neurobiol.* **5,** 559–565
2. Chen, M. S., Obar, R. A., Schroeder, C. C., Austin, T. W., Poodry, C. A., Wadsworth, S. C., and Vallee, R. B. (1991) *Nature* **351,** 583–586
3. van der Bliek, A. M., and Meyerowitz, E. M. (1991) *Nature* **351,** 411–414
4. Herskovits, J. S., Burgess, C. C., Obar, R. A., and Vallee, R. B. (1993) *J. Cell Biol.* **122,** 565–578
5. 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
6. Damke, H., Baba, T., Warnock, D. E., and Schmid, S. L. (1994) *J. Cell Biol.* **127,** 915–934
7. Gout, I., Dhand, R., Hiles, I. D., Fry, M. J., Panayotou, G., Das, P., Truong, O., Totty, N. F., Hsuan, J., Booker, G. W., Campbell, I. D., and Waterfield, M. D. (1993) *Cell* **75,** 25–36
8. David, C., McPherson, P. S., Mundigl, O., and De Camilli, P. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 331–335
9. Grabs, D., Slepnev, V. I., Songyang, Z., David, C., Lynch, M., Cantley, L. C., and De Camilli, P. (1997) *J. Biol. Chem.* **272,** 13419–13425
10. David, C., Solimena, M., and De Camilli, P. (1994) *FEBS Lett.* **351,** 73–79
11. Munn, A. L., Stevenson, B. J., Geli, M. I., and Riezman, H. (1995) *Mol. Biol. Cell* **6,** 1721–1742
12. Shpetner, H. S., Herskovits, J. S., and Vallee, R. B. (1996) *J. Biol. Chem.* **271,** 13–16
13. Shupliakov, O., Löw, P., Grabs, D., Gad, H., Chen, H., David, C., Takei, K., De Camilli, P., and Brodin, L. (1997) *Science* **276,** 259–263

14. Wang, L.-H., Südhof, T. C., and Anderson, R. G. W. (1995) *J. Biol. Chem.* **270,** 10079–10083
15. Leprince, C., Romero, F., Cussac, D., Vayssiere, B., Berger, R., Tavitian, A., and Camois, J. H. (1997) *J. Biol. Chem.* **272,** 15101–15105
16. Butler, M. H., David, C., Ochoa, G.-C., Freyberg, Z., Daniell, L., Grabs, D., Cremona, O., and De Camilli, P. (1997) *J. Cell Biol.* **137,** 1355–1367
17. Ramjaun, A. R., Micheva, K. D., Bouchelet, I., and McPherson, P. S. (1997) *J. Biol. Chem.* **272,** 16700–16706
18. Tsutsui, K., Maeda, Y., Tsutsui, K., Seki, S., and Tokunaga, A. (1997) *Biochem. Biophys. Res. Commun.* **236,** 178–183
19. McPherson, P. S., Czernic, A. J., Chilcote, T. J., Onofri, F., Benfenati, F., Greengard, P., Schlessinger, J., and De Camilli, P. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 6486–6490
20. McPherson, P. S., Takei, K., Schmid, S. L., and De Camilli, P. (1994) *J. Biol. Chem.* **269,** 30132–30139
21. McPherson, P. S., Garcia, E. P., Slepnev, V. I., David, C., Zhang, X., Grabs, D., Sossin, W. S., Bauerfeind, R., Nemoto, Y., and De Camilli, P. (1996) *Nature* **379,** 353–357
22. Woscholski, R., Finan, P. M., Radley, E., Totty, N. F., Sterling, A. E., Hsuan, J. J., Waterfield, M. D., and Parker, P. J. (1997) *J. Biol. Chem.* **272,** 9625–9628
23. De Camilli, P., Emr, S. D., McPherson, P. S., and Novick, P. (1996) *Science* **271,** 1533–1539
24. Kearns, B. G., McGee, T. P., Mayinger, P., Gedvilaite, A., Phillips, S. E., Kagiwada, S., and Bankaitis, V. A. (1997) *Nature* **387,** 101–105
25. Sparks, A. B., Hoffman, N. G., McConnell, S. J., Fowlkes, D. M., and Kay, B. K. (1996) *Nature Biotechnol.* **14,** 741–744
26. de Heuvel, E., Bell, A. W., Ramjaun, A. R., Wong, K., Sossin, W. S., and McPherson, P. S. (1997) *J. Biol. Chem.* **272,** 8710–8716
27. Schneider, W. J., Slaughter, C. J., Goldstein, J. L., Anderson, R. G. W., Capra, J. D., and Brown, M. S. (1983) *J. Cell Biol.* **97,** 1635–1640
28. Sharp, A. H., McPherson, P. S., Dawson, T. M., Aoki, C., Campbell, K. P., and Snyder, S. H. (1993) *J. Neurosci.* **13,** 3051–3063
29. De Camilli, P., Harris, J. S. M., Huttner, W. B., and Greengard, P. (1983) *J. Cell Biol.* **96,** 1355–1373
30. Jahn, R., Schiebler, W., Ouimet, C., and Greengard, P. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 4137–4141
31. McPherson, P. S., and Campbell, K. P. (1990) *J. Biol. Chem.* **265,** 18454–18460
32. Warnock, D. E., Hinshaw, J. E., and Schmid, S. L. (1996) *J. Biol. Chem.* **271,** 22310–22314
33. Huttner, W. B., Schiebler, W., Greengard, P., and De Camilli, P. (1983) *J. Cell Biol.* **96,** 1374–1388
34. Ramjaun, A. R., and McPherson, P. S. (1996) *J. Biol. Chem.* **271,** 24856–24861
35. Jung, M. W., and McNaughton, B. L. (1993) *Hippocampus* **3,** 165–182
36. Blackstad, T. W., and Kjaerheim, A. (1961) *J. Comp. Neurol.* **117,** 113–159
37. Okamoto, P. M., Herskovits, J. S., and Vallee, R. B. (1997) *J. Biol. Chem.* **272,** 11629–11635
38. Sparks, A. B., Rider, J. E., Hoffman, N. G., Fowlkes, D. M., Quillam, L. A., and Kay, B. K. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 1540–1544
39. Gaidarov, I., Chen, Q., Falck, J. R., Reddy, K. K., and Keen, J. H. (1996) *J. Biol. Chem.* **271,** 20922–20929
40. Hao, W., Tan, Z., Prasad, K., Reddy, K. K., Chen, J., Prestwich, G. D., Falck, J. R., Shears, S. B., and Lafer, E. M. (1997) *J. Biol. Chem.* **272,** 6393–6398
41. Chung, J.-K., Sekiya, F., Kang, H.-S., Lee, C., Han, J.-S., Kim, S. R., Bae, Y. S., Morris, A. J., and Rhee, S. G. (1997) *J. Biol. Chem.* **272,** 15980–15985
42. Sakisaka, T., Itoh, T., Miura, K., and Takenawa, T. (1997) *Mol. Cell Biol.* **17,** 3841–3849
43. Robinson, P. J., Haupttschein, R., Lovenburg, W., and Dunkley, P. R. (1987) *J. Neurochem.* **48,** 187–195
44. Robinson, P. J., Sontag, J.-M., Liu, J.-P., Fyske, M., Slaughter, C., McMahon, H., and Südhof, T. C. (1993) *Nature* **365,** 163–166
45. Micheva, K. D., Ramjaun, A. R., Kay, B. K., and McPherson, P. S. (1997) *FEBS Lett.* **414,** 308–312