Identification of the Major Synaptojanin-binding Proteins in Brain*

Elaine de Heuvel, Alexander W. Bell‡, Antoine R. Ramjaun, Kenny Wong, Wayne S. Sossin§, and Peter S. McPherson¶

From the Department of Neurology and Neurosurgery, Montreal Neurological Institute and the ¶Department of Anatomy and Cell Biology, McGill University, Montreal, Quebec H3A 2B4, Canada

Synaptojanin is a nerve-terminal enriched inositol 5-phosphatase thought to function in synaptic vesicle endocytosis, in part through interactions with the Src homology 3 domain of amphiphysin. We have used synaptojanin purified from SF9 cells after baculovirus mediated expression in overlay assays to identify two major synaptojanin-binding proteins in rat brain. The first, at 125 kDa, is amphiphysin. The second, at 40 kDa, is the major synaptojanin-binding protein detected, is highly enriched in brain, is concentrated in a soluble synaptic fraction, and co-immunoprecipitates with synaptojanin. The 40-kDa protein does not bind to a synaptojanin construct lacking the proline-rich C terminus, suggesting that its interaction with synaptojanin is mediated through an Src homology 3 domain. The 40-kDa synaptojanin-binding protein was partially purified from rat brain cytosol through a three-step procedure involving ammonium sulfate precipitation, sucrose density gradient centrifugation, and DEAE ion-exchange chromatography. Peptide sequence analysis identified the 40-kDa protein as SH3P4, a member of a novel family of Src homology 3 domain-containing proteins. These data suggest an important role for SH3P4 in synaptic vesicle endocytosis.
Synaptopin-binding Proteins

8711

synaptopin. Purification and peptide sequence analysis revealed the 40-kDa protein as SH3P3, a novel SH3 domain-containing protein that was identified from a mouse library screened with a Src SH3 ligand peptide (27). SH3P3, along with SH3P8 and SH3P13, define a family of similar proteins of unknown function (27). Our data strongly implicate SH3P3, and perhaps other family members, in synaptic vesicle endocytosis.

EXPERIMENTAL PROCEDURES

Synaptopin Baculovirus Transfer Vector Constructs—A BamHI-HindIII fragment from nucleotides 181 to 747, digested from a full-length synaptopin cDNA (clone 9) (6), was subcloned into the BamHI-HindIII sites of pBluBac 4 (Invitrogen) (generating clone 1-18). A 5′ fragment encoding the N-terminal of synaptopin in with a His tag was then generated by PCR with Vent Polymerase (New England Biolabs) using the forward oligonucleotide 5′-GCGGATCCATGCATCACCACCAT-CCACCCACCGCTTACGAAAGCTTTCG-3′, which encodes a BamHI site, and the reverse oligonucleotide 5′-ATGGGTTCCATCTATCAAAAGAA-3′ corresponding to nucleotide positions 2449–2552 of clone 1B7 (generating clone synaptopin). For the deletion construct, PCR was performed on clone 9 with Vent Polymerase using the forward primer 5′-GGTATGTCGGACGACTAC corresponding to nucleotide positions 3184–3195 spanning a HindIII site, and the reverse primer R2 5′-GCGGATCCCGAACAGCTTCTGGCGAGTACTCTGGTG-3′ corresponding to nucleotide positions 3307–3325 encoding a SalI site flanking a HindIII site. The PCR product was digested with SalI and subcloned into SalI-digested clone 9 generating clone Bluescript R-2, which was digested with HindIII and the liberated fragment cloned into clone 1B7 generating clone synaptopin R-2. At each step for both constructs, the junctions of the clones were confirmed by sequence analysis.

Expression and Purification of Synaptopin Baculovirus Constructs—Spodoptera frugiperda (Sf9; Invitrogen) cells were grown at 27 °C in suspension cultures in SF-900 II SFM optimized serum-free medium (Life Technologies, Inc.) supplemented with gentamycin. The baculovirus transfer vectors were co-transfected with linear baculovirus into Sf9 cells, and recombinant baculovirus was selected by plaque assay as described (26). Positive colonies were confirmed by Southern blot purification and Western blot, and high titer stocks (10^8–10^9 plaque forming units/ml) were generated as described (28). For purification of synaptopin constructs, 200-ml cultures of Sf9 cells (1.5 × 10^7 cells/ml) were infected with 1 × 10^8 plaque forming units/ml of recombinant baculovirus R-2 (27). Freshly prepared 100 mM CNBr cleavage solution (30% trifluoroacetic acid, 0.83% benzamidine, 0.23% phenylmethylsulfonyl fluoride, 0.5 μg/ml aprotinin, 0.5 μg/ml leupeptin, 20 mM HEPES-OH, pH 7.4) was added to the cell suspension, followed by centrifugation for 5 min at 800 × g_{max}. The supernatant fractions were separated on SDS-PAGE on 5–16% or 3–12% gradient gels. Subcellular fractionation of brain homogenates to generate synaptic fractions was performed as described (5).

Immunoprecipitation Analysis—Amphiphysin immunoprecipitations were performed as described (14). For synaptopin immunoprecipitations, a rat brain was homogenized at 1:10 (w/v) in buffer D (0.3 M sucrose, 0.83 mM benzamidine, 0.23 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml aprotinin, 0.5 μg/ml leupeptin, 10 mM HEPES-OH, pH 7.4) with a polycarbonate glass Teflon homogenizer, followed by centrifugation for 5 min at 800 × g_{max}. The supernatant fractions were separated on SDS-PAGE on 5–16% or 3–12% gradient gels. Subcellular fractionation of brain homogenates to generate synaptic fractions was performed as described (5).

Preparation of Membrane Fractions—Various tissues were dissected from adult male rats and were homogenized at 1:10 (w/v) in buffer C (0.83 mM benzamidine, 0.23 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml aprotinin, 0.5 μg/ml leupeptin, 20 mM HEPES-OH, pH 7.4) with a polycarbonate glass Teflon homogenizer, followed by centrifugation for 5 min at 800 × g_{max}. The supernatant fractions were separated on SDS-PAGE on 5–16% or 3–12% gradient gels. Subcellular fractionation of brain homogenates to generate synaptic fractions was performed as described (5).

Purification and Identification of the 40-kDa Synaptopin-binding Protein—Adult rat brain synaptosomal membranes were homogenized at 1:10 (w/v) in buffer C with a polycarbonate, and the extracts were centrifuged at 180,000 × g_{max} for 1 h. Ammonium sulfate powder was added slowly to the soluble supernatant with stirring until 20% saturation. After 45 min on ice, the sample was centrifuged at 2700 × g_{max} for 30 min, and the supernatant was removed and precipitated with ammonium sulfate to 40% saturation. The 20–40% ammonium sulfate precipitate was resuspended in 16 ml of buffer C and was loaded on four 40-ml 2.5–15% linear sucrose gradients prepared in buffer C. The gradients were centrifuged in a Beckman VTI 50 rotor for 6 h at 45,000 rpm with slow acceleration and no brake. Gradient fractions (20 × 2 ml) were analyzed by synaptopin overlay assay, and peak 40-kDa synaptopin-binding protein fractions were pooled and passed over a 5-ml column of DEAE-Sephasel (Pharmacia Biotech Inc.) equilibrated in buffer C. Samples were recirculated at the column at a flow rate of 0.2 ml/min for 16 h, and the column was then eluted into 20 4-ml fractions at 2 ml/min with an 80-ml linear gradient of 0–0.5 M NaCl prepared in buffer C. Eluted fractions (80 μl/fraction) were analyzed for the 40-kDa synaptopin-binding protein by overlay assay. Alternately, proteins from eluted fractions (1 ml/fraction) were precipitated with 75% (v/v) cold trichloroacetic acid with 0.03% sodium deoxycholate as a carrier and analyzed by Coomassie Blue staining of protein gels. The peak 40-kDa synaptopin-binding protein fraction was concentrated, run on SDS-PAGE, and transferred to PVDF membranes. The 40-kDa protein was excised and subjected to Edman degradation but was found to have a blocked N terminus. Therefore, the sample was treated with cyanogen bromide (CNBr) to affect peptide bond cleavage at the C-terminal side of histidine residues. CNBr cleavage solution (70 μg CNBr/ml in 70% formic acid), washed with argon, sealed, and left 18 h at room temperature. The cleavage mixture was then dried in a speed vacuum centrifuge, and the PVDF pieces were subjected to sequence analysis in an Applied Biosystems model 470A protein sequencing equipped with an on-line Applied Biosystems model 120A phenylthiodyantoin analyzer (31) according to procedures as recommended by the manufacturer. Sequence analysis revealed multiple sequencing signals which were manually analyzed by overlaying successive high pressure liquid chromatography traces. The strengths of the multiple signals were ranked, and probable sequences were searched against protein data bases employing the Blastp algorithm.

Antibodies—A polyclonal anti-synaptopin antibody (1852) was prepared by injection of a rabbit with 200 μg of synaptopin R-2 deletion construct in Titer-Max® adjuvant (CytRx Corporation) using standard protocols. Serum was tested for immunoreactivity by Western blot against brain extracts and purified synaptopin. Antibodies were affinity purified from serum against purified synaptopin on PVDF membranes as described (5). A polyclonal antibody against synaptopin R-2 deletion construct was affinity purified from rat brain extracts and purified synaptopin as described (5). Polyclonal antibodies against amphiphysin were prepared as described (15) and were a generous gift of Drs. Carol David and Pietro De Camilli (Yale University). The monoclonal antibody against synaptopin was raised against a glutathione S-transferase fusion protein encoded amino acids 1156–1286 of synaptopin in the laboratory of Dr. Pietro De Camilli and was a generous gift of Drs. Amy Hudson and Pietro De
Expression and Purification of Synaptojanin Constructs in Sf9 Cells—To study the SH3 domain-binding properties of synaptojanin, we generated full-length synaptojanin and a synaptojanin deletion construct (synaptojanin R-2) lacking the proline-rich C terminus for expression in Sf9 cells using baculovirus. The constructs had six histidine residues introduced at the N terminus to allow for their purification with nickel-agarose. Approximately 4 μg of protein from each sample were separated on SDS-PAGE and stained with Coomassie Blue (Coomassie) or were transferred to nitrocellulose and blotted with a polyclonal antibody against synaptojanin purified from rat brain (5) (Milo Western) or with a polyclonal antibody raised against synaptojanin R-2 (1852 Western).

RESULTS

Amphiphysin Overlay of Synaptojanin and Dynamin—Purified synaptojanin and purified dynamin (500–20 ng as indicated) were separated on SDS-PAGE, transferred to nitrocellulose, and overlaid with glutathione S-transferase/amphiphysin SH3 domain (amphiphysin overlay) as described (4). Transfers were stained with ponceau S to ensure even electrophoretic transfer. The arrows on the right indicate the migratory position of the proteins detected on the blots.

Synaptojanin Overlay of a Rat Brain Extract—Rat brain post-nuclear supernatant fractions were separated on SDS-PAGE, transferred to nitrocellulose, and overlaid with synaptojanin (synaptojanin overlay) or with protein purified from mock infected Sf9 cells (control overlay). The symbols on the right denote the migratory positions of the two major (stars) and two minor (arrowheads) synaptojanin-binding proteins detected on the blot. The diamond denotes the migratory position of synaptojanin, which is also detected in this assay.

Identification of a Major Synaptojanin-binding Protein as Amphiphysin—Based on previous results (6, 14), we predicted that amphiphysin would be detected in the synaptojanin overlay assay. One of the major synaptojanin-binding proteins migrates at approximately 125 kDa, consistent with the molecular mass of amphiphysin (13, 14). In fact, amphiphysin and the 125-kDa synaptojanin-binding protein have an identical mobility on SDS-PAGE (Fig. 4A). To confirm the identity of this protein, we performed an immunoprecipitation assay using two different amphiphysin antibodies (CD5 and CD6). As seen in Fig. 4B, and in agreement with previous data (14), both amphiphysin antibodies immunoprecipitate amphiphysin from a rat brain extract, although CD5 is much more effective than CD6. A synaptojanin overlay assay of the amphiphysin immunoprecipitates demonstrates that the 125-kDa synaptojanin-

FIG. 1. Purification of baculovirus expressed synaptojanin constructs. Sf9 cells were infected with baculovirus encoding full-length synaptojanin (synaptojanin) or a synaptojanin deletion construct lacking the proline-rich C terminus (synaptojanin R-2), and the synaptojanin proteins were purified with nickel-agarose. Approximately 4 μg of protein from each sample were separated on SDS-PAGE and stained with Coomassie Blue (Coomassie) or were transferred to nitrocellulose and blotted with a polyclonal antibody against synaptojanin purified from rat brain (5) (Milo Western) or with a polyclonal antibody raised against synaptojanin R-2 (1852 Western).

FIG. 2. Amphiphysin overlay of synaptojanin and dynamin. Purified synaptojanin and purified dynamin (500–20 ng as indicated) were separated on SDS-PAGE, transferred to nitrocellulose, and overlaid with glutathione S-transferase/amphiphysin SH3 domain (amphiphysin overlay) as described (4). Transfers were stained with ponceau S to ensure even electrophoretic transfer. The arrows on the right indicate the migratory position of the proteins detected on the blots.

FIG. 3. Synaptojanin overlay of a rat brain extract. Rat brain post-nuclear supernatant fractions were separated on SDS-PAGE, transferred to nitrocellulose, and overlaid with synaptojanin (synaptojanin overlay) or with protein purified from mock infected Sf9 cells (control overlay). The symbols on the right denote the migratory positions of the two major (stars) and two minor (arrowheads) synaptojanin-binding proteins detected on the blot. The diamond denotes the migratory position of synaptojanin, which is also detected in this assay.

with the polyclonal antiserum raised against full-length synaptojanin (5) (Milo Western, Fig. 1), indicating that the antibodies are directed entirely against the last 231 amino acids of the proline-rich C terminus of synaptojanin. However, a rabbit antiserum raised against synaptojanin R-2 reacted strongly with both synaptojanin constructs (1852 Western, Fig. 1).

To further characterize the baculovirus expressed synaptojanin, various dilutions of dynamin and synaptojanin, both purified from baculovirus infected Sf9 cells, were run on SDS-PAGE and overlaid (4) with a glutathione S-transferase/fusion protein encoding the SH3 domain of amphiphysin. The synaptojanin construct strongly binds the SH3 domain of amphiphysin in this assay. Interestingly, when equal amounts of the two proteins are compared directly, amphiphysin demonstrates a greater relative affinity for synaptojanin than dynamin (Fig. 2).
A protein at approximately 40 kDa is the strongest synaptojanin-binding protein in brain (Figs. 3 and 4A). As determined by overlay, the 40-kDa protein is enriched in brain, although it is also detected in extracts from rat testis (Fig. 5A). Synaptojanin is also enriched in adult brain (Fig. 5A) although lower levels are seen in a wide variety of tissues (12). Synaptojanin, which is concentrated in presynaptic nerve terminals (5), is enriched in synaptic membrane fractions (Fig. 5B, LP2). As determined by overlay, the 40-kDa synaptojanin-binding protein is enriched in soluble fractions and is concentrated in the LS2 fraction that corresponds to cytosol isolated from lysed synaptosomes (Fig. 5B).

Co-immunoprecipitation of Synaptojanin and the 40-kDa Synaptojanin-binding Protein—We used a monoclonal antibody against synaptojanin to immunoprecipitate the protein from soluble fractions of rat brain. Synaptojanin is enriched in the precipitated material (Fig. 6). The 125-kDa synaptojanin-binding protein, which we identified as amphiphysin, does not co-immunoprecipitate with synaptojanin (Fig. 6; see “Discussion”). However, the 40-kDa synaptojanin-binding protein does co-immunoprecipitate with synaptojanin and is enriched in the synaptojanin immunoprecipitate as compared with the starting material (Fig. 6). These data confirm the interaction between synaptojanin and the 40-kDa synaptojanin-binding protein in the brain.

Identification of the 40-kDa Synaptojanin-binding Protein—Rat brain cytosol was fractionated using various concentrations of ammonium sulfate, and the 40-kDa synaptojanin-binding protein was found exclusively in the 20–40% ammonium sulfate precipitate (data not shown). This fraction was then subjected to size fractionation on 2.5–15% linear sucrose density gradients, and the 40-kDa protein was found in a narrow peak near the top of the gradient (data not shown). Peak

**FIG. 4.** Identification of a major synaptojanin-binding protein as amphiphysin. A, rat brain post-nuclear fractions were separated on SDS-PAGE, transferred to nitrocellulose, and overlaid with synaptojanin (synaptojanin overlay) or were blotted with a polyclonal antibody against amphiphysin (amphiphysin Western). B, a rat brain Triton X-100 soluble extract was subject to immunoprecipitation with two different polyclonal antibodies against amphiphysin (CD5 and CD6) or with control normal rabbit serum (NRS) conjugated to protein A-Sepharose. Precipitated proteins were separated on SDS-PAGE along with an aliquot of the soluble extract (starting material, SM), transferred to nitrocellulose, and subjected to an amphiphysin Western blot (amphiphysin Western; top panel) or a synaptojanin overlay (bottom panel). The arrows on the right indicate the migratory position of amphiphysin detected on the blots.

**FIG. 5.** Characterization of the 40-kDa synaptojanin-binding protein. A, post-nuclear tissue extracts from a variety of tissues as indicated were separated on SDS-PAGE, transferred to nitrocellulose, and were subjected to a synaptojanin Western blot (synaptojanin, top panel) or a synaptojanin overlay (bottom panel). The arrows on the right indicate the migratory positions of the proteins detected on the blots. B, proteins of brain subcellular fractions were separated by SDS-PAGE, transferred to nitrocellulose, and subjected to a synaptojanin Western blot (synaptojanin, top panel) or a synaptojanin overlay (bottom panel). Subcellular fractions were prepared as described (5). H, homogenate; P, pellet; S, supernatant; LP, lysed pellet; LS, lysed supernatant; CPG, controlled pore glass. The eluted fractions from the CPG column were pooled into three fractions with purified synaptic vesicles in CPG-3. The arrows on the right indicate the molecular masses of the proteins detected on the blots.

**FIG. 6.** Co-immunoprecipitation of synaptojanin and the 40-kDa synaptojanin-binding protein. A soluble fraction from rat brain was subjected to immunoprecipitation with a monoclonal antibody against p75 cAMP (anti-p75) or a monoclonal antibody against synaptojanin (anti-synaptojanin). Precipitated proteins were separated on SDS-PAGE along with an aliquot of the soluble extract (starting material, SM), transferred to nitrocellulose and subjected to a synaptojanin Western blot (top panel) or a synaptojanin overlay (middle and bottom panels). The arrows on the right indicate the molecular masses of the proteins detected on the blots.
sucrose density gradient fractions were pooled and subjected to anion exchange chromatography on DEAE-Sephacel. The column was eluted with a linear gradient of NaCl from 0 to 0.5 M. A Coomassie Blue-stained gel of the proteins eluted from the DEAE column is shown in Fig. 7A. A band at 40-kDa was apparent that was strongly reactive in the synaptojanin overlay assay (Fig. 7A, synaptojanin overlay).

To further characterize the 40-kDa protein, partially purified samples were overlaid with synaptojanin or synaptojanin R-2 deletion mutant (Fig. 7B) using the antiserum that recognizes the N-terminal domain of synaptojanin (1852 Western, Fig. 1). Synaptojanin, but not synaptojanin R-2, binds to the 40-kDa synaptojanin-binding protein. This demonstrates that the interaction of the 40-kDa protein with synaptojanin is mediated through synaptojanin's proline-rich C terminus and suggests that the 40-kDa protein contains an SH3 domain.

To identify the 40-kDa protein, fraction 14 from the DEAE column elution (Fig. 7A) was concentrated and transferred to PVDF membranes, and the 40-kDa protein band was subjected to peptide sequence analysis. The sample was refractive to automated Edman degradation, suggesting a blocked N terminus. Therefore, the sample was cleaved at methionyl residues with CNBr and resubjected to sequence analysis. The mixture resequencing revealed 2–3 major sets of sequencing signals. The strengths of the multiple signals were ranked, and the best guess sequence predicted from the major mixture sequencing data is shown in bold. The aligned sequences from SH3P4, SH3P8, and SH3P13 (27) are indicated and matches to the best guess sequence are in bold. The sequence of a second region of SH3P4, which was also identified in the sequencing mixture, is indicated, and amino acids that align with three of the five mismatches from the best guess sequence are in bold.

FIG. 7. Partial purification and identification of the 40-kDa synaptojanin-binding protein. The 40-kDa synaptojanin-binding protein was partially purified using a combination of ammonium sulfate precipitation, sucrose density gradient centrifugation, and DEAE anion-exchange chromatography. A, the top panel shows a Coomassie Blue-stained gel (Coomassie) of the proteins eluted from the DEAE column with a 0–0.5 M NaCl gradient. A distinct protein band at 40 kDa (arrow, top panel) binds synaptojanin by overlay assay (synaptojanin overlay, bottom panel). B, the partially purified 40-kDa synaptojanin-binding protein was overlaid with synaptojanin or with the synaptojanin construct lacking the proline-rich C terminus (synaptojanin R-2 overlay). C, the best guess sequence predicted from the major mixture sequencing data is shown in bold. The aligned sequences from SH3P4, SH3P8, and SH3P13 (27) are indicated and matches to the best guess sequence are in bold. The sequence of a second region of SH3P4, which was also identified in the sequencing mixture, is indicated, and amino acids that align with three of the five mismatches from the best guess sequence are in bold.
were then purified on nickel-agarose using a His6 tag engineered into the N terminus of the constructs. To characterize the baculovirus expressed synaptojanin, we compared the affinity of amphiphysin binding to synaptojanin versus dynam. When amphiphysin or Grb2 are used as substrates for the purification of SH3 domain-binding proteins from brain extracts, greater amounts of dynam than synaptojanin are isolated (5, 14), likely owing to higher levels of dynam expression in brain. However, as shown here, when equal amounts of purified dynam and synaptojanin are analyzed, amphiphysin shows stronger binding to synaptojanin than dynam. It has been proposed (14) that amphiphysin may serve to target dynam to sites of synaptic vesicle endocytosis via its dual interactions with AP2 (14, 19) and dynam. Amphiphysin may also play a role in targeting synaptojanin to endocytic sites. The higher affinity of synaptojanin than dynam for amphiphysin binding may be important to allow for synaptojanin targeting in the presence of high dynam concentrations in the nerve terminal.

We used synaptojanin purified from S9 cells in a gel overlay assay to identify two major synaptojanin-binding proteins with molecular masses of approximately 125 and 40 kDa. The 125-kDa synaptojanin-binding protein was identified as amphiphysin based on its co-migration with amphiphysin on SDS-PAGE and its precipitation with amphiphysin antibodies. The identification of amphiphysin as a major synaptojanin-binding protein strongly suggests that the assay is effective in identifying relevant synaptojanin-binding partners in vitro and further suggests that amphiphysin and the 40-kDa protein are the major synaptojanin-binding proteins in vivo.

Further characterization of the 40-kDa synaptojanin-binding protein demonstrates that it is highly concentrated in brain and is predominantly a soluble protein that is enriched in cytosol isolated from lysed synaptosomes. Proteins that function in clathrin-mediated endocytosis are often expressed at levels 10–50-fold higher in neuronal versus non-neuronal cells (33). For example, both dynam and synaptojanin are highly expressed in neurons, whereas these proteins or related isoforms are expressed at lower levels in non-neuronal cells (12, 34–36). The 40-kDa synaptojanin-binding protein is concentrated in brain but is also detected in testis, a tissue with little or no expression of the 145-kDa isoform of synaptojanin (12). However, the testis does express the 170-kDa synaptojanin isoform (12), and this protein also binds strongly to the 40-kDa synaptojanin-binding protein (data not shown). An important role for the 40-kDa synaptojanin-binding protein is also supported by the observation that it co-immunoprecipitates with synaptojanin from rat brain cytosol. This is in contrast to amphiphysin, which does not co-immunoprecipitate with synaptojanin (Fig. 6). The reason for the lack of amphiphysin/synaptojanin co-immunoprecipitation is unclear, but it may be due to a technical reason such as steric interference of the synaptojanin antibody with the site of amphiphysin binding.

A more interesting explanation may be that the binding of synaptojanin to the 40-kDa synaptojanin-binding protein excludes amphiphysin binding. Thus, it is possible that the 40-kDa synaptojanin-binding protein could regulate the ability of synaptojanin to bind to amphiphysin, and this could play a key role in regulating the targeting of synaptojanin to sites of endocytosis.

To identify the 40-kDa protein, we purified it from rat brain cytosol and subjected it to peptide sequence analysis. The sequence analysis identifies the 40-kDa synaptojanin-binding protein as SH3P4, a novel SH3 domain-containing protein with a predicted molecular mass of 39,880 Da (27). The identification of the 40-kDa synaptojanin-binding protein as an SH3 domain-containing protein is consistent with our observation that the 40-kDa protein does not bind to a synaptojanin deletion construct lacking the proline-rich C terminus. Further, the predicted isoelectric point of 5.3 for SH3P4 (27) is consistent with its elution from the DEAE ion exchange column in high salt. SH3P4, which was identified from a mouse library screened with a Src SH3 ligand peptide, is 75 and 63% identical to SH3P8 and SH3P13, respectively, two other proteins identified in the same screen (27). These three proteins define a novel protein family of unknown function. Our data strongly implicate SH3P4, and perhaps other family members, in synaptic vesicle endocytosis. It will be of interest to determine if the interaction of SH3P4 can regulate the ability of synaptojanin to bind to amphiphysin and thus regulate the targeting of synaptojanin to sites of endocytosis.

Acknowledgments—We thank Drs. Pietro De Camilli, Carol David, and Amy Hudson (Yale University), Dale Warnock and Dr. Sandra Schmid (Scripps University), Dr. Phil Barker (Montreal Neurological Institute), and Eric Shooter (Stanford University) for providing important reagents used in this study. We also thank WeiHua Lu and France Dumas for expert technical assistance and Drs. Pietro De Camilli, Sandra McPherson, Sandra Schmid, Phil Barker, and Philippe Seguela for support and discussion. Protein sequencing was performed at the Biotechnology Research Institute, Montreal.

REFERENCES

1. McPherson P. S., and De Camilli, P (1994) Semin. Neurosci. 6, 137–147
2. Baverfeind, R., David, C., Galli, T., McPherson, P. S., Takei, K., and De Camilli, P. (1995) Protein Kinesin: The Dynamics of Protein Trafficking and Stability. Vol. LX, pp. 387–404, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
3. Ryan, T. A., Smith, S. J., and Reuter, H. (1996) Proc. Natl. Acad. Sci. 93, 5567–5571
4. McPherson, P. S., Czernik, A. J., Chilcoat, T. J., Onodri, P., Benfenati, F., Greengard, P., Schlessinger, J., and De Camilli, P. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 6486–6490
5. McPherson P. S., Takei, K., Schmid, S. L., and De Camilli, P. (1994) J. Biol. Chem. 269, 30312–30319
6. McPherson, P. S., Garcia, E., Slepnev, V. I., David, C., Zhang, X., Grabs, D., Sosnin, W. S., Bauerfeind, R., Nemoto, Y., and De Camilli, P. (1996) Nature 379, 532–537
7. De Camilli, P., Emr, S. D., McPherson, P. S., and Novick, P. (1996) Science 271, 1533–1539
8. Novick, P., Osmond, B. C., and Botstein, D. (1989) Genetics 121, 659–674
9. Cleves, A. E., Novick, P. J., and Barkaitis, V. A. (1989) J. Cell Biol. 109, 2939–2950
10. Mayinger, P., Barkaitis, V. A., and Meyer, D. I. (1995) J. Cell Biol. 131, 1777–1786
11. Mayer, B. J., and Eck, M. J. (1995) Curr. Biol. 5, 364–367
12. Ramjaun, A. R., and McPherson, P. S. (1996) J. Biol. Chem. 271, 24856–24861
13. Lischte, V., Vah, R. W., Meyer, H. E., and Kiliann, M. W. (1992) EMBO J. 11, 2521–2530
14. David, C., McPherson, P. S., Mundigal, O., and De Camilli, P. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 331–335
15. David, C., Solimena, M., and De Camilli, P. (1994) FEBS Lett. 351, 73–79
16. Cruozza, M., Urbaci, M., Dulau, L., and Aigle, M (1991) J. Biol. Chem. 266, 727–743
17. Bauer, F., Urbaci, M., Aigle, M., and Cruozza, M. (1993) Mol. Cell. Biol. 13, 5070–5084
18. Munn, A. L., Stevenson, B. J., Gelli, M. I., and Riezman, H. (1995) J. Cell. Biol. 270,
Synaptojanin-binding Proteins

10079–10083
20. Robinson, M. S. (1994) Curr. Opin. Cell Biol. 6, 538–544
21. 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
22. van der Bliek, A. M., and Meyerowitz, E. M. (1991) Nature 351, 411–414
23. Kosaka, T., and Ikeda, K. (1983) J. Neurobiol. 14, 207–225
24. Takei, K., McPherson, P. S., Schmid, S. L., and De Camilli, P. (1995) Nature 374, 186–190
25. Hinshaw, J. E., and Schmid, S. L. (1995) Nature 374, 190–192
26. Wang, Z., and Moran, M. F. (1996) Science 272, 1935–1939
27. Sparks, A. B., Hoffman, N. G., McConnell, S. J., Fowlkes, D. M., and Kay, B. K. (1996) Nat. Biotech. 14, 741–744
28. Sossin, W. S., Fan, X., and Saberi, F. (1996) J. Neurosci. 16, 10–18
29. Cater, L. L., Redelmeier, T. E., Woolenweber, L. A., and Schmid, S. L. (1993) J. Cell Biol. 120, 37–45
30. Gross, E. (1967) Methods Enzymol. 11, 403–410
31. Hewick, R. M., Hunkapiller, M. W., Hood, L. E., and Dreyer, W. J. (1981) J. Biol. Chem. 256, 7990–7997
32. Chandler, C. E., Parsons, L. M., Hosang, M., and Shooter, E. M. (1984) J. Biol. Chem. 259, 6882–6889
33. Morris, S. A., and Schmid, S. L. (1995) Curr. Biol. 5, 1–3
34. Nakata, T., Takenura, R., and Hirokawa, N. (1993) J. Cell Sci. 105, 1–5
35. Sontag, J.-M., Fyske, E. M., Ushkaryov, Y., Liu, J.-P., Robinson, P. J., and Sudhof, T. C. (1994) J. Biol. Chem. 269, 4547–4554
36. Cook, T. A., Urrutia, R., and McNiven, M. A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 644–648