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The SH3p4/Sh3p8/SH3p13 protein family: Binding partners for synaptojanin and dynamin via a Grb2-like Src homology 3 domain

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ABSTRACT **The GTPase dynamin I and the inositol 5-phosphatase synaptojanin are nerve terminal proteins implicated in synaptic vesicle recycling. Both proteins contain COOH-terminal proline-rich domains that can interact with a variety of Src homology 3 (SH3) domains. A major physiological binding partner for dynamin I and synaptojanin in the nervous system is amphiphysin I, an SH3 domain-containing protein also concentrated in nerve terminals. We have used the proline-rich tail of synaptojanin to screen a rat brain library by the two-hybrid method to identify additional interacting partners of synaptojanin. Three related proteins containing SH3 domains that are closely related to the SH3 domains of Grb2 were isolated: SH3p4, SH3p8, and SH3p13. Further biochemical studies demonstrated that the SH3p4/8/13 proteins bind to both synaptojanin and dynamin I. The SH3p4/8/13 transcripts are differentially expressed in tissues: SH3p4 mRNA was detected only in brain, SH3p13 mRNA was present in brain and testis, and the SH3p8 transcript was detected at similar levels in multiple tissues. Members of the SH3p4/8/13 protein family were found to be concentrated in nerve terminals, and pools of synaptojanin and dynamin I were coprecipitated from brain extracts with antibodies recognizing SH3p4/8/13. These findings underscore the important role of SH3-mediated interactions in synaptic vesicle recycling.**

Many intracellular proteins contain proline-rich sequences that serve as binding sites for Src homology 3 (SH3) domains and other protein modules. These interactions often mediate the generation of dynamic macromolecular complexes such as those involved in signaling at the cell surface or the organization of cytoskeletal matrices (1). These interactions also are implicated in the function of the presynaptic nerve terminal, as three major nerve terminal proteins with a putative role in the trafficking of synaptic vesicles, synapsin I, dynamin I, and synaptojanin, contain proline-rich sequences that have been demonstrated to bind SH3 domains (2, 3). Synapsin I has been linked to the recruitment of synaptic vesicles to the active zone (4–6). Dynamin I is a GTPase that oligomerizes at the stalk of clathrin-coated vesicles budding from the plasmalemma and participates in the fission reaction required for synaptic vesicle recycling (7, 8). Synaptojanin is an inositol-5-phosphatase that is thought to act in concert with dynamin in the endocytic reaction (3, 9).

An SH3 domain-containing protein that has been strongly implicated in the synaptic vesicle cycle via its interactions with dynamin I and synaptojanin is amphiphysin I, a member of the Rvs/amphiphysin protein family (3, 10, 11). Disruption of interactions mediated by the SH3 domain of amphiphysin I in living nerve terminal preparations produces a strong inhibition of synaptic vesicle endocytosis at the stage of invaginated clathrin-coated pits, i.e., an inhibition of the fission reaction (12). To date, no other SH3 domain-containing protein other than amphiphysin I has been reported to be selectively concentrated in presynaptic nerve terminals. We now have performed a search for synaptojanin-interacting partners in rat brain and have isolated a family of three recently identified SH3 domain-containing proteins (13), SH3p4, SH3p8, and SH3p13 (collectively referred to as the SH3p4/8/13 protein family). We demonstrate that members of this family have differential tissue distributions and that in brain they are concentrated in nerve terminals where they interact selectively with synaptojanin and dynamin I.

MATERIALS AND METHODS

Yeast Two-Hybrid Screen. The yeast two-hybrid screen was performed using the protocol of Vojtek *et al.* (14). Briefly, a portion of the rat synaptojanin cDNA encoding amino acids 1,015–1,308 (the proline-rich tail of the 145-kDa isoform of synaptojanin) was subcloned into the pLexA vector, creating a fusion protein with the lexA DNA binding domain. This construct includes a 16-aa alternative splice found between amino acids 1,140 and 1,155. The yeast strain L40 (partial genotype *MAT***a** *trp1 leu2 his3 LYS2*::*lexA-HIS3 URA3*::*lexA-lacZ*) was transformed with this construct to create the strain L40:pLexAPro. Expression of the fusion protein was confirmed by Western blot of cell lysates. As a positive control, L40:pLexAPro was transformed with the SH3 domain of human amphiphysin I subcloned into the pACT vector, generating a fusion of the amphiphysin SH3 domain with the GAL4 activation domain. Double transformants were selected and screened for His^+ $lacZ^+$ phenotype. Activation of the reporter genes in double transformants demonstrated that the L40:pLexA strain was expressing a functional ligand for the amphiphysin SH3 domain. L40:pLexAPro was then transformed with a rat brain cDNA library maintained in the pGAD10 vector (CLONTECH). Of approximately 70×10^6 clones plated, 108 were His^+ and 30 demonstrated expression of the $lacZ^+$ reporter gene by a filter assay. These 30 clones represented 11 distinct cDNAs as determined by restriction analysis of the cDNA inserts. The clones isolated from the primary screen were screened a second time by a mating assay, allowing us to discard clones that activated reporter genes when paired with a pLexA:Lamin construct. Six distinct clones fulfilled the requirements for specificity of this assay. These cDNAs were sequenced entirely on both strands.

PCR Cloning of 5′ Regions of SH3p4 and SH3p13 cDNAs. Five micrograms of the rat brain cDNA library described above was used as a template to amplify the missing 5′ regions of the SH3p4 and the SH3p13 cDNAs. 5′ and 3′ primers were directed against the pGAD10 vector and the cDNA to be amplified, respectively. Standard PCR conditions were used with the exception that a 5:1 mixture of *Taq* (Promega) and PFU (Stratagene) polymerases was used. PCR products were subcloned into the pGex 4T2 vector and sequenced.

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Abbreviations: SH3, Src homology 3; GST, glutathione *S*-transferase.
Data deposition: The sequences in this paper have been deposited in the GenBank database (accession nos. AF009602, AF009603, and AF009604).

Northern Blot Analysis. Commercial multiple tissue Northern blots were purchased from CLONTECH and probed with dsDNA derived from clones isolated in the two-hybrid screen. Probes for SH3p4 and SH3p13 transcripts were PCR products of 530 and 1,300 nucleotides, respectively. The SH3p8 probe was an *Eco*RI fragment from the library plasmid that consists of the entire cDNA (see Fig. 1*A*). Fifty nanograms of gel-purified probe was labeled using a random primed labeling kit (Boehringer Mannheim), and blots were probed under standard high-stringency conditions (15) and exposed for 24 hr. Blots were dehybridized by boiling in 0.1× standard saline citrate for 5 min and reprobed after verification of dehybridization.

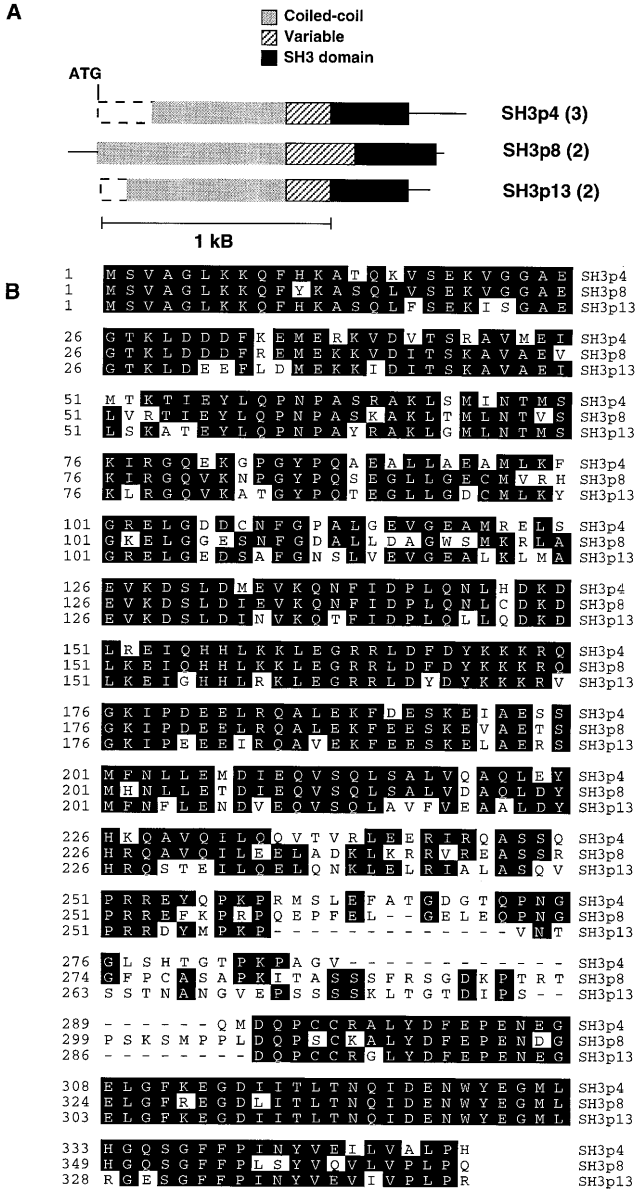

FIG. 1. Structure of the rat SH3p4, SH3p8, and SH3p13 proteins isolated by the two-hybrid screen. (*A*) Domain cartoon of the cDNAs isolated by the screen. Dashed boxes represent portions of the ORF that were not contained in the isolated cDNAs and subsequently were cloned by PCR. Thin lines represent untranslated regions. Numbers in parentheses refer to the number of clones isolated for each cDNA by the two-hybrid screen. (*B*) Sequence alignment of SH3p4, SH3p8, and SH3p13 proteins. The complete amino acid sequences of the three proteins was aligned by the clustal method using the Megalign module of the Lasergene package (DNAstar, Madison, WI). Regions of identity between the three proteins are outlined by black boxes. Note the high similarity among the three proteins, which is interrupted only by a highly variable amino acid stretch that separates the NH_2-terminal coiled-coil domain portion from the COOH-terminal SH3 domain.

Production of Recombinant Proteins. Portions of the coding regions of the SH3p4/8/13 proteins corresponding to the SH3 domains and variable regions of SH3p4, SH3p8, and SH3p13 were amplified by PCR using Vent polymerase (NEB, Beverly, MA). PCR fragments were digested by appropriate restriction enzymes and subcloned into the pGex4T series of vectors (Pharmacia) or the pET21a vector (Invitrogen) that expresses the recombinant protein with a polyhistidine tag. Constructs were verified by sequencing. The host strain BL21(DE3) was transformed with the resulting expression vectors, and the recombinant protein was purified on the appropriate affinity matrix using standard methods.

Affinity Chromatography. Triton X-100 extracted brain homogenate was prepared as follows. Male Sprague–Dawley rats were euthanized and decapitated. One brain was homogenized in 10 ml of homogenization buffer (20 mM Hepes, pH 7.4/300 mM sucrose/150 mM NaCl) to which a protease inhibitor cocktail was added (4 μg/ml each of pepstatin, aprotinin, leupeptin, and phenylmethylsulfonyl fluoride). Total homogenate was spun at 2,600 rpm for 10 min at 4°C in an SS-34 rotor (Beckman). Twenty percent Triton X-100 (wt/vol) was added to the supernatant to a final concentration of 1%. After a 30-min incubation at 4°C the sample was spun for 1 hr at 35,000 rpm at 4°C in a Ti-70 rotor (Beckman). The supernatant from the high-speed centrifugation was saved as brain extract.

Two hundred micrograms of recombinant glutathione *S*-transferase (GST) fusion proteins was incubated with glutathione-Sepharose beads for 2–3 hr and washed three times with PBS containing 1% Triton X-100 (wt/vol). The beads were equilibrated with homogenization buffer and then added to 10 ml of brain extract. After 3 hr of incubation at 4°C the beads were washed four times for 20 min in PBS/1% Triton X-100 (wt/vol). Proteins on the beads were eluted into SDS/PAGE sample buffer, separated by SDS/PAGE, and either visualized by Coomassie brilliant blue stain or processed for Western blotting.

Antibodies. Three pairs of rabbits were immunized with GST fusion proteins comprising COOH-terminal fragments of SH3p4 (97 amino acids), SH3p8 (113 amino acids), or SH3p13 (103 amino acids), respectively. Sera were affinity-purified on GST fusion proteins immobilized on a 1-ml HiTrap column (Pharmacia). Polyclonal antibodies raised against dynamin I (DG1) and glutamic acid decarboxylase were previously described (16, 17). A mAb raised against the proline-rich tail of synaptojanin (AC1) was a kind gift of Amy Hudson and Christof Haffner in our laboratory. An anti-synaptophysin mAb (C7.2) was a kind gift of R. Jahn (Yale University), and anti-dynamin mAb (HUDY-1) was purchased from Signal Transduction Laboratories (Lake Placid, New York).

Immunoprecipitation. One milliliter of the Triton X-100 brain extract used for affinity chromatography (see above) was precleared by incubation at 4°C for 2 hr with 10 μg of nonimmune rabbit IgGs followed by two sequential precipitations with 100 μl of protein A-Sepharose. Five micrograms of affinity-purified antibodies then were added to the precleared extract and incubated for 3 hr at 4°C. Immune complexes were precipitated with 50 μl of protein A Sepharose and eluted into 200 μl of SDS/PAGE sample buffer.

Miscellaneous Procedures. Cultured hippocampal neurons were prepared as previously described (18). Immunofluorescence of brain tissue and cultured hippocampal neurons was performed as described (18, 19). SDS/PAGE and Western blotting were performed according to the methods of Laemmli *et al.* (20) and Towbin *et al.* (21), respectively. Preparation of total brain homogenate and gel overlays with recombinant fusion proteins (far Western blots) were performed as previously reported (10).

RESULTS

Isolation of SH3p4/8/13 Proteins. A portion of the rat synaptojanin cDNA encoding the proline-rich tail of the 145-kDa isoform (amino acids 1,015–1,308) was used as the bait in a two-hybrid screen to identify interacting partners from a rat brain library. Both the bait construct and the brain library were transformed into the yeast strain L40, which contains two selectable markers for the two-hybrid interaction: the yeast *HIS3* and the bacterial *lacZ* genes. The screen led to the isolation of 11 clones encoding five distinct cDNAs, which activated both reporter genes in the presence of the bait construct and not in the presence of a control plasmid.

Sequencing revealed that four cDNAs encoded SH3 domain containing proteins. Three of the cDNAs encoded rat homologues of recently identified mouse proteins SH3p4, SH3p8, and SH3p13 (13). Comparison of these cDNAs with the sequence of the mouse homologues revealed that two identical clones contained the entire ORF of Sh3p8, including 3′ and 5′ untranslated regions (Fig. 1*A*). The clones corresponding to SH3p4 (two identical clones) and Sh3p13 (three identical clones) lacked the starting methionine but extended beyond the COOH terminus, which encodes the SH3 domain. The other SH3 domain containing protein isolated by the screen (two independent clones containing the COOH-terminal fragment of the molecule) was amphiphysin II. This protein interacts *in vitro* with synaptojanin and dynamin (22) and has been extensively characterized by our lab and others (13, 22, 23). Because SH3p4, SH3p8, and Sh3p13 were uncharacterized, we focused our further analysis on this protein family.

The missing 5′ regions of the ORFs of SH3p4 and SH3p13 were cloned by PCR (see *Materials and Methods*) and found to correspond to the sequences of the mouse homologues present in the database. The complete ORFs of rat SH3p4, SH3p8, and SH3p13 encode proteins of 352, 368, and 347 amino acids, respectively. An alignment of the three sequences (Fig. 1*B*) revealed that they are highly similar to each other (68% average identity) with two blocks of strong similarity corresponding to the NH_2-terminal two-thirds of the molecule, which is predicted to form a coiled-coil structure, and to the COOH-terminal SH3 domains. These two regions are linked by a sequence that is substantially divergent among family members. A high degree of identity is observed between rat and mouse proteins along their entire sequences (96% average identity). The three proteins do not contain hydrophobic stretches predicted to form transmembrane regions.

Binding of SH3p4/8/13 Proteins to Synaptojanin and to Dynamin I. To verify the interaction between the SH3p4/8/13 proteins and synaptojanin, the COOH-terminal moieties (variable region plus SH3 domain) of the SH3p4/8/13 proteins were expressed as GST fusion proteins and used to probe brain extract in an overlay (far Western blot) assay. As shown in Fig. 2*A* each of the SH3 domains binds primarily to bands with electrophoretic mobilities of ≈100 and 150 kDa. These bands also are recognized by the SH3 domain of amphiphysin I and previously have been identified as dynamin I and synaptojanin (10). Accordingly, bands of the same motility were recognized by anti-dynamin I and anti-synaptojanin antibodies (Fig. 2*B*). Note that in comparison to the SH3 domain of amphiphysin I these SH3 domains label synaptojanin relatively more intensely than dynamin I.

The fusion proteins comprising the COOH-terminal regions of SH3p4 and SH3p13 also were used to affinity-purify binding proteins from brain extract. As shown in Fig. 3 *A* and *B*, these fusion proteins specifically purified two bands that by Western blotting were shown to be dynamin I and synaptojanin, respectively (Fig. 3*C*).

Having established that the SH3p4/8/13 proteins bind to synaptojanin and dynamin I, we characterized the cellular and subcellular distribution of these proteins.

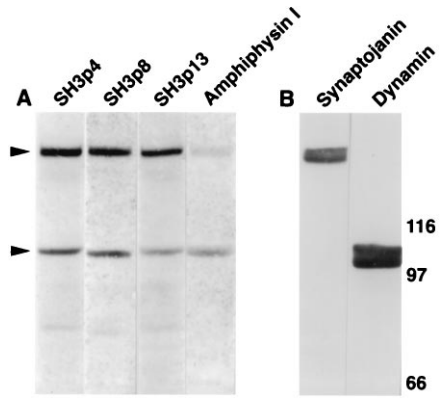

FIG. 2. Overlay assays of a total brain homogenate. (*A*) GST fusion proteins comprising either the COOH-terminal fragments of the SH3p4/8/13 proteins (including both the variable and the SH3 domains) or the SH3 domain of amphiphysin I were used to probe blots of a total brain homogenate by overlay (far Western blotting). (*B*) The same homogenate was probed by Western blotting with antibodies directed against synaptojanin and dynamin. Bands corresponding to synaptojanin and dynamin are indicated by arrowheads. The figure demonstrates the specific and direct interaction of the SH3p4/8/13 proteins with synaptojanin and dynamin I.

Tissue Distribution of SH3p4/8/13 Proteins. The pattern of expression of SH3p4, SH3p8, and SH3p13 mRNAs in different tissues was determined by Northern blot analysis (Fig. 4). The message for SH3p4 (single band around 3 kb) is expressed almost exclusively in brain, with extremely low levels detectable in kidney (Fig. 4*A*). The Sh3p8 message (single band at 2.4 kb) is found in all tissues (Fig. 4*B*). The message for SH3p13 (single band around 1.8 kb) is most abundant in testis, but also detected at lower levels in brain and liver (Fig. 4*C*).

Antibodies were raised against GST fusion proteins of C-terminal fragments of the SH3p proteins comprising the variable domains plus the SH3 domains. Two sera were used for further experiments due to their high titers and specificity for the

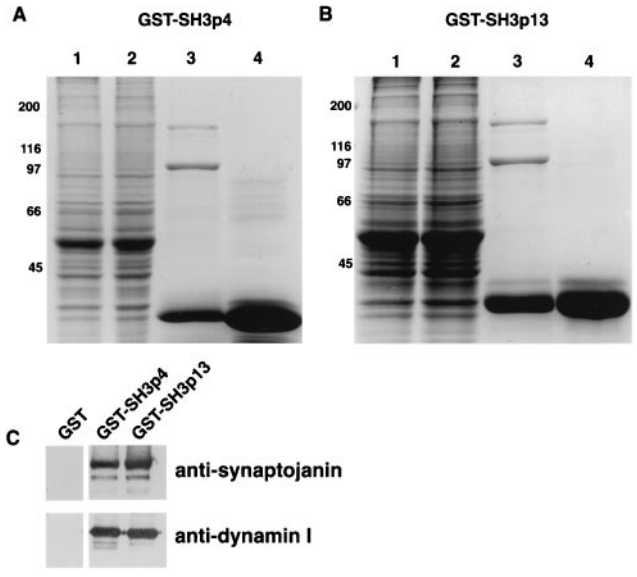

FIG. 3. Affinity purification of a brain extract onto immobilized SH3 domains of the SH3p4 and SH3p13 proteins. (*A*) Protein staining of the starting material (lane 1), flow through (lane 2), bound material (lane 3), and GST fusion protein alone (lane 4). An excess of control fusion proteins was loaded on the control lane. (*B*) Western blot analysis of material affinity-purified from the brain extract with the SH3 domains of the SH3p4 and SH3p13 proteins or with GST alone, demonstrating that the affinity-purified bands of 100 and 150 kDa correspond to dynamin I and synaptojanin, respectively.

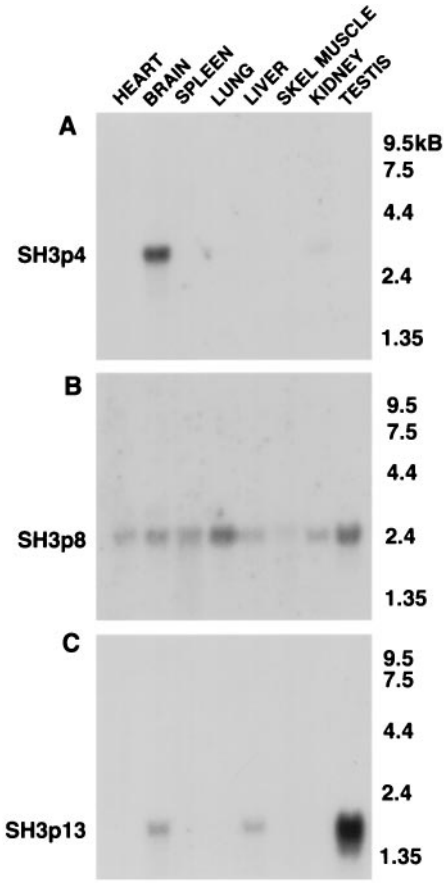

FIG. 4. Northern blot analysis of the pattern of expression of SH3p4/8/13 mRNAs in different tissues. Oligonucleotide probes derived from the cDNAs of SH3p4, SH3p8, and SH3p13 were used to probe a multiple tissue blot. Quantity and integrity of mRNA present on the filters was verified by probing for glyceraldehyde-3-phosphate dehydrogenase transcripts (data not shown).

SH3p4/8/13 proteins. Serum 1 that was raised against SH3p13 proved to be specific for SH3p13 whereas serum 2 that was raised against SH3p4 recognized all three SH3p4/8/13 proteins by Western blot (Fig. 5*A*). Antibodies affinity-purified from serum 1 recognized a single band of 40 kDa in brain and affinity-purified antibodies from serum 2 recognized a doublet of 40 and 45 kDa in brain (Fig. 5*B*). These electrophoretic mobilities agree with the predicted molecular masses of the SH3p4/8/13 proteins. Based on a comparison of data obtained with serum 1 and serum 2 as well as other sera (data not shown), the 40-kDa immunoreactivity corresponds to the SH3p4 and SH3p13 proteins whereas the 45-kDa immunoreactivity corresponds to SH3p8. In conclusion, all three proteins are expressed in brain although only SH3p4 seems to be brain specific.

Subcellular Localization of SH3p4/8/13 Proteins. Western blot analysis of primary subfractions of rat brain demonstrated that all three proteins were present in both soluble and particulate fractions. The overall distribution of these proteins during subcellular fractionation was similar to that of dynamin I and synaptojanin (data not shown). We next investigated by immunofluorescence whether the cellular and subcellular localization of the SH3p4/8/13 proteins in brain is consistent with an interaction *in situ* with synaptojanin and dynamin I.

Immunostaining of rat brain sections (Fig. 6) and cultured hippocampal neurons (Fig. 7) was performed using the antibodies affinity-purified from serum 1 and serum 2 (see Fig. 5 *A* and *B*). Both in brain sections and cultured neurons immunostaining was mainly concentrated in nerve terminals as demonstrated by a colocalization with immunoreactivity for synaptophysin, a marker

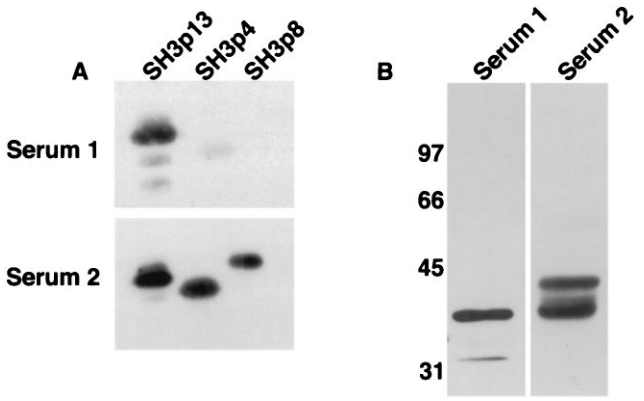

FIG. 5. Western blot analysis of SH3p4/8/13 proteins in brain extract. (*A*) Polyclonal rabbit antibodies raised against the COOH-terminal regions of SH3p4 (serum 1) and SH3p13 (serum 2), respectively were affinity-purified and used by Western blotting to label His-tagged COOH-terminal regions of the three SH3p proteins corresponding to the immunogens used to raise antisera. Serum 1 recognizes primarily SH3p13 whereas serum 2 recognizes intensely all three proteins. (*B*) The same antibodies were used to blot a total homogenate of rat brain. Serum 2 labels two bands, whereas serum 1 labels only the lower band. The electrophoretic motility of the two bands, 40 and 45 kDa, respectively, is in good agreement with the molecular masses of the SH3p4/8/13 proteins.

of presynaptic nerve terminals (24, 25). A diffuse cytosolic staining also was observed in both brain sections and cultured neurons, consistent with the existence of a soluble pool of the SH3p4/8/13 proteins. Generally the immunoreactivity for the SH3p4/8/13 proteins was highly overlapping with the immunoreactivity for dynamin I, synaptojanin, and amphiphysin I immunostaining.

Coprecipitation of Synaptojanin and Dynamin I with SH3p4/8/13 Proteins. To further confirm the association of the SH3p4/8/13 proteins with synaptojanin and dynamin I in the neuronal cytoplasm we investigated whether immunoprecipitation of the SH3p4/8/13 proteins from brain extract resulted in coprecipitation of these two proteins. As shown in Fig. 8, a Western blot analysis of the immunoprecipitates formed by antibodies affinity-purified from serum 2 demonstrated that both dynamin I and synaptojanin, but not a cytosolic control protein, glutamic acid decarboxylase, were present in a complex with the SH3p4/8/13 proteins. Note that the immunoprecipitate is relatively more enriched with synaptojanin in comparison to dynamin I consistent with the preferential binding of these proteins to synaptojanin (see above).

DISCUSSION

We report here the identification of three similar proteins, SH3p4, SH3p8, and SH3p13, as binding partners for synaptojanin and dynamin I. We identified these proteins as interacting partners of synaptojanin in a yeast two-hybrid screen and then confirmed the interaction of the SH3p4/8/13 proteins with synaptojanin by gel overlay and affinity chromatography. We also observed that these proteins bind to dynamin I. The results of the gel overlay assay demonstrate that the binding of the SH3p4/8/13 proteins to dynamin I and synaptojanin is specific and direct. SH3p4/8/13 immunoreactivity is concentrated in the nerve terminals where it is colocalized with dynamin I and synaptojanin immunoreactivities. Finally, we demonstrate that pools of dynamin I and synaptojanin are coprecipitated with SH3p4/8/13 proteins from brain extract. It therefore is likely that the SH3p4/8/13 proteins are physiological ligands of synaptojanin and dynamin I.

Given the large number of SH3 domain-containing proteins expressed in brain, the observation that the majority of the clones isolated by our screen encode SH3p4/8/13 proteins suggests that

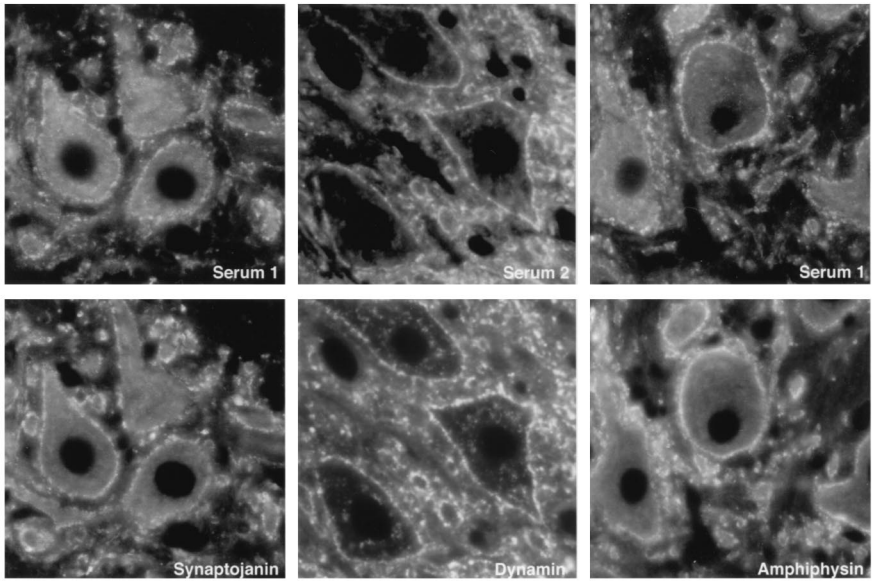

FIG. 6. Colocalization of SH3p4/8/13 immunoreactivity with dynamin I, synaptojanin, and amphiphysin I in nerve terminals in rat brain. Indirect immunofluorescence of frozen sections of rat brain stem using serum 1 and serum 2 and mAbs directed against dynamin I, synaptojanin, and amphiphysin I. In all cases immunoreactivity is represented by a punctate nerve terminal pattern, superimposed on a diffuse cytosolic staining. Slight differences between staining patterns (*Upper*) can be accounted for by variability within the neurons in the region of the brain examined.

they are preferred partners of synaptojanin. Accordingly, both in the overlay assay and in coprecipitation experiments these proteins demonstrate a preference for synaptojanin over dynamin I. As noted above, the SH3 domain of amphiphysin I recognizes dynamin I more than synaptojanin in gel overlay experiments (see Fig. 2). Therefore the SH3p4/8/13 proteins do not exactly recapitulate the protein interactions mediated by the SH3 domain of amphiphysin I. The identification of members of this protein family as interacting partners of synaptojanin is convergent with the recent independent identification of SH3p4 as a major synaptojanin binding protein in brain by an overlay assay using recombinant synaptojanin as the ligard (26).

The mouse homologues of the SH3p4/8/13 proteins were identified previously in a systematic screen for SH3 domain containing proteins (13). Comparison of the mouse sequences with the rat sequences reported here demonstrates a high degree of conservation between the species in each of the three main domains: the coiled-coil NH_2-terminal moiety, the COOH-terminal SH3 domain, and the variable linker region. The tripartite domain structure of SH3p4/8/13 is reminiscent of the domain structure of amphiphysin I and II, which possesses an evolutionarily conserved coiled-coil domain at its NH_2 terminus and a COOH-terminal SH3 domain (22, 27, 28). Despite this similarity, the primary sequence similarity between Rvs/amphiphysin family members and SH3p4/8/13 proteins is very low. In contrast, as shown in Fig. 9, the SH3 domains of these

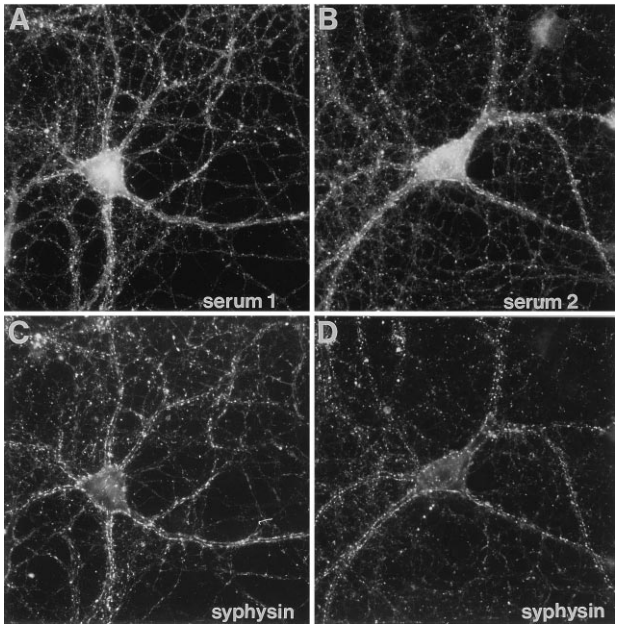

FIG. 7. Colocalization of SH3p4/8/13 immunoreactivity with the synaptic vesicle marker synaptophysin in cultured hippocampal neurons. Two-week-old cultures of hippocampal neurons were immunostained with serum 1 (*A*) and serum 2 (*B*) and counterstained with the anti-synaptophysin mAb C7.2 (*C* and *D*). SH3p4/8/13 and synaptophysin immunoreactivities colocalize in presynaptic nerve terminals visible as puncta surrounding the perikarya and processes of the cultured neurons.

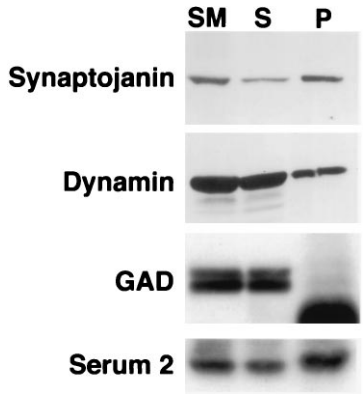

FIG. 8. Coimmunoprecipitation of dynamin and synaptojanin with SH3p4/8/13 proteins. Material immunoprecipitated using anti-SH3p4/8/13 antibodies (serum 2) from a brain extract was processed for Western blotting with antibodies directed against synaptojanin, dynamin, the SH3p4/8/13 proteins (serum 1), and, as a control, with antibodies directed against the nerve terminal protein glutamic acid decarboxylase (GAD). Starting material (SM), supernatant (S), and pellet (P) fractions are shown. Anti-dynamin and synaptojanin Western blots were processed using an alkaline phosphatase detection system while anti-glutamic acid decarboxylase and anti-SH3p4/8/13 immunoreactivity was detected by ECL.

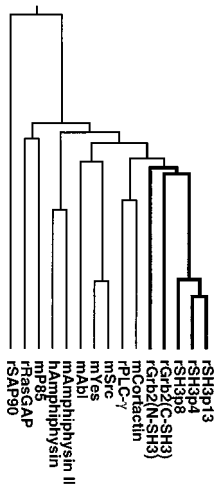

FIG. 9. The SH3 domains of the SH3p4/8/13 proteins are closely related to the SH3 domains of Grb2. SH3 domains of various proteins were aligned using the clustal method and displayed as a phylogenetic tree.

three proteins are closely related to the two SH3 domains of Grb2 (an average of 59% identity). We previously have shown that the three major Grb2 binding proteins in brain are synaptojanin, dynamin I, and synapsin I (2, 9), but have failed to demonstrate a significant concentration of Grb2 in nerve terminals. The SH3p4/8/13 proteins therefore may represent physiological ligands for Grb2 binding sites in synaptojanin and dynamin I. Although the experiments described here do not demonstrate any appreciable binding of the SH3p4/8/13 proteins to synapsin I, binding assays performed under lower stringency conditions do detect an interaction between the SH3 domains of the SH3p4/8/13 proteins and synapsin I (data not shown).

Based on mRNA levels, SH3p4 is predominantly expressed in brain, SH3p13 is expressed primarily in the testis, and SH3p8 has a widespread tissue distribution. All three proteins are expressed in brain. The patterns of expression of the SH3p4/8/13 mRNAs are reminiscent of the patterns of expression of the mRNAs for the three dynamin isoforms. Dynamin I is a brain-specific isoform, dynamin II is expressed in all tissues, and dynamin III is most abundant in testis but also expressed in brain and lung (reviewed in ref. 29). It is possible that SH3p4, Sh3p8, and Sh3p13 are preferred partners for dynamin I, II, and III, respectively.

The identification of the SH3p4/8/13 proteins as physiological binding protein for synaptojanin and dynamin I adds further support to the hypothesis that SH3-mediated interactions play important roles in the dynamics of synaptic vesicles (2, 9, 10). Recent studies suggest that one of the functions of these interactions is to recruit dynamin I, and possibly synaptojanin, to clathrin-coated vesicular buds (10, 12). Studies on the interacting partners of the SH3p4/8/13 proteins via regions distinct from their SH3 domains will help to determine whether the primary function of these proteins is to act as adaptors for dynamin, synaptojanin, and other effectors of membrane trafficking in the nerve terminal. The GTPase activity of dynamin is regulated by SH3 domain binding, raising the possibility that the SH3p4/8/13 proteins also may modulate the enzymatic activities of their ligands. It will be of interest to determine if the functions of the SH3p4/8/13 proteins overlap with the function of amphiphysin I or whether they play a distinct role in the presynaptic nerve terminal more directly linked to the function synaptojanin.

A growing number of proteins appear to play dual roles in cell signaling and endocytosis (3, 30–35). These include Grb2, a protein known to participate in signal transduction, which also may serve as an adaptor between activated receptor tyrosine kinases and components of the endocytic machinery (1, 33, 36). Members of the SH3p4/8/13 protein family are concentrated in a neuronal compartment specialized for vesicular transport and have partial similarity to Grb2. Elucidation of their functions in the nerve terminal and in nonneuronal cells may shed further light on the relationship between cell signaling and endocytosis.

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