Identification and Characterization of a Nerve Terminal-enriched Amphiphysin Isoform*

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Amphiphysin is a nerve terminal-enriched protein thought to function in synaptic vesicle endocytosis, in part through Src homology 3 (SH3) domain-mediated interactions with dynamin and synaptojanin. Here, we report the characterization of a novel amphiphysin isofrom (termed amphiphysin II) that was identified through a homologous search of the data base of expressed sequence tags. Antibodies specific to amphiphysin II recognize a 90-kDa protein on Western blot that is brain-specific and highly enriched in nerve terminals. Like amphiphysin (now referred to as amphiphysin I), amphiphysin II binds to dynamin and synaptojanin through its SH3 domain. Further, both proteins bind directly to clathrin in an SH3 domain-independent manner. Taken together, these data suggest that amphiphysin II may participate with amphiphysin I in the regulation of synaptic vesicle endocytosis.

Following their exocytosis at the nerve terminal, synaptic vesicle membranes are retrieved by internalization through clathrin-coated pits and vesicles (1). A number of proteins have been identified that are thought to be involved in this process, including dynamin, which is similar to the product of the Drosophila shibire gene (2, 3). A temperature-sensitive mutation in this gene leads to a block in endocytosis of synaptic vesicles (4), and recent data suggest that dynamin functions in the nerve terminal by mediating the fission of endocytic vesicles (5, 6). Another protein putatively involved in synaptic vesicle endocytosis is synaptojanin, which was identified based on its ability to bind to the Src homology 3 (SH3) domains of Grb2 (7). Synaptojanin is enriched in brain and is concentrated in the nerve terminal, where it precisely co-localizes with dynamin (8). Both dynamin and synaptojanin are associated with synaptojanin-positive membrane fractions on the endocytic limb of the synaptic vesicle cycle, and both proteins undergo dephosphorylation in response to synaptic vesicle mobilization (8). Cloning of synaptojanin revealed a domain homologous to a family of enzymes that dephosphorylate inositol phospholipids and inositol polyphosphates at the 5′-position of the inositol ring (5-phosphatases) as well as a proline-rich C terminus with consensus binding sites for SH3 domains (9). We have recently identified a major binding partner for synaptojanin as SH3P4 (10), a member of a novel family of SH3 domain-containing proteins (11).

Another protein with an important role in synaptic vesicle endocytosis is the SH3 domain-containing protein amphiphysin (12–14). Amphiphysin co-localizes with dynamin and synaptojanin in nerve terminals, where it interacts with proline-rich regions in each of these proteins through its SH3 domain (9, 10, 14). Amphiphysin has also been reported to bind to the clathrin assembly protein 2 (AP2), a component of the plasma membrane clathrin coat (14–16), and mutations in RVS 161 and RVS 167, the yeast homologues of amphiphysin (14, 17, 18), lead to defects in endocytosis (19).

Recently, two mammalian amphiphysin homologues have been identified, BIN1 (20) and SH3P9 (11). BIN1, which is localized to the nucleus, was identified as a Myc-binding protein (20). The Myc oncoprotein contains functionally critical N-terminal Myc box regions that have been implicated in cell growth, apoptosis, and malignancy (21, 22), and BIN1 binding to these domains inhibits malignant cell transformation by Myc (20). Little information is available on SH3P9, which was discovered along with SH3P4 in a library screen isolating SH3 domain-containing proteins (11). BIN1 and SH3P9, which are very similar in sequence, are homologous to amphiphysin at their N termini as well as at their C-terminal SH3 domains but do not contain a large central domain found in amphiphysin.

In this paper, we have characterized a third amphiphysin homologue, which appears to be an alternatively spliced product of the gene encoding BIN1 and SH3P9. This protein, which we refer to as amphiphysin II, contains a large central insert domain that is partially homologous to the central domain of amphiphysin (now referred to as amphiphysin I) but that is absent from BIN1 and SH3P9. We generated amphiphysin II-specific antibodies and determined that the protein is brain-specific and highly enriched in nerve terminals. Like amphiphysin I, amphiphysin II binds through its SH3 domain to dynamin and synaptojanin. Further, amphiphysin I and II both bind to clathrin through a region outside of their SH3 domains. Taken together, these data suggest that amphiphysin II may function in synaptic vesicle endocytosis in the nerve terminal.

EXPERIMENTAL PROCEDURES

Identification of Amphiphysin II—A search of the data base of expressed sequence tags (dbEST) with the sequence of human amphiphysin I (13) revealed several homologous human brain cDNAs. An I.M.A.G.E. consortium clone (I.M.A.G.E. consortium clone I.D. number 183571, GenBank™ number R12992) overlapping with human amphiphysin I from amino acid 169 to 695 was purchased and sequenced.
To obtain the amino terminus of amphiphysin II, we performed PCR on cDNA prepared from adult human cortex (23). The forward primer (5’-GGCGGATCCATGGGAGATGGGCCGATTAAG) corresponded to nucleotides 1–22 of the coding sequence of BIN1 (20) (from GenBank™ clone) and the reverse primer (5’-GCAAATTGCGACGAGTCCTGGCCTGCG) corresponded to nucleotides 1083–1066 of amphiphysin II in a region of the amphiphysin II-specific insert domain. A specific PCR product was subcloned, sequenced, and found to have a 100% overlap with 564 base pairs at the 5’-end of the I.M.A.G.E. consortium clone and to extend the clone by an additional 519 base pairs.

Southern Blot Analysis of Amphiphysin II—Human genomic DNA (8 μg/sample) was digested with BamHI or EcoRI and the HindIII-digested DNA was separated on an agarose gel and transferred to Hybond N+ membrane (Amersham Corp.). A cDNA probe from nucleotides 1396–1671 of amphiphysin II was prepared by PCR, labeled with [32P]dCTP by random priming, and hybridized to immobilized DNA under high stringency using standard protocols.

Production of Amphiphysin II-Specific Antibodies—To generate an amphiphysin II antibody, the I.M.A.G.E. consortium clone was used as a template in PCR reactions with Vent Polymerase (New England Biolabs) with the forward primer, 5’-GGCGGATCTCCCTACGTTGGAGGAGGCAGGCCCG (nucleotides 1035–1050 of amphiphysin II) and the reverse primer, 5’-GGCGGAATTCATGGCAGAGATGGGCAGTAAAG (forward primer, 5’-GGCGGAATTCACACAGCAAAGGTGCCTCG (nucleotides 1122–1140 of amphiphysin II) and the reverse primer, 5’-GGCGGAATTCTCATGTGACAGCCCTCCAC (nucleotides 981–998); reverse primer, 5’-GGCGGAATTCACACAGCAAAGGTGCCTCG (nucleotides 1145–1429). In all cases, the forward primer encoded a BamHI site, and the reverse primer encoded an EcoRI site. The primer pairs were used in PCR reactions with Vent Polymerase using cDNA clones encoding amphiphysin I (13) or amphiphysin II as the templates. All PCR products were cloned in frame into the BamHI/EcoRI sites of pGEX-2T, and GST fusion proteins were expressed and purified as described (8). GST-amphiphysin I SH3 domain was prepared as described previously (13). Rat brain postnuclear supernatants, prepared as described above, were incubated with 1% Triton X-100 for 1 h at 4 °C, and insoluble material was removed by centrifugation at 12,000 × g for 10 min. The soluble supernatant (1 mg of protein) was incubated overnight at 4 °C with 25 μl each of various amphiphysin fusion proteins coupled to glutathione-Sepharose. Samples were then washed extensively in 20 mM HEPES-PO4, 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, and bound proteins were eluted with SDS-PAGE sample buffer.

Purification of Clathrin—Clathrin-coated vesicles were purified from rat brain as described (24). Clathrin was extracted by incubating the purified vesicles with 0.5 mM Tris-Cl, pH 7.4, for 30 min at 4 °C followed by removal of insoluble material by centrifugation in a Beckman TLA 100.1 rotor at 50,000 rpm. for 30 min (25). The supernatant fraction was then incubated overnight at 4 °C with 25 μg each of various amphiphysin fusion proteins bound to glutathione-Sepharose. Samples were washed in 0.5 mM Tris, pH 7.4, and bound proteins were eluted with SDS-PAGE sample buffer.

Other Antibodies—Other antibodies used in this study were raised against the following proteins as described: amphiphysin I (13), a gift from Drs. Carol David and Pietro De Camilli, Yale University; synaptophin (26), a gift of Dr. Reinhard Jahn, Yale University; clathrin (27) and α-adaptin (28), gifts of Dr. Margaret Robinson, Cambridge University; synaptotagmin (8); and dynamin (8).

RESULTS

Amino Acid Sequence of Amphiphysin II—A partial amino acid sequence for amphiphysin II was determined from an I.M.A.G.E. consortium clone identified through an amphiphysin I homology search of the dbEST. Analysis of the protein suggested that it was an alternatively spliced version of the amphiphysin homologue BIN1 (20). To obtain the amino terminus of amphiphysin II, we performed PCR on cDNA prepared from adult human cortex (25) using a forward primer encoding the N terminus of BIN1 (from GenBank™ clone U68465) and a reverse primer in a region of the I.M.A.G.E. consortium clone unique to amphiphysin II. A specific PCR product was generated that overlapped the I.M.A.G.E. consortium clone by 188 amino acids and extended the amino terminus of amphiphysin II by 173 amino acids. The amino acid sequence of amphiphysin II is shown aligned to amphiphysin I, BIN1, and SH3P9 (Fig. 1A). Amphiphysin II, BIN1, and SH3P9 are essentially identical at their N termini and their SH3 domain-containing C termini. Minor differences between SH3P9 and the other two sequences are due to interspecies variations (20) (amphiphysin II and BIN1 are from humans, whereas SH3P9 is from mice). The central domain of amphiphysin II contains a 123-amino acid insert that is not found in SH3P9 or BIN1. The amphiphysin II insert domain is homologous to amphiphysin I through its N-terminal half, but the homology is decreased in the C terminus of the insert domain.
A 15-amino acid insert, which is unique to the BIN1 sequence, encodes a nuclear localization signal (Fig. 1A; Ref. 20). A 31-amino acid insert specific to amphiphysin II is located between amino acids 174 and 204. The pattern of homology seen for amphiphysin II with BIN1 and SH3P9, in which regions of 100% identity are interrupted by regions unique to each protein, suggests that the three proteins arise from alternative splicing of the same gene. To confirm this, we performed Southern blot analysis with human genomic DNA digested with BamHI or EcoRI and hybridized under high stringency with a cDNA probe encoding the SH3 domain of the amphiphysin II gene. The migratory positions of DNA markers are shown on the left.

**Detection of Amphiphysin II in Brain Homogenate**—To begin to characterize the amphiphysin II protein, a polyclonal antiserum (antiserum 1874) was produced against a GST fusion protein.
protein encoding the C-terminal 217 amino acids of amphiphysin II. To generate an antibody reactive with amphiphysin II but not BIN1 or SH3P9, the serum was affinity-purified against a fusion protein encoding a region of the amphiphysin II-specific insert domain (amino acids 376–444). When tested against strips of crude rat brain homogenate, the affinity-purified antibody was found to react strongly with bands at 90 and 125 kDa (Fig. 2). The band at 125 kDa co-migrated with amphiphysin I (CD5). The antibody was then incubated with a fusion protein against amphiphysin I to remove cross-reactive antibodies.

Tissue and Subcellular Distribution of Amphiphysin II—Western blot analysis of tissue extracts with the doubly affinity-purified amphiphysin II antibody revealed expression of amphiphysin II in brain only (Fig. 3A). In contrast, amphiphysin I, although enriched in brain, was detected in testis extracts as well (Fig. 3A). This is in contrast to BIN1, which is present in a wide variety of tissues and most highly expressed in skeletal muscle (20). Upon subcellular fractionation of brain extracts, amphiphysin I and II demonstrate a similar distribution pattern, with both proteins present in soluble and membrane-associated fractions (Fig. 3B). The subcellular localization of amphiphysin II was then studied using immunofluorescence microscopy. In all brain regions examined, amphiphysin II immunoreactivity had a punctate appearance, suggesting a concentration of the antigen in synaptic terminals. Thus, a rim of bright puncta were seen surrounding cell bodies and primary dendrites, which themselves were not immunolabeled (Fig. 4, A and B). In the cerebellum, the molecular layer, which is abundant in synaptic terminals, was brightly stained, while the granule cell layer, which is densely packed with granule cell bodies, exhibited immunoreactivity only in discrete clusters corresponding to the mossy fiber terminals. Axons in the white matter were not immunolabeled. Similarly, in the spinal cord, immunonegative cell bodies and dendrites of motor neurons were surrounded by brightly stained puncta, while axonal bundles did not exhibit any immunoreactivity. We never observed labeling of nuclei that are enriched in BIN1 (20). The staining pattern for amphiphysin II was practically identical with the immunolabeling observed with an antibody against synaptojanin, a synaptic terminal protein (Fig. 4, E and F). Amphiphysin I immunolabeling (Fig. 4, C and D) also demonstrated a similar punctate synaptic-like distribution (14) although labeling was also present at moderate levels in the cell body cytoplasm.

Interaction of Amphiphysin II with Dynamin and Synaptojanin—Amphiphysin I binds through its SH3 domain to proline-rich sequences in synaptojanin and dynamin. To examine the binding properties of amphiphysin II, we used GST-amphiphysin II in overlay assays on rat brain postnuclear supernatant fractions (7). The fusion protein binds specifically to bands of 145 and 100 kDa (Fig. 5A), a pattern identical to that seen for Grb2 binding (7, 8), suggesting that the bands are synaptojanin and dynamin. To confirm the identity of these proteins and to determine if the interactions were SH3 domain-mediated, we performed affinity purifications from rat brain extracts using fusion proteins encoding the SH3 domains of amphiphysin I and II. As a control, we also used fusion proteins encoding a non-SH3 domain region of amphiphysin I and II. The SH3 domain fusion proteins from both amphiphysin I and II strongly bind to dynamin and synaptojanin, whereas the non-SH3 domain amphiphysin I and II fusion proteins do not bind (Fig. 5B).
noticed the presence of a 170-kDa protein that bound to the non-SH3 domain fusion protein. In Fig. 6A, we demonstrate that the 170-kDa band is clathrin and that it binds to the non-SH3 domain fusion proteins of amphiphysin I and II but does not bind to the SH3 domain of either of these proteins. To further study the clathrin binding, we generated clathrin-coated vesicles from rat brain and extracted the clathrin using 0.5 M Tris (25). Clathrin prepared in this manner bound to the non-SH3 domain fusion proteins from amphiphysin I and II but did not bind to the fusion proteins encoding the SH3 domains (Fig. 6B). It has previously been demonstrated that amphiphysin I binds to the α-subunit of AP2 (14, 16). Therefore, we examined the binding of AP2 to our amphiphysin fusion proteins. The two α-adaptin subunits (α1 and α2) of AP2 bound weakly to the SH3 domain of amphiphysin II but did not bind to the SH3 domain of amphiphysin I or to the non-SH3 domain fusion proteins (Fig. 6A). To further explore this issue, we generated a GST fusion protein against the insert domain of amphiphysin II and a corresponding fusion protein from amphiphysin I. Interestingly, the amphiphysin I but not the amphiphysin II fusion protein bound α-adaptin (Fig. 6C).

DISCUSSION

Amphiphysin I is a nerve terminal-enriched protein that is thought to function in the endocytosis of synaptic vesicles. Here, we report the amino sequence of amphiphysin II, which we identified based on the sequence of a cDNA clone from the dbEST. Recently, the amino acid sequence of two other amphiphysin I homologues, BIN1 (20) and SH3P9 (11), were reported. BIN1, SH3P9, and amphiphysin II align with long blocks of virtually identical amino acid sequence interrupted by stretches of sequence specific to the individual proteins. Southern blot analysis of human genomic DNA suggests that the three proteins are generated from a single gene. Thus, it seems likely that the three proteins are encoded by alternative splicing leading to the generation of unique sequences that impart distinct properties upon each protein. For example, the only apparent difference between BIN1 and SH3P9 is the presence of a small insert sequence (RKKSKLFSRLRRKKN) in BIN1, which encodes a nuclear localization signal. This is consistent with the function of BIN1 as a Myc-binding protein localized in the nucleus (20) and would suggest that SH3P9 may share a common function with BIN1 but be localized outside of the nucleus. A striking feature of amphiphysin II is that it contains a large central domain of 123 amino acids that is not present in either of the other two molecules. This region is strongly homologous to amphiphysin I throughout its N-terminal half, including the presence of a highly conserved proline-rich sequence that forms a potential SH3 domain-binding site (14, 29). The insert domain also contains a binding site for clathrin as discussed below.

To begin to characterize amphiphysin II, we generated a polyclonal antiserum against an amphiphysin II-GST fusion protein. To avoid reactivity against BIN1 and SH3P9, we affinity-purified the serum using a fusion protein encoding a region of amphiphysin II restricted to the insert domain. The affinity-purified antibodies reacted with both amphiphysin I and II. Therefore, antibodies against amphiphysin I were absorbed out using an immobilized amphiphysin I fusion protein generating an amphiphysin II-specific antibody. This antibody was used to determine that amphiphysin II is highly enriched in the brain and is concentrated in the presynaptic nerve ter-
minal. This is in contrast to BIN1, which has its highest expression levels in skeletal muscle and is enriched in nuclei (20). The tissue distribution and subcellular localization of SH3P9 is unknown. A number of proteins involved in synaptic vesicle recycling have been shown to be concentrated in the nerve terminal including clathrin (5), AP2 (14), dynamin and synaptojanin (8), and amphiphysin I (14). Further, recent morphological data suggest that dynamin, synaptojanin, and amphiphysin I are present with AP2 at clathrin coats in the nerve terminal and in nonneuronal cells (5, 30, 31).

Synaptojanin and dynamin are the major brain proteins that interact with the SH3 domains of amphiphysin I (14). Based on the conservation of the SH3 domains of amphiphysin I and II, we decided to test the ability of amphiphysin II to interact with these proteins. By overlay and column chromatography assays, dynamin and synaptojanin appear to be the major brain proteins that interact with the SH3 domain of amphiphysin II. As a control for the experiments, we used a fusion protein to a non-SH3 domain region of amphiphysin II that encoded a portion of the insert domain as well as the corresponding fusion protein from amphiphysin I. Interestingly, while these fusion proteins did not bind to synaptojanin or dynamin, they were both able to specifically precipitate clathrin from a crude Triton X-100 brain extract. To further study the interaction of clathrin with amphiphysin, we purified clathrin-coated vesicles from rat brain and extracted the clathrin with 0.5 M Tris-Cl, pH 7.4. This procedure releases clathrin as soluble triskelia and leads to an enrichment in clathrin relative to the clathrin adaptor protein AP2 (25). Under these conditions, clathrin retains its strong binding to the amphiphysin isoforms. Thus, both amphiphysin I and II bind to synaptojanin and dynamin through their SH3 domains and bind to clathrin in an SH3 domain-independent manner.

It has previously been demonstrated that amphiphysin I can interact with AP2 (14, 16). Thus, the interaction of amphiphysin I and II with clathrin observed here could be indirect and mediated through AP2. However, as determined by immunoblot with an antibody against the α-adaptin subunit (28), AP2 was not present in the non-SH3 domain fusion protein precipitates in which clathrin was highly enriched. In contrast, AP2 did bind to an amphiphysin I fusion protein encoding a region of amphiphysin I from amino acids 291–445. The non-SH3 domain amphiphysin I fusion protein, which does not bind clathrin, encodes a region of amphiphysin I from amino acids 338–565, suggesting the presence of a binding site for α-adaptin between amino acids 291 and 338 of amphiphysin I. In addition, AP2 was found to bind to the SH3 domain of amphiphysin II but not amphiphysin I. It was previously demonstrated that AP2 could interact with the SH3 domains of Grb2 (14). The interaction of the SH3 domain of amphiphysin II with the α-adaptin subunit of AP2 reported here could be direct, through proline-rich sequences in the α-adaptin subunit (32).

A model has been proposed (14) that suggests that amphiphysin I, which is concentrated on clathrin coats in the

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**FIG. 6. Clathrin binding.** A, Triton X-100-soluble proteins from brain were incubated with GST fusion proteins (GST-amphiphysin I non-SH3 domain, GST-amphiphysin I SH3 domain, GST-amphiphysin II non-SH3 domain, and GST-amphiphysin II SH3 domain as indicated) which were conjugated to glutathione-Sepharose, and material specifically bound to the beads was eluted with SDS-PAGE sample buffer. The panels show immunoblots with an antibody against clathrin or α-adaptin isoforms (αa and αc) as indicated. B, clathrin-coated vesicles (CCVs) were purified from rat brain, and the clathrin was extracted with 0.5 M Tris-Cl, pH 7.4. The clathrin extract (extract) was incubated with amphiphysin I and II fusion proteins as indicated, and the material specifically bound to the beads was eluted with SDS-PAGE sample buffer. The panel in B shows a Coomassie-stained SDS-gel with the migratory position of clathrin noted. C, Triton X-100-soluble proteins from brain were incubated with GST fusion proteins (GST-amphiphysin I insert domain and GST-amphiphysin II insert domain as indicated) which were conjugated to glutathione-Sepharose, and material specifically bound to the beads was eluted with SDS-PAGE sample buffer. The panel in C shows an immunoblot of equal aliquots of starting material (SM), unbound material (void), and bead fraction (beads) stained with an antibody against α-adaptin.
nerve terminal (31), is recruited to these sites through its SH3 domain-independent interactions with AP2. The SH3 domain of amphiphysin I then serves to target dynamin to the endocytic site (14, 30, 33). Our data would suggest an additional role for amphiphysin II in the recruitment of dynamin and synaptojanin through SH3 domain-independent interactions leading to the observed concentration of both of these proteins on clathrin-coated structures (5, 30, 31). Further, interactions of the SH3-independent domains of both proteins with clathrin could provide an additional targeting substrate for the amphiphysin isoforms. Cycles of phosphorylation and dephosphorylation of dynamin (34) and synaptojanin (8), which are regulated by the polarization state of the nerve terminal, may play an important role in regulating these targeting events. Further, SH3P4, which is a major SH3 domain-containing synaptojanin-binding protein in brain (10), may play a role in regulating the association of synaptojanin with the amphiphysin isoforms. Once at the endocytic site, dynamin functions in fission of the clathrin-coated vesicles (5, 6), and synaptojanin may function to regulate local concentrations of inositol polyphosphates or inositol phospholipids, leading to changes in the function of adaptins (35) or dynamin (36, 37). Regardless, the data presented here suggest an important role for amphiphysin II in synaptic vesicle endocytosis.

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