The SH3 Domain of Amphiphysin Binds the Proline-rich Domain of Dynamin at a Single Site That Defines a New SH3 Binding Consensus Sequence*

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Amphiphysin is an SH3 domain-containing neuronal protein that is highly concentrated in nerve terminals where it interacts via its SH3 domain with dynamin I, a GTPase implicated in synaptic vesicle endocytosis. We show here that the SH3 domain of amphiphysin, but not a mutant SH3 domain, bound with high affinity to a single site in the long proline-rich region of human dynamin I, that this site was distinct from the binding sites for other SH3 domains, and that the mutation of two adjacent amino acids in dynamin I was sufficient to abolish binding. The dynamin I sequence critically required for amphiphysin binding (PSRPNR) fits in the novel SH3 binding consensus identified for the SH3 domain of amphiphysin via a combinatorial peptide library approach: PXRPXR(H)R(H). Our data demonstrate that the long proline-rich stretch present in dynamin I contained multiple SH3 domain binding sites that recognize interacting proteins with high specificity.

Dynam I is a neuronal GTPase concentrated in nerve terminals that plays an essential role in synaptic vesicle endocytosis and recycling (for reviews, see Refs. 1 and 2). A temperature-sensitive mutation in the dynamin I gene of Drosophila leads to rapid and massive block of synaptic vesicle endocytosis resulting in a paralytic phenotype (3–5). Ultrastructural studies have shown that dynamin I forms rings at the neck of clathrin-coated pits, and it has been hypothesized that a conformational change of the ring that correlates with GTP hydrolysis represents a key step leading to vesicle fission from the plasmalemma (6, 7). In addition, dynamin I has also been implicated in rapid endocytosis, a form of Ca^{2+}-triggered endocytosis detectable by capacitance measurement in neuroendocrine cells (8). Dynamin isoforms (dynamin II and dynamin III) are expressed in non-neuronal cells (9–11) where they are thought to play a general role in clathrin-mediated endocytosis (12, 13).

The COOH-terminal region of dynamin I contains a 100-amino acid-long proline-rich domain (14), which undergoes regulation by protein phosphorylation and binds a variety of SH3 domains (13, 15–18). An abundant SH3 domain-containing protein, which is a major binding partner for dynamin I in nerve terminals (19), is amphiphysin, the dominant autoantigen in paraneoplastic stiff-man syndrome (20–22). Amphiphysin is closely colocalized with dynamin I at synapses where, in addition to dynamin I, it binds the presynaptic inositol-5-phosphatase synaptojanin (23). Amphiphysin binds the plasmalemmal clathrin adaptor AP2 via a region distinct from its SH3 domain (19, 24), further supporting an involvement of amphiphysin in endocytosis. In addition, amphiphysin contains regions of similarity to two yeast proteins, Rvs167 and Rvs161 (20, 25, 26), which genetic studies have shown to be implicated both in endocytosis and in the function of the actin cytoskeleton (26, 27).

As a premise to further elucidate the functional interconnections between amphiphysin and dynamin I, we investigated regions that are crucial for reciprocal interactions in the two proteins. We report that the SH3 domain of amphiphysin bound with high affinity to a short amino acid stretch in dynamin I. The amino acid sequence of the binding site fits the novel consensus for the SH3 domain of amphiphysin, which we have identified by a combinatorial peptide library.

EXPERIMENTAL PROCEDURES

Antibodies

Affinity-purified rabbit antibodies directed against amphiphysin (CD5) were generated in our laboratory (19). Anti-dynamin and anti-Grb2 monoclonal antibodies were purchased from Transduction Laboratories. Anti-cortactin (4F11) and anti-PLC1-y (F-7-2) monoclonal antibodies were the kind gifts of Dr. T. Parson (University of Virginia) and Dr. S. G. Rhee (National Institutes of Health), respectively.

Recombinant Proteins

Full-length polyhistidine-tagged human amphiphysin and a GST-fusion protein containing its wild type SH3 domain (construct V (amino acids 545–695) of Ref. 20) were generated as described previously (19, 20). A mutant amphiphysin SH3 domain was constructed as follows. A primer matching the 5’ end (see Ref. 20) of the wild type SH3 domain fragment and a primer partially matching its 3’ end (5’-AGG ATG ATC ATG TAA GCC TGA GCC TGT GAA GCG TTC TTA GGT GCC-3’), which included two base changes resulting in the substitution of two amino acids at position 684 (glycine to arginine) and 687 (proline to leucine), were used in polymerase chain reactions

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1 The abbreviations used are: PLC, phospholipase C; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; PRD, proline-rich domain.
using the wild type SH3 domain as the template. The resulting DNA fragment was cloned into pGEX2T and expressed using the *Escherichia coli* strain BL21 as a GST-fusion protein.

A polyhistidine human dynamin deletion construct missing the entire proline-rich domain (amino acid 1750) was expressed in the pET23c vector (Novagen). GST and GST-fusion proteins containing either the full-length proline-rich domain (PRD) or the COOH-terminal truncated forms of this domain (see Fig. 2A) were expressed in pGEX2T. The construct harboring point mutations at positions 835 (arginine to aspartic acid) and 836 (proline to alanine) (Fig. 3, construct 5) was obtained by polymerase chain reaction amplification using mutant primers. All dynamin constructs were partial sequences of the human dynamin isofrom Iaa (11). Whereas the NH2-terminal primer for most constructs was the same (5'-GGC GTA ACC ACC TTC ACC CCC GC-3'), the COOH-terminal primer used to construct the dynamin-PRD GST-fusion proteins (see Figs. 2A and 3A) was as follows: full-length PRD, 5'-CCG GAA TTC CGC CGA AAG GGT CAG GGG-3'; 751–848 (Fig. 2A, construct 4), 5'-CCG GAA TTC ACC CCG CTG GGG GAC C-3'; 751–852 (Figs. 2A and 3A, construct 5), 5'-CCG GAA TTC ACC CGC CCC-3'; 751–854 (Fig. 2A, construct 6), 5'-GGT GGC GTC CGA GGG CAC C-3'; 751–798 (Fig. 2A, construct 7), 5'-GGT GGC GAT ACC AGC CCC GCC CCC-3'; 751–836 (Fig. 2A, construct 8), 5'-GGT GGC GAT ACC AGC CCC GCC CCC-3'; 751–854 (Fig. 2A, construct 9), 5'-GGT GGC GAT ACC AGC CCC GCC CCC-3'; 751–858 (Fig. 3A, construct 3), 5'-GGC GGC AAC GTT CGC GCC GTT GGC GTC CGA GCC CCC-3'; 751–829 (Fig. 3A, construct 4), 5'-GGC GGC AAC GTT CGC GCC GTT GGC GTC CGA GCC CCC-3'; 751–838 (Fig. 3A, construct 5), 5'-GGC GGC AAC GTT CGC GCC GTT GGC GTC CGA GCC CCC-3'; 751–858 (Fig. 3A, construct 6), 5'-GGC GGC AAC GTT CGC GCC GTT GGC GTC CGA GCC CCC-3'; 751–858 (Fig. 3A, construct 7), 5'-GGC GGC AAC GTT CGC GCC GTT GGC GTC CGA GCC CCC-3'; 751–858 (Fig. 3A, construct 8), 5'-GGC GGC AAC GTT CGC GCC GTT GGC GTC CGA GCC CCC-3'; 751–858 (Fig. 3A, construct 9), 5'-GGC GGC AAC GTT CGC GCC GTT GGC GTC CGA GCC CCC-3'.

**Protein Interaction Studies**

**Affinity Chromatography of Brain Extracts**—Soluble brain extracts were prepared as described (19). Recombinant proteins to be used as adsorbers were immobilized on glutathione-Sepharose 4B beads (Pharmacia Biotech Inc.) or on nickel-nitrilotriacetic acid-agarose (Qia-Link Biosupport medium (Pierce) at a concentration of 0.3 mg/ml. The radiolabeled GST-SH3 domain of amphiphysin was incubated with 5 μl of PRD beads in 100 μl of 20 mM HEPES, pH 7.2, 150 mM NaCl, 5 mM MgCl2 bovine serum albumin, and 0.1% Tween 20 for 1 h at room temperature. Beads were collected by centrifugation in the Eppendorf microcentrifuge and washed once with ice-cold reaction buffer. The radioactivity of the supernatant of the centrifugation (free) and beads (bound) was determined using a γ-radiation counter. Specific binding was determined as the difference in binding in the absence and presence of a 100-molar excess of unlabeled amphiphysin SH3 domain. The affinity constant was calculated from the slope of the Scatchard plot of the binding data.

**Miscellaneous Procedures**

Preparation of total tissue homogenates and SDS-PAGE were performed according to Laemmli (30). Protein was measured using the BCA and Coomassie Plus protein assay kit (Pierce).

**RESULTS**

To determine whether the binding of amphiphysin to dynamin I is critically dependent on the classical SH3 domain binding interface, we generated a mutant amphiphysin SH3 domain harboring point mutations at amino acid positions 684 (glycine to arginine) and 687 (proline to leucine). Each of these two mutations was found to abolish the binding properties of the SH3 domains of the Grb2Sem5Drk protein (31). GST-fusion proteins comprising either the wild type or the mutant amphiphysin SH3 domains were incubated with brain extracts, and the proteins that selectively bound to these constructs were analyzed by SDS-PAGE and protein staining. As shown in Fig. 1, both dynamin I and synaptojanin were specifically affinity-purified by the wild type but not by the mutant SH3 domain.

To identify the binding site for amphiphysin SH3 domain in dynamin I, a variety of dynamin I deletion constructs were generated (Fig. 2, A and B), and their ability to affinity-purify amphiphysin from brain extract was assessed by Western blotting of the affinity-purified material (Fig. 2C). As shown in Fig. 2, B and C (lane 3), a GST-fusion protein containing the entire proline-rich domain of dynamin I binds amphiphysin, while GST alone or a truncated form of dynamin I missing the entire proline-rich region does not (Fig. 2, B and C, lanes 1 and 2), confirming that the interaction is mediated as expected by the proline-rich region of dynamin I. Progressive deletion of the COOH-terminal portion of the proline-rich region of dynamin I defined the segment comprising amino acids 832 and 848 as

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**Fig. 1. Affinity purification of total rat brain homogenate on GST-fusion proteins containing the wild type or mutant (Gly684→Arg and Pro687→Leu) SH3 domain of amphiphysin.** Material specifically retained by the beads was separated by SDS-PAGE and stained with Coomassie Blue. The wild type (wt) but not the mutant (mut) SH3 domain binds dynamin I and synaptojanin. The identity of the two proteins was confirmed by Western blotting (not shown).
essential for amphiphysin binding (Fig. 2, compare lanes 4 and 5). Since amphiphysin is the major band selectively enriched in the material, which is affinity-purified by constructs 3 and 4 (lanes 3 and 4 of Fig. 2B, arrows), these data also confirm that amphiphysin is indeed one of the major dynamin-binding proteins in rat brain.

Further deletions within the proline-rich domain of dynamin I allowed definition of the sequence PSRPNR as the amino acid stretch required for the interaction (Fig. 3A, constructs 2 and 5; Fig. 3B, lanes 2 and 5). This result was corroborated by the demonstration that mutation of the arginine and proline within this segment into an aspartate and alanine, respectively (construct 3 of Fig. 3A), also abolished amphiphysin binding (Fig. 3B, lane 3).

A common characteristic of SH3 domain binding sites is the presence of the core motif PXXP, which is often followed by an arginine at position +5 (the first P of the core PXXP being position 0) in class II binding ligands for SH3 domains (32–35). Disruption of amphiphysin binding by the mutation of constructs 3 and 5 is therefore compatible with a binding of amphiphysin either to the sequence PSRPNR or to the overlapping sequence PQVPSR. However, amphiphysin was still able to bind (albeit less efficiently) to construct 4, which includes only the PSRPNR motif (Fig. 3A, construct 4; Fig. 3B, lane 4), confirming the importance of this amino acid sequence. The less effective binding of the amphiphysin to this construct is not surprising since there is evidence that SH3 domain binding can be modulated by amino acid sequences flanking the main SH3 domain binding site (34).

In contrast to these findings, all Grb2 binding activity was lost in constructs 4, 5, and 6, indicating that the PQVPSR motif is the major Grb2 binding site (Fig. 3B). Mutation or deletion of the PSRPNR sequence did not decrease binding of the SH3-containing proteins PLC-y1 (36) (Fig. 3B) and cortactin (37) (not shown), which therefore must bind to sequences NH₂-terminal to this site. The crucial role of the PSRPNR region for the amphiphysin-dynamin I interaction was confirmed by overlaying blots of dynamin I constructs (Fig. 3A) with recombinant polyhistidine-tagged amphiphysin (Fig. 3C). This experiment rules out the possibility that the interactions revealed by affinity chromatography of brain extracts (Fig. 2, B and C, and Fig. 3B) may be indirect.
We next used a combinatorial peptide library approach to identify the preferred binding motif of the SH3 domain of amphiphysin and its relation to the binding region identified in dynamin I. A combinatorial peptide library was incubated with the GST-amphiphysin SH3 domain, and the consensus amino acid sequence that binds the SH3 domain was established by sequencing of the bound peptides (Fig. 4). The consensus obtained was P\(\times\)RP\(\times\)R(H)R(H) in good agreement with the results of our binding studies.

To determine the affinity of the interaction between the proline-rich domain of dynamin I and the SH3 domain of amphiphysin we tested a\(^{125}\)I-labeled GST-SH3 domain of amphiphysin for its binding to immobilized GST-fusion protein comprising the proline-rich domain of dynamin I (construct 3 of Fig. 2A). The results are shown in Fig. 5. They are consistent with a single-site binding model and demonstrate a high affinity interaction with a dissociation constant of 190 ± 10 nM.

Since the putative preferred binding sites for amphiphysin and Grb2 are partially overlapping in the proline-rich domain of dynamin, we tested whether the SH3 domain of amphiphysin and Grb2 can compete for binding to dynamin I. As shown in Fig. 6, Grb2 prevented the binding of amphiphysin, confirming the close localization of the binding sites for these two proteins in the dynamin I molecule.

**DISCUSSION**

Dynamin I contains a proline-rich COOH-terminal region of about 100 amino acids that interacts in vitro and in vivo with a variety of SH3 domain-containing proteins, including PLC-\(\gamma\), the p85 subunit of phosphatidylinositol 3-kinase, Src, Grb2, and amphiphysin (15, 16, 19, 23, 37). The multiplicity of the binding partners for this domain revealed by in vitro and in vivo studies may be explained by either a promiscuous interaction of various SH3 domains with several proline-rich stretches or by the presence in the tail of dynamin I of an array of distinct sites, each of which specifically binds certain SH3 domains. We report here that amphiphysin, a binding partner for dynamin I in nerve endings (19), binds with remarkable specificity and high affinity to a unique site in its long proline-rich domain.

The sequence PSRPNR at the amino acid position from 833 to 838 was found to be critically required for amphiphysin binding. Strikingly, this sequence matches the consensus binding motif of the amphiphysin-SH3 domain very well as revealed by a combinatorial peptide library: P\(\times\)RP\(\times\)R(H)R(H). The only difference between the dynamin I binding site and this consensus is the presence of an alanine rather than an arginine at position +6 (see Fig. 3A). However, the position +6 is clearly not essential for dynamin I binding as shown by the similar binding of constructs 1 and 2 of Fig. 3A. This is a novel SH3 binding consensus that would predict a class II type orientation of the peptide, given the presence of an arginine at the COOH-terminal side (position +5) of the core (32–35). We note that an amino acid sequence that fits the consensus P\(\times\)RP\(\times\)R is present in synaptojanin, the other major amphiphysin binding protein in brain (23).

The PSRPNR sequence is placed at the COOH-terminal end of a longer proline-rich stretch in dynamin I (Fig. 3A) that contains the sequence PQVPSR. This sequence was recently reported to fit the consensus for binding to the NH\(_2\)-terminal SH3 domain of Grb2 and to the SH3 domain of Src (35). While the core of these other consensus motifs is the PQVP sequence, our results suggest that the core of the amphiphysin binding site is located three amino acids closer to the COOH terminus and corresponds to the PSRP sequence. We do, however, provide support to the hypothesis that the PQVP motif represents the core of the major Grb2 binding site, which is consistent with
the mapping of the Grb2 binding region in dynamin I reported by other groups (15, 18). Accordingly, the ability of Grb2 to compete with amphiphysin for binding to dynamin I supports a close localization of the binding sites for these two proteins.

The sequence PSRPNR is clearly not required for the binding of dynamin I to the SH3 domain of PLC-γ, in agreement with

**FIG. 4. Peptide specificity (PXRPX(H)R(H)) of the amphiphysin SH3 domain.** The first fixed proline (underlined) in the library (MAXXXXXPXXPXXAKKK) is considered as the 0 position. Panels A–F show selectivity values of amino acids in positions -1, +1, +2, +4, +5, and +6, respectively. Values that are higher than 1.5 indicate significant selection. No significant preference of amino acids was detected at other degenerate positions (not shown).
Dynamin-Amphiphysin Interaction

pothesis that the long proline-rich COOH-terminal region of dynamin I represents a template for the recruitment of different SH3-containing proteins via highly specific interactions mediated by distinct binding sites. The proline-rich domain of dynamin I undergoes phosphorylation/dephosphorylation (38). It is possible that the phosphorylation of specific amino acid positions may serve to selectively regulate the interactions of dynamin I with one or a subset of SH3 domains.

Many of the SH3 domain-containing proteins that interact with dynamin I can also interact via distinct domains with proteins that are concentrated at plasmalemmal clathrin-coated pits. For example, PLC-γ, the p85 subunit of phosphatidylinositol 3-kinase and Grb2, also contains SH2 domains that bind to tyrosine-phosphorylated growth factor receptors (39). Amphiphysin interacts via a region distinct from its SH3 domain with the clathrin adaptor AP2 (19, 24). The presence of binding sites for these various proteins in the tail of dynamin I may serve to guarantee an efficient recruitment of dynamin I at clathrin-coated buds (19) where dynamin I plays a key role in the fission reaction (13). Conversely, due to its property of interacting with multiple SH3 domains dynamin I itself may serve to further recruit other SH3 domain-containing proteins in a positive feedback loop. In agreement with these considerations, we have recently shown that disruptions of the SH3-mediated interactions of dynamin in living nerve terminal preparations potently inhibits the fission reaction of clathrin-coated vesicles (40). Furthermore, a previous study has shown that an SH3 domain binding motif in dynamin I is required for its targeting to clathrin-coated pits in COS7 cells (41). This motif was mapped to a site upstream to the sequence PSRPN described here, which could be deleted without abolishing dynamin I targeting in these cells (41). However, the SH3 domain of amphiphysin may play a dominant role in the recruitment of dynamin I at clathrin-coated pits involved in synaptic vesicle reformation, while other SH3 domain-containing proteins may play a dominant role at other types of clathrin-coated pits.

The data here open the possibility of generating mutant dynamin I molecules that are selectively impaired in their property of binding the SH3 domain of amphiphysin and therefore studying the precise role of this interaction in nerve terminal function.

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