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Specificity of the Binding of Synapsin I to Src Homology 3 Domains*

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Synapsins are synaptic vesicle-associated phosphoproteins involved in synapse formation and regulation of neurotransmitter release. Recently, synapsin I has been found to bind the Src homology 3 (SH3) domains of Grb2 and c-Src. In this work we have analyzed the interactions between synapsins and an array of SH3 domains belonging to proteins involved in signal transduction, cytoskeleton assembly, or endocytosis. The binding of synapsin I was specific for a subset of SH3 domains. The highest binding was observed with SH3 domains of c-Src, phospholipase C-γ, p85 subunit of phosphatidylinositol 3-kinase, full-length and NH_2-terminal Grb2, whereas binding was moderate with the SH3 domains of amphiphysins I/II, Crk, α-spectrin, and NADPH oxidase factor $p47^{phox}$ and negligible with the SH3 domains of $p21^{ras}$ GTPase-activating protein and COOH-terminal Grb2. Distinct sites in the proline-rich COOH-terminal region of synapsin I were found to be involved in binding to the various SH3 domains. Synapsin II also interacted with SH3 domains with a partly distinct binding pattern. Phosphorylation of synapsin I in the COOH-terminal region by Ca^{2+}/calmodulin-dependent protein kinase II or mitogen-activated protein kinase modulated the binding to the SH3 domains of amphiphysins I/II, Crk, and α-spectrin without affecting the high affinity interactions. The SH3-mediated interaction of synapsin I with amphiphysins affected the ability of synapsin I to interact with actin and synaptic vesicles, and pools of synapsin I and amphiphysin I were shown to associate in isolated nerve terminals. The ability to bind multiple SH3 domains further implicates the synapsins in signal transduction and protein-protein interactions at the nerve terminal level.

Src homology 3 (SH3)[1] domains are noncatalytic multi-purpose modules that regulate a variety of cellular processes by mediating the formation and dissociation of specific intra- and intermolecular protein-protein interactions. First identified in the Src family of protein tyrosine kinases, SH3 domains were subsequently found to be present in proteins involved in the transduction of signals originating from growth factor receptors, in cytoskeletal components (*e.g.* spectrin and myosin I), in adapter proteins (*e.g.* Grb2 and Crk), and in signal transduction enzymes (*e.g.* nonreceptor tyrosine kinases and phospholipase C-γ) (1). SH3 domains consist of approximately 60 amino acids that, despite differences in their primary structure, exhibit the same three-dimensional topology and bind with high affinity proline-rich protein sequences containing the minimal consensus sequence *X*P*XX*P. However, SH3 domain-mediated interactions are thought to be very specific because of both the high specificity of the domain-acceptor binding and the compartmentalization of both the domain-containing protein and the acceptor protein (1–3).

An involvement of SH3 domains in presynaptic physiology was originally suggested by the demonstration that in nerve terminals, three major proteins are excellent ligands for the SH3 domains of the adapter protein Grb2, namely dynamin I, synaptojanin I, and synapsin I (4). Subsequently, a series of physiologically relevant interactions involving SH3 domains among proteins involved in the exo-endocytotic cycle of synaptic vesicles (SV) and in nerve terminal signal transduction have been demonstrated (5, 6).

The search for endogenous SH3 domain-containing proteins has led to the identification of amphiphysin, existing in two similar isoforms, amphiphysins I and II, that can form heterodimers and interact with dynamin I and synaptojanin I through their SH3 domain. Amphiphysins also bind to clathrin and the clathrin adaptor AP2 via a region distinct from the SH3 domain and may serve to recruit dynamin I and synaptojanin I at clathrin-coated buds where they play a major role in the fission reaction (for review see Refs. 5, 7, and 8). A key role of the SH3 domain-mediated interactions in endocytosis of SV was recently demonstrated. The SH3-binding motif of dynamin I is required for its targeting to clathrin-coated pits in COS7 cells (9). Furthermore, disruption of SH3 domain-mediated in-

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[1] The abbreviations used are: SH3, Src homology 3; CaMPKII, Ca^{2+}/calmodulin-dependent protein kinase II; C- and N-Grb2 SH3 domains, COOH- and NH_2-terminal SH3 domains of Grb2; GST, glutathione *S*-transferase; MAPK, mitogen-activated protein kinase Erk; NTCB, 2-nitro-5-thiocyanobenzoic acid; PI3K, p85 subunit of phosphatidylinositol 3-kinase; PKA, cAMP-dependent protein kinase; PLCγ, phospholipase C-γ; RasGAP, $p21^{ras}$ GTPase-activating protein; PAGE, polyacrylamide gel electrophoresis; SV, synaptic vesicles; mAb, monoclonal antibody.

teractions of dynamin I in living nerve terminals by the microinjection of the SH3 domain of amphiphysin or of the dynamin SH3-binding sequence potently inhibited the fission reaction of clathrin-coated vesicles (10). Recently, another family of SH3 domain containing proteins, endophilins 1–3 (SH3p4, SH3p8, and SH3p13), have been found to bind dynamin I and synaptojanin I and to be implicated in the invagination of clathrin-coated pits that precedes the fission reaction (11–13).

The search for SH3 domain-containing proteins binding to SV proteins has demonstrated that synapsin I is the major binding protein for the SH3 domain of the tyrosine kinase $pp60^{c\text{-}src}$ (c-Src) in highly purified SV (Ref. 14; see also Ref. 15). This interaction displayed very high affinity and resulted in a severalfold stimulation of the tyrosine kinase activity of c-Src that was competitively antagonized by an excess of recombinant c-Src SH3 domain. Synapsin I was also found to stimulate endogenous SV-associated c-Src activity and tyrosine phosphorylation of SV substrates, suggesting that Src activity may be physiologically regulated by the extent of association of synapsin I with the vesicle membrane (6, 14, 16).

In this paper, we have analyzed the specificity of the binding of the synapsins, SV-specific phosphoproteins involved in synapse formation and in the regulation of neurotransmitter release (17, 18), with an array of SH3 domains belonging to proteins implicated in signal transduction or cytoskeleton assembly to get insights into the specificity of the synapsin-SH3 domain interactions and to identify new potential synapsin partners at the nerve terminal level. The results suggest that the synapsins may be implicated in previously unidentified signal transduction pathways and protein-protein interactions at the nerve terminal level.

EXPERIMENTAL PROCEDURES

Materials—^{125}I-Labeled anti-rabbit IgGs (Fab fragments) were from Amersham Pharmacia Biotech; the Renaissance enhanced chemiluminescence detection system was from NEN Life Science Products. The polyclonal antibodies against synapsin I, dynamin I, synaptojanin I, and glutathione S-transferase (GST) and the monoclonal antibodies (mAb) against amphiphysin I (mAb 8) and synapsin I (mAbs 19.11 and 10.22) were made in our laboratories. The SH3 domain-containing constructs of amphiphysins I and II (amino acids 588–695 and 516–612, respectively) were made as described (19). The other SH3 domain pGEX-2T constructs used in this study, generously provided by Dr. I. Gout (Ludwig Institute for Cancer Research, University College, London, UK) were: chicken c-Src SH3 domain (amino acids 84–148), bovine PI3K SH3 domain (amino acids 1–86), human full-length Grb2 (amino acids 1–217), N-Grb2 and C-Grb2 SH3 domains (amino acids 1–58 and 159–217, respectively), human RasGAP SH3 domain (amino acids 275–345), human PLCγ SH3 domain (amino acids 792–851), chicken α-spectrin SH3 domain (amino acids 967–1025), Crk SH3 domain (amino acids 369–429), and COOH-terminal SH3 domain of the $p47^{phox}$ subunit of NADPH oxidase (amino acids 226–286). Glutathione-Sepharose, protein G-Sepharose, goat anti-His_6 antibodies, and pGEX-2T were from Amersham Pharmacia Biotech, and 2-nitro-5-thiocyanobenzoic acid (NTCB) was from Sigma. *Staphylococcus aureus* V8 protease was from Roche Molecular Biochemicals. The pET-30a/pET-30c vectors and the S-protein HRP conjugate were from Novagen Inc. (Madison, WI). Nickel-nitrilotriacetic acid-agarose was from Qiagen (Valencia, CA). *N*-(l-pyrenyl)iodoacetamide was from Molecular Probes (Eugene, OR). The following peptides from the COOH-terminal region of rat synapsin I were synthesized by the Modena University Oligomer Synthesis Facility: C1 peptide ($syn^{531–562}$), C2 peptide ($syn^{585–607}$), C3 peptide ($syn^{593–602}$), and C4 peptide ($syn^{619–634}$). All other materials were obtained from standard commercial suppliers.

Protein Expression and Purification—Bacterial cells were transformed to ampicillin resistance by electroporation with constructs containing pGEX-2T alone or pGEX-2T in frame with sequences encoding for SH3 domains. Large scale cultures of Luria broth containing ampicillin (100 μg/ml) were inoculated with small overnight cultures, grown at 37 °C to log phase, and induced with isopropyl β-D-thiogalactopyranoside (100 μM) for 3–5 h. GST and GST-SH3 domain fusion proteins were extracted from bacterial lysates, purified to homogeneity by affinity chromatography on glutathione-Sepharose, and dialyzed against 25 mM Tris-Cl, 50 mM NaCl, pH 7.4 (4, 14).

Fusion proteins containing truncations from the COOH terminus of domains DE of rat synapsin Ia were made using the vector pET-30a or pET-30c. The DNAs corresponding to truncated regions were amplified by polymerase chain reaction, and the amplified products were subcloned into pET-30c using standard recombinant DNA techniques (20). The resulting expression plasmids were then verified by sequencing and transformed into *Escherichia coli* BL21(DE3) Lys E. Sequencing was performed by the Rockefeller University Protein/DNA Technology Center. The following mutants, containing His_6 and S tags at the NH_2 terminus and a decreasing number of *XPXXP* motifs, were made: DE peptide ($syn^{413–704}$; 11 *XPXXP*), D peptide ($syn^{413–652}$; 9 *XPXXP*), D1 peptide ($syn^{413–617}$; 7 *XPXXP*), D2 peptide ($syn^{413–585}$; 5 *XPXXP*), D3 peptide ($syn^{413–556}$; 4 *XPXXP*), and D4 peptide ($syn^{413–538}$; 2 *XPXXP*). After sequencing, a mutation was found in the cDNA of the D1 peptide affecting the deduced sequence of the seventh consensus sequence in which a histidine residue substituted for Pro^{598} (K**P**PG**P**AGHIR instead of K**P**PG**P**AG**P**IR). Recombinant proteins were induced in BL21 as described above. The truncation mutants of synapsin domain DE were then extracted from bacterial lysates and purified to homogeneity on nitrilotriacetic acid-agarose affinity columns following the manufacturer's procedure. Truncation mutants were detected in immunoblotting assays by anti-His_6 antibodies, protein S, or monoclonal antibody 10.22 (21) recognizing single sites on their NH_2 terminus and detecting all mutants with the same sensitivity.

Synapsin I was purified from bovine brain (22). Synapsin IIa was expressed in Sf9 cells infected with the baculovirus expression vector pVLSynIIa encoding for rat synapsin IIa and purified by affinity chromatography (23, 24). Purified synapsin I was stoichiometrically phosphorylated *in vitro* using purified protein kinases as described previously (24–26). Purified synapsin I was subjected to cysteine-specific cleavage with NTCB, and the resulting fragments were dialyzed against 4 M urea in preparation for SDS-polyacrylamide gel electrophoresis (PAGE) or purified as described previously (27). Cleavage of purified synapsin I with *S. aureus* V8 protease was performed as described previously (4). Actin was prepared from an acetone powder of rabbit skeletal muscle in buffer A (0.2 mM $CaCl_2$, 0.2 mM ATP, 0.5 mM NaN_3, 0.5 mM β-mercaptoethanol, 2 mM Tris-HCl, pH 8.0), further purified by gel filtration on a Sephadex G-150 column, and stored in liquid nitrogen (28, 29). Purified gel filtered actin was fluorescently labeled with *N*-(l-pyrenyl)iodoacetamide as described previously (30) at a pyrene/actin molar ratio of 0.8.

SH3 Domain Far Western Overlays—Protein fractions (50 μg of protein), purified synapsins Ia/Ib and IIa (0.75 μg), or synapsin I NTCB or V8 protease fragments resulting from 5 μg of digested synapsin I were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated for 1 h at room temperature in blocking buffer (150 mM NaCl, 25 mM Tris-Cl, pH 7.4, 5% (w/v) nonfat dry milk), rinsed with Tris-buffered saline (150 mM NaCl, 25 mM Tris-Cl, pH 7.4) and incubated overnight at 4 °C in overlay buffer (3% bovine serum albumin/20 mM Tris-Cl, pH 7.4, 1 mM dithiothreitol, 0.1% v/v Tween 20) containing 5 μg/ml of GST or GST-SH3 domain fusion protein. The membranes were then washed and incubated for 2 h at room temperature with a rabbit anti-GST antibody and revealed with ^{125}I-labeled anti-rabbit Fab fragments.

SH3 Binding Assays and Affinity Chromatography—The binding in solution of GST-SH3 domains to purified synapsin I, to the purified COOH-terminal fragments generated by cysteine-specific cleavage of synapsins Ia/Ib (fragments γ_a/γ_b; Ref. 27) or to truncation mutants of the COOH-terminal region of synapsin I was assessed by co-precipitation experiments as follows. SH3 domain-GST fusion proteins or GST alone were coupled to glutathione-Sepharose (0.1 nmol of fusion protein/μl of settled Sepharose beads) in binding buffer (10 mM Hepes, 150 mM NaCl, 1% (v/v) Triton X-100, pH 7.4). After extensive wash, 12 μl of fusion protein-coupled beads were incubated with 200–400 ng of purified dephosphorylated or phosphorylated synapsin I in 500 μl of binding buffer for 3–5 h at 4 °C. After the incubation, the beads were pelleted by centrifugation, washed three times in binding buffer, resuspended in Laemmli sample buffer, and boiled for 2 min. Affinity resins for the isolation of SH3 domain-binding proteins from brain extracts were prepared by immobilizing either GST alone or GST-SH3 domain fusion proteins (10 nmol of fusion protein) on glutathione-Sepharose (100 μl of settled beads) by an overnight incubation at 4 °C in binding buffer under gentle rotation in small columns. Columns were washed with 20 volumes of binding buffer and loaded with 7 ml of a 1% (v/v) Triton X-100 extract of crude synaptosomes (P2 fraction; 1 mg/ml protein). The extract was incubated with the resins for 3–5 h at 4 °C under gentle

rotation, and then the columns were extensively washed with binding buffer followed by detergent-free binding buffer. Elution of the bound proteins was performed with Laemmli sample buffer. The eluted proteins were separated by SDS-PAGE and analyzed by both Coomassie Blue staining of the gels and immunoblotting.

Actin Polymerization Assays—Before the experiments, actin was rapidly thawed in a 37 °C water bath, prespun at 100,000 rpm for 15 min (Beckman TLA 100.3 rotor), kept on ice, and used within 8 h. The polymerization of actin was monitored by recording the enhancement of pyrenyl-actin fluorescence as described previously (30, 31) using a Perkin-Elmer LS50 spectrofluorometer equipped with a water-jacketed cuvette holder and a circulating water bath. The sample temperature was carefully maintained at 25 °C, and the samples were allowed to equilibrate in the cuvette for 10 min. Polymerization was triggered at time 0 by the addition of 300 nM synapsin I that had been preincubated for 10 min at room temperature with 2 μM of either c-Src, PI3K, amphiphysin I, or amphiphysin II SH3 domain-GST fusion proteins or GST alone in buffer A containing 30 mM NaCl (sample size, 0.6 ml). Control curves were obtained by adding the corresponding fusion proteins in the absence of synapsin I. The fluorescence was measured at excitation and emission wavelengths of 365 and 407 nm, respectively, with excitation and emission slits set at 2.5 and 10 nm, respectively. Fluorescence data were plotted as fluorescence increase (in arbitrary units) with respect to the basal fluorescence before polymerization.

Acting Bundling Assays—For light scattering experiments, unlabeled G-actin was first polymerized at room temperature for 1 h by the addition of 100 mM KCl, 1.2 mM $MgCl_2$ and subsequently incubated (final concentration, 1.5 μM) in the presence or absence of dephosphorylated synapsin I (final concentration, 0.5 μM) or GST or GST/SH3 domains (final concentration, 1–5 μM) in disposable cuvettes for 45 min at room temperature in buffer A containing 90 mM KCl, 1.2 mM $MgCl_2$, 10 mM NaCl. The extent of light scattering was measured spectrofluorometrically with both excitation and emission wavelengths set at 400 nm with 5-nm slits and a 2% attenuation filter, as described previously (22, 27). The light scattering of the samples incubated in the absence of F-actin was subtracted, and the extent of bundling was calculated as the fold increase with respect to the scattering of F-actin alone. For low speed sedimentation experiments, samples containing 3 μM G-actin were incubated under the same conditions used for the light scattering assay and centrifuged at 10,000 × *g* for 10 min at room temperature (22). The recovered actin pellets were solubilized in Laemmli sample buffer and analyzed by SDS-PAGE.

Immunoprecipitation—Nonidet P-40 (2% v/v) extracts of Percoll-purified synaptosomes obtained from rat cerebral cortex were incubated for 3 h at 4 °C with anti-synapsin antibodies (mAb 19.11 or polyclonal G177), an anti-amphiphysin-I antibody (mAb 8), or a control polyclonal antibody not recognizing any specific protein in synaptosomal extracts (10 μg/sample). Immunoprecipitation was performed as described previously (14) using protein G-Sepharose (25 μl of settled prewashed beads) to precipitate the immunocomplexes.

Miscellaneous Procedures—Subcellular fractionation of rat forebrain tissue from homogenate to purified SV was carried out as described (32). Synaptosomes were purified from P2 fractions by centrifugation on discontinuous Percoll gradients as described previously (33). The binding of synapsin I or of its COOH-terminal fragment generated by cysteine-specific cleavage to purified, synapsin-depleted SV was carried out by using the high speed sedimentation assay as described (25). Protein concentrations were determined using the BCA (Pierce) or Bradford (Bio-Rad) assay using bovine serum albumin as standard. SDS-PAGE was performed according to Laemmli (34). Electrophoretic transfer of proteins to nitrocellulose membranes was performed as described (35). The semi-quantitative analysis of Far Western overlays was carried out by laser scanning densitometry of the autoradiograms obtained in the linear range of the emulsion response (Ultroscan XL, LKB, Bromma, Sweden). Quantitation of ^{125}I-labeled immunoblots obtained from co-precipitation experiments was carried out by direct counting of the nitrocellulose strips, followed by interpolation of the values into a suitable standard curve made from various amounts of purified synapsin I.

RESULTS

Specificity of the Binding of the Synapsins to SH3 Domains—The binding to synapsins of an array of SH3 domains expressed as fusion proteins with GST was first evaluated by Far Western overlays of nitrocellulose membranes on which purified bovine synapsin Ia/Ib and purified recombinant rat synapsin IIa had been immobilized (Fig. 1). Binding appeared to be specific for

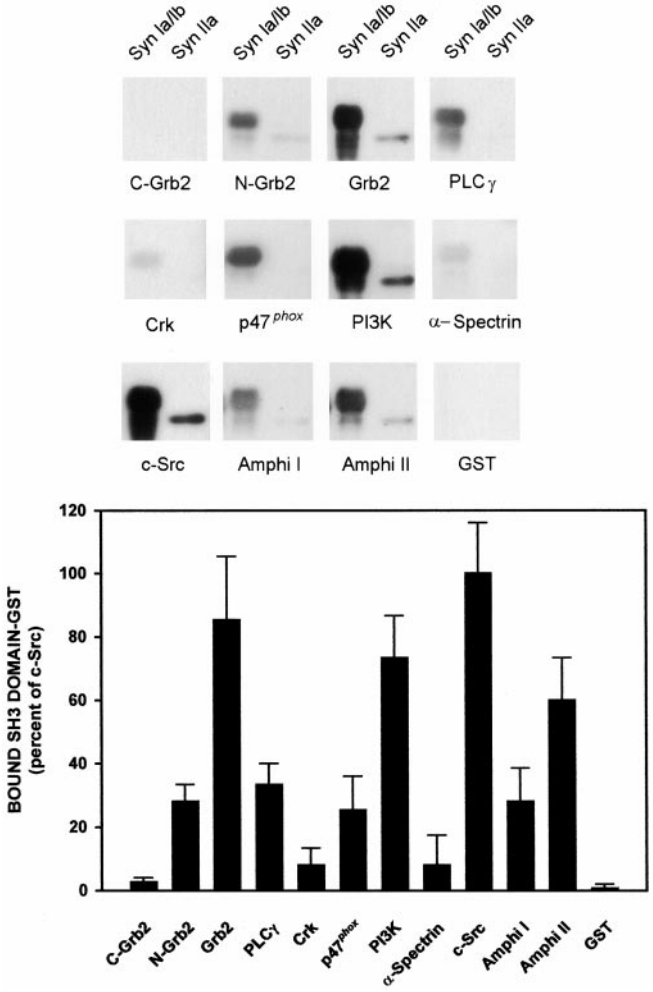

FIG. 1. **Binding of SH3 domains to purified synapsins I and II as evaluated by Far Western overlay.** Synapsin I purified from calf brain and purified recombinant rat synapsin IIa expressed in baculovirus-infected insect cells (0.75 μg/lane) were immobilized on nitrocellulose membranes after SDS-PAGE and subjected to Far Western overlay as described under "Experimental Procedures" using either the various SH3 domain-GST fusion proteins or GST alone. *Upper panel*, autoradiography of the Far Western overlays in which binding was revealed by immunoblotting with rabbit anti-GST antibodies and ^{125}I-labeled anti-rabbit Fab fragments. *Lower panel*, semi-quantitative analysis of the SH3 binding of synapsin I by densitometric scanning of the autoradiograms. The amounts of binding are expressed as percentages of the binding of the c-Src SH3 domain and shown as the means ± S.E. (n = 4).

SH3 domains, because no detectable labeling was observed when the overlays were performed with GST alone. In the case of synapsin I, the highest binding levels were found with the SH3 domains of c-Src, PI3K, and Grb2; somewhat lower binding levels were found with the SH3 domains of amphiphysin II, PLCγ, amphiphysin I, and N-Grb2; low binding levels were observed with the SH3 domains of p47^{phox}, Crk, and α-spectrin, whereas binding was absent with the C-Grb2 SH3 domain. When binding to equal amounts of immobilized synapsin IIa was measured, the SH3 binding was significantly lower. However, the binding pattern was similar to that observed with synapsin I, with the noticeable exceptions of the SH3 domains of PLCγ, Crk, p47^{phox}, and α-spectrin that did not exhibit detectable synapsin IIa binding (Fig. 1).

To determine whether a similar SH3 binding pattern was found with endogenous brain proteins and to compare synapsin labeling with the labeling of other abundant SH3-binding proteins such as dynamin I and synaptojanin I, proteins from

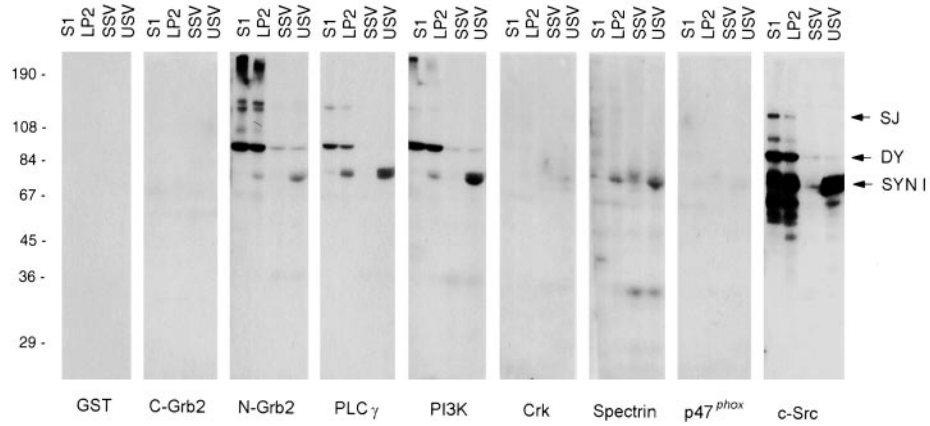

FIG. 2. **Binding of SH3 domains to proteins in subcellular fractions from rat forebrain.** Proteins from rat forebrain subcellular fractions (60 μg) were separated by SDS-PAGE, transferred to nitrocellulose membranes, and overlaid with the indicated SH3 domain-GST fusion proteins or with GST alone. Far Western overlay was carried out as described in the legend to Fig. 1. The major proteins labeled by the procedure were synaptojanin I (*SJ*), dynamin I (*DY*), and synapsin I (*SYN I*), as identified by immunodetection of parallel lanes (not shown). *S1*, post-nuclear supernatant; *LP2*, crude synaptic vesicle pellet; *SSV*, synapsin I-depleted highly purified SV; *USV*, untreated highly purified SV.

subcellular fractions obtained from rat forebrain including post-nuclear supernatant of homogenate, crude SV, and highly purified native and synapsin-depleted SV were analyzed for their ability to bind to the various SH3 domains using the overlay assay (Fig. 2). The labeling of a 80–86-kDa doublet co-migrating with synapsins Ia/Ib was qualitatively and quantitatively in agreement with the data obtained with purified synapsin I. Endogenous synapsin I was strongly labeled by the SH3 domains of c-Src, PLCγ, PI3K, and N-Grb2. A progressive enrichment of labeled synapsin I was observed from homogenate to SV, whereas a virtually complete disappearance of the SH3 domain labeling was observed in SV that had been depleted of endogenous synapsin I by mild salt treatment. The SH3 domains of α-spectrin and Crk bound synapsin I with lower affinity and the labeled protein was only detected in highly purified SV, whereas very low or absent labeling was observed in all fractions with the SH3 domains of p47^{phox} and C-Grb2. The SH3 domains of N-Grb2, PLCγ, PI3K, and c-Src also labeled dynamin I and synaptojanin I in homogenate and crude SV fractions, although they were always less intensely labeled than synapsin I in highly purified SV. In addition, the synapsin I/dynamin I binding ratio was different for the various SH3 domains, with the highest value found for the c-Src SH3 domain, which labeled synapsin I very intensely also in S1 and LP2 fractions. Very low or no significant dynamin I and synaptojanin I labeling was observed with the SH3 domains of C-Grb2, Crk, p47^{phox}, and α-spectrin (Fig. 2).

Although the overlay assay appears to be specific in detecting differences in binding affinity among various SH3 domains, SH3 ligands undergo denaturation during SDS-PAGE separation and may not reacquire the native conformation once immobilized onto a solid support. For these reasons, we analyzed the binding of the SH3 domains/GST fusion proteins immobilized on glutathione beads with purified synapsin I in solution. All the analyzed SH3 domains bound synapsin I to various extents, whereas GST did not show any appreciable binding (Fig. 3). The highest binding, with very high synapsin I recoveries, was observed with the SH3 domains of c-Src, PI3K, PLCγ, Grb2, and N-Grb2. Moderate binding was found with amphiphysin II, Crk, and amphiphysin I SH3 domains, whereas low binding was observed with the SH3 domains of α-spectrin, RasGAP, p47^{phox}, and C-Grb2 (Fig. 3, *upper panel*).

We also investigated whether synapsin I could be purified using SH3 domain affinity columns from an extract of crude synaptosomal fractions (P2) obtained from rat forebrain, in which competition with other SH3-binding proteins occurs. In substantial agreement with the results obtained above, the c-Src, PI3K, PLCγ, and Grb2 SH3 domain affinity columns purified synapsin I with high efficiency. Lower recoveries of synapsin I were observed from the N-Grb2, amphiphysins I and II, Crk, α-spectrin, and p47^{phox} SH3 domain affinity columns, whereas no significant recovery was found from RasGAP and C-Grb2 SH3 domain and GST columns (Fig. 4). Synapsins IIa/IIb were also eluted from the SH3 domain affinity columns with an elution pattern similar to that of synapsin I, albeit with lower recoveries. The elution patterns of dynamin I and synaptojanin I were qualitatively different from those of synapsin I, with high recoveries of both proteins from amphiphysin I, amphiphysin II, PI3K, c-Src, PLCγ, Grb2, and N-Grb2 SH3 domain columns, recovery of dynamin I from the Crk SH3 domain column, and negligible recoveries of both proteins from the other SH3 domain affinity columns (Fig. 4).

Effects of Site-specific Phosphorylation of Synapsin I on the Interactions with SH3 Domains—We have previously shown that site-specific phosphorylation of synapsin I does not affect its binding to the SH3 domain of c-Src. Using the same co-precipitation technique described above, we analyzed whether phosphorylation of synapsin I in the COOH-terminal region by Ca^{2+}/calmodulin-dependent kinase II (CaMPKII; sites 2 and 3; Ser^{568} and Ser^{605}, respectively) or mitogen-activated protein kinase Erk 1/2 (MAPK; sites 4, 5, and 6; Ser^{62}, Ser^{67}, and Ser^{551}, respectively) was able to affect the binding to any of the SH3 domains analyzed and compared the observed effects with those of phosphorylation by cAMP-dependent protein kinase (PKA; site 1, Ser^{9}) involving the NH_2-terminal region of synapsin I. Although phosphorylation of synapsin I by PKA did not markedly affect the interactions of synapsin I with any of the SH3 domains analyzed, phosphorylation by CaMPKII and MAPK had complex effects. As shown in Fig. 3 (*lower panel*), phosphorylation by either kinase was unable to alter the high affinity interactions of synapsin I with the SH3 domains of c-Src, PI3K, PLCγ, Grb2, and N-Grb2 but negatively modulated the interactions with the SH3 domains of amphiphysins I and II, spectrin, Crk, and p47^{phox} characterized by a lower affinity. The effects were particularly significant on the binding of the SH3 domains of amphiphysin II, spectrin, and Crk and were more intense after CaMPKII phosphorylation than after MAPK phosphorylation of synapsin I. No effects of phosphorylation were found with the very low affinity interactions of synapsin I with RasGAP and C-Grb2 SH3 domains (Fig. 3, *lower panel*).

Localization in Synapsin I of the Sites of Interaction with SH3 Domains—Protein-protein interactions mediated by SH3-binding sequences appear to be very specific and depend on residues flanking the *XPXXP* motif, often including a basic residue (most frequently an arginine). Because SH3-binding peptides can bind SH3 domains in either parallel or antiparallel orientation, the basic residue can be located either NH_2-terminal (position −3, the first P of the core being position 0; Class I ligand, R*XXPXXP*) or COOH-terminal (position +5;

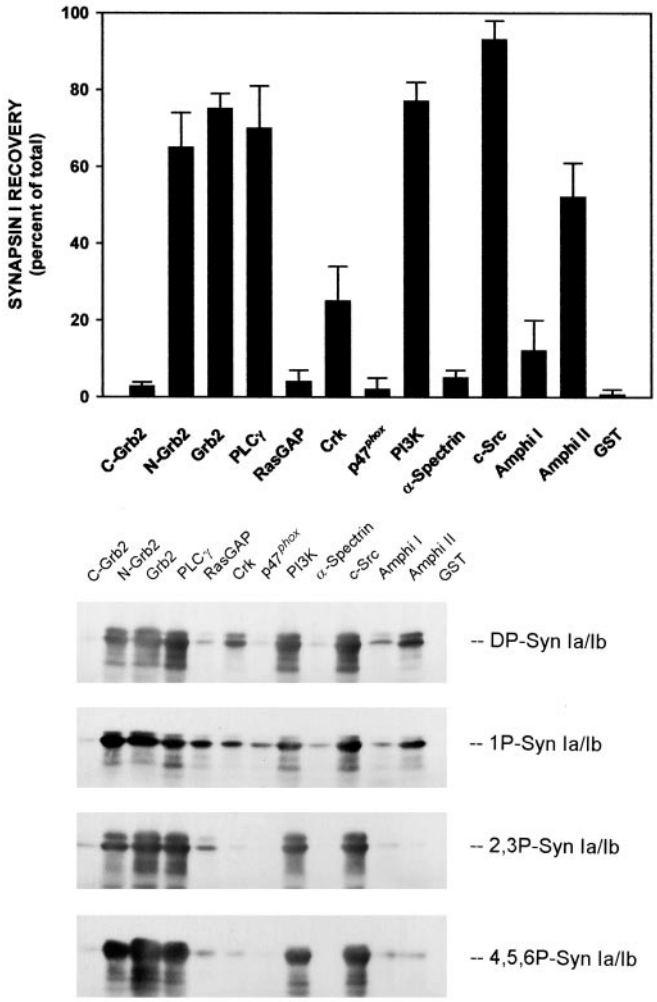

FIG. 3. **Interactions of dephosphorylated and site-specific phosphorylated synapsin I with SH3 domains as evaluated by co-precipitation.** Purified dephosphorylated synapsin I or synapsin I phosphorylated by PKA, CaMPKII, or MAPK was incubated with either GST alone or with the various SH3 domain GST fusion proteins that had been coupled to glutathione-Sepharose as described under "Experimental Procedures." Synapsin I recovered in the pellet was separated by SDS-PAGE and analyzed by quantitative immunoblotting using ^{125}I-secondary antibodies and radioactivity counting. *Upper panel*, the binding of dephosphorylated synapsin I (*DP-Syn Ia/Ib*) to SH3 domain-GST fusion proteins or to GST alone is expressed as a percentage of recovery of the protein in the pellet with respect to the total amount added to the samples and shown as the means ± S.E. (n = 5). *Lower panel*, autoradiography of a representative experiment showing the effects of phosphorylation of synapsin I by PKA on site 1 (*1P-Syn Ia/Ib*), by CaMPKII on sites 2 and 3 (*2,3P-Syn Ia/Ib*), and by MAPK on sites 4–6 (*4,5,6P-Syn Ia/Ib*) on SH3 domain binding.

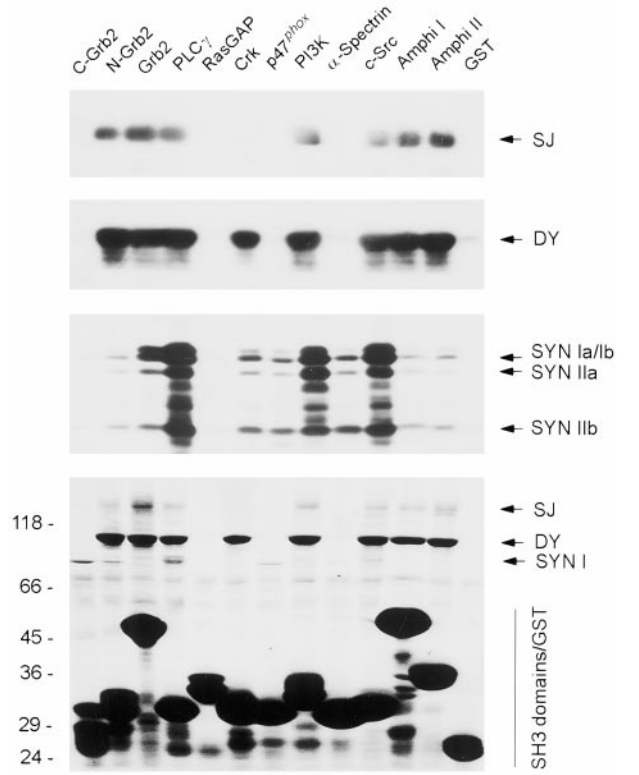

FIG. 4. **Affinity purification of SH3 domain-binding proteins from an extract of crude synaptosomes.** Affinity resins were prepared by coupling either GST-SH3 domains or GST alone to glutathione-Sepharose. Columns were loaded with a Triton X-100 extract of crude synaptosomal fraction (P2) from rat forebrain. Bound proteins were eluted with SDS, separated by SDS-PAGE, and analyzed by Coomassie staining and immunoblotting. The *upper three panels* show the elution patterns of synaptojanin I (*SJ*), dynamin I (*DY*), and synapsins (*SYN*) Ia/Ib and IIa/IIb as revealed by immunoblotting with specific antibodies. The *bottom panel* shows the general elution pattern after Coomassie staining of the gel in which, in addition to the GST fusion proteins, synaptojanin I, dynamin I, and synapsin I are visible. Because of its lower abundance, synapsin I is visible only in the eluates from the columns to which its binding was highest (c-Src, PI3K, and PLCγ SH3 domain affinity columns).

Class II ligand, *X*P*XX*P*X*R) with respect to the *X*P*XX*P motif (1, 3, 36). Although the sequences RPQPPPP (syn I$^{27-33}$, domain A-B) and PAGPTR (syn I$^{595-600}$, domain D) of synapsin I conform exactly to the minimal consensus sequences described for Class I and Class II ligands, respectively, several other *X*P*XX*P motifs are present (37). Previous studies have shown that, despite the presence of *X*P*XX*P consensus sequences in both the NH_2- and the COOH-terminal regions of synapsin I, only the COOH-terminal region was involved in binding the SH3 domains of c-Src and Grb2 (4, 14). Because different SH3 domains may have distinct preferences in terms of binding motifs and binding orientation of the proline-rich stretch (1, 3, 38), we examined the location of the synapsin SH3 domain-binding site(s) by using various approaches.

First, synapsin I was subjected to cysteine-specific chemical cleavage or to digestion with *S. aureus* V8 protease, and the binding of the SH3 domains to the resulting fragments was analyzed by the overlay assay. As found for the c-Src SH3 domain, virtually all SH3 domains interacting with synapsin I bound to the peptides containing the COOH-terminal proline-rich domain D, namely the COOH-terminal fragments (peptides γ_a/γ_b), the middle/COOH-terminal fragment (peptide $\beta\gamma_a/\beta\gamma_b$), and holo-synapsins Ia/Ib (Fig. 5). The peptides α and $\alpha\beta$ containing domains A/B were never labeled by any of the SH3 domains studied, whereas both synapsin I isoforms as well as both γ_a/γ_b and $\beta\gamma_a/\beta\gamma_b$ fragments, originating from synapsins Ia and Ib, were equally labeled in Far Western overlays, excluding a participation of the consensus sequences in domains A/B and of the nonconserved sequences of domains E/F in the interaction with SH3 domains. In agreement with these data, virtually all SH3 domains bound to the V8-generated 35-kDa COOH-terminal fragment, whereas no significant binding was observed to the 10-kDa NH_2-terminal fragment (data not shown).

To identify the sites of interaction with SH3 domains, we have analyzed the SH3 domain binding activity of truncation mutants of the COOH-terminal region of synapsin I as well as the ability of synapsin I peptides encoding some of the putative SH3-binding motifs to inhibit the interactions with various SH3 domains. Recombinant peptides were made corresponding

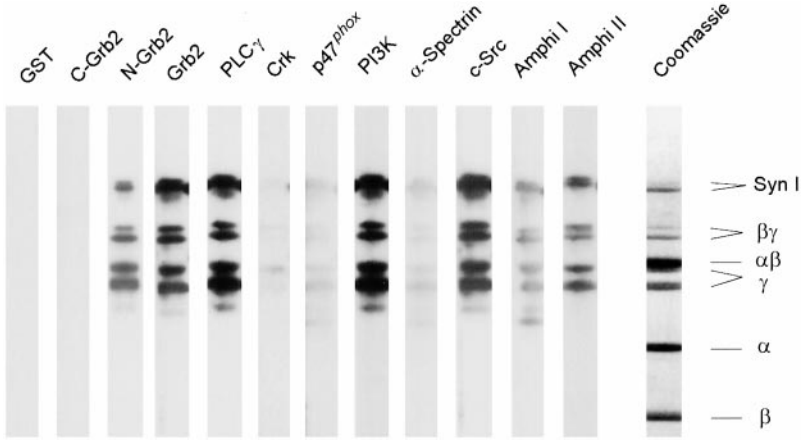

FIG. 5. **Binding of SH3 domains to synapsin I involves its COOH-terminal region.** Synapsin I was cleaved at cysteine residues (Cys^{223}, Cys^{360}, and/or Cys^{370}) with NTCB as described (25), and the resulting fragments were separated by SDS-PAGE on 7–15% polyacrylamide gradient gels and electrophoretically transferred to nitrocellulose membranes. The membranes were then processed for Far Western overlay with the various SH3 domain-GST fusion proteins as described in the legend to Fig. 1. The Coomassie stain of the gel (*right lane*) shows the presence of three fragments spanning the synapsin I molecule, namely fragments α (residues 1–222), β (residues 224–359/369), and γ (residues 359/369–704 and 359/369–668 for synapsins Ia and Ib, respectively), together with undigested synapsin I (*Syn I*) and two additional fragments ($\alpha\beta$ and $\beta\gamma$) resulting from partial cleavage.

to domains DE, domain D, and four distinct truncation mutants of domain D, containing a progressively decreasing number of *XPXXP* motifs (DE peptide, 11; D peptide, 9; D1 peptide, 7, with the last one bearing a mutation; D2 peptide, 5; D3 peptide, 4; and D4 peptide, 2) (Fig. 6, *upper panel*). The various SH3 domains exhibited a distinct binding pattern indicating the presence of multiple SH3-binding sites in the COOH-terminal region of synapsin I. The SH3 domains of c-Src, full-length Grb2, and PI3K bound equally well to DE, D, and D1 peptides, whereas their binding to D2, D3, and D4 peptides was virtually absent. On the other hand, the SH3 domains of amphiphysins and PLCγ exhibited a distinct binding pattern with the amphiphysin I SH3 domain binding only to the DE peptide, the amphiphysin II SH3 domain binding equally well to the DE and D peptides, and the PLCγ SH3 domain binding to all peptides except the D4 mutant (Fig. 6, *lower panel*).

The different binding patterns indicate that the various SH3 domains interact with distinct sites in the synapsin I molecule. The SH3 domains of c-Src, Grb2, and PI3K are likely to interact with the sequence RQGPPQKPPGP (syn $I^{585–595}$; sixth *XPXXP*) as they bound to the D1 mutant (whose Class II binding site was mutated in the second critical proline residue) but not to the shorter mutant D2. On the other hand, the SH3 domains of amphiphysins I and II bound to more COOH-terminal motifs. The amphiphysin I SH3 may interact with either of the two *XPXXP* sequences present in domain E, because its binding was only present with the DE peptide. Because the more NH_2-terminal motif is conserved also in the F domain of synapsin Ib and the amphiphysin I SH3 domain also bound the b isoform of synapsin I in both co-precipitation, affinity chromatography and Far Western overlays, the most likely binding sequence is GGPPHPQL (syn $I^{652–659}$; tenth *XPXXP*). The amphiphysin II SH3 domain interacts with a more NH_2-terminal sequence including either the seventh (PGPAGPIR conforming to the minimal sequence of Class II ligands), the eighth (PRPSGPGP) or the ninth (GRPTKPQL) *XPXXP* motif, because it bound to DE and D peptides but not to the D1 peptide mutated in the seventh *XPXXP* motif. Finally the binding of the PLCγ SH3 domain was preserved up to the D3 peptide and therefore can be localized to the third or fourth *XPXXP* motif (GGPGAPPA or RPPASPSP) (Fig. 6, *upper panel*).

Next we investigated the ability of an array of peptides encompassing one or more of the 11 *XPXXP* motifs present in the COOH-terminal region of synapsin I (C1 peptide, syn $^{531–562}$; C2 peptide, syn $^{585–607}$; C3 peptide. syn $^{593–602}$; and C4 peptide, syn $^{619–634}$, containing the third and fourth; sixth and seventh; seventh; and eighth and ninth *XPXXP* motifs, respectively) to inhibit the binding of the SH3 domains of c-Src, Grb2, PI3K, and PLCγ to the purified synapsin COOH-terminal fragment. Although the C3 peptide encompassing the seventh *XPXXP* Class II binding motif was ineffective, a longer peptide including the sixth *XPXXP* motif (syn $I^{585–607}$) inhibited the binding of the c-Src, Grb2, and PI3K SH3 domains to the synapsin COOH-terminal fragments, confirming that the latter motif is involved in the interaction. The only peptide that was effective in inhibiting the binding of the PLCγ SH3 domain to the synapsin COOH-terminal fragments was the C1 peptide, including the more NH_2-terminal third and fourth *XPXXP* motifs. The C4 peptide was ineffective (Fig. 7).

Effects of the Interactions between Synapsin I and the SH3 Domains of Amphiphysins—We examined whether the interactions of synapsin I with the SH3 domains of amphiphysins I and II are able to affect the ability of synapsin I to interact with actin. Using the sensitive fluorometric assay of pyrenyl-actin polymerization, we found that preincubation of synapsin I with the GST-SH3 domain of either amphiphysin I or amphiphysin II significantly impaired the ability of synapsin I to trigger actin polymerization in the absence of K^+ and Mg^{2+} with an effect that was more pronounced for the amphiphysin I SH3 domain (Fig. 8). The effect was specific for the SH3 domains of amphiphysins, because preincubation of synapsin I with GST alone or with the SH3 domain of either c-Src (Fig. 8) or PI3K (not shown) was completely ineffective.

The ability of amphiphysin SH3 domains to affect the actin filament bundling activity of synapsin I was investigated using both low speed sedimentation and light scattering assays (Fig. 9, *upper* and *lower panels*, respectively). Although neither the c-Src SH3 domain nor GST alone markedly affected the amount of actin bundles recovered in the pellet after incubation with synapsin I or the light scattering of an F-actin/synapsin solution, the SH3 domains of both amphiphysins I and II caused a noticeable inhibition of the synapsin I-induced actin filament bundling that was slightly more pronounced with the amphiphysin I SH3 domain.

We next tested whether the interaction of the COOH-terminal region of synapsin I with the SH3 domains of amphiphysins affected the binding of synapsin I and of its COOH-terminal fragments (peptides γ_a/γ_b) to SV (Fig. 10). It is known that

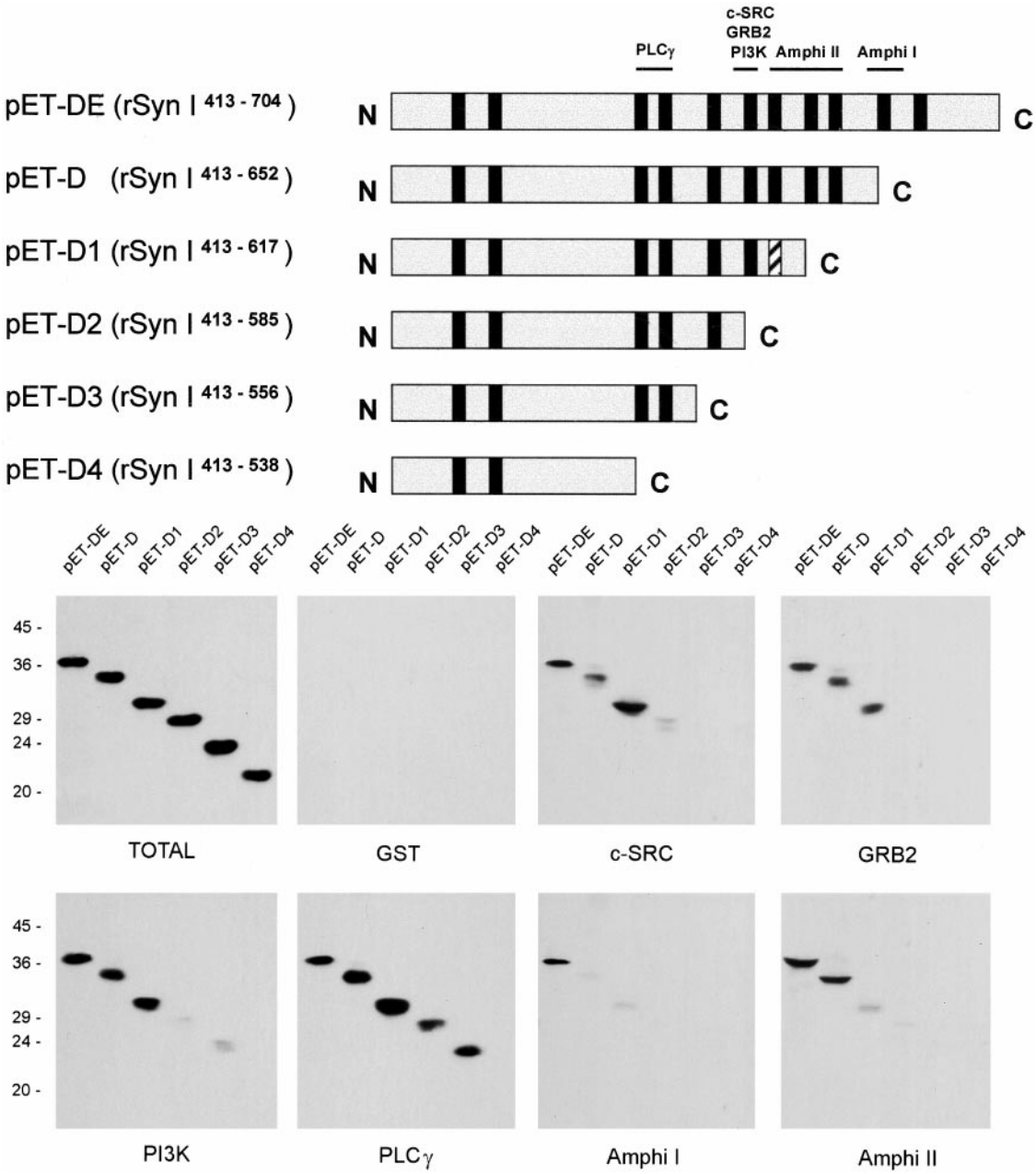

FIG. 6. **Binding of truncation mutants of the COOH-terminal region of synapsin I to SH3 domains.** *Upper panel*, schematic representation of the recombinant rat synapsin Ia domains DE, domain D, and of the four truncation mutants of domain D (D1–D4) progressively missing *XPXXP* motifs (*black rectangles*). The *hatched rectangle* represents the Class II *XPXXP* motif in which one of the critical prolines was mutated. The His_6 and protein S site sequences were fused to the NH_2 terminus of the proteins. The deduced location of the SH3-binding sites is reported on the DE peptide. *Lower panel*, the binding of the recombinant and truncated synapsin domains to GST or to the SH3 domains of c-Src, Grb2, PI3K, PLCγ, and amphiphysins I/II immobilized on glutathione-Sepharose were evaluated by co-precipitation assays as described under "Experimental Procedures." The synapsin peptides recovered in the pellet were separated by SDS-PAGE and analyzed by immunoblotting assays using anti-His_6 antibodies and the chemiluminescence detection system. Aliquots of the synapsin peptides added to the samples are also shown (*TOTAL*).

synapsin I binds to SV through multiple sites and that its COOH-terminal region interacts with protein components of SV, including a vesicle-associated form of CaMPKII and contributes to the binding of the holoprotein (25, 39). Indeed, the SH3 domain of amphiphysin I, but not the SH3 domain of amphiphysin II or c-Src or GST alone, decreased the binding of the COOH-terminal fragment of synapsin I to SV without markedly affecting the binding of holosynapsin I (Fig. 10).

To determine whether an association between synapsin I and amphiphysin I occurs in intact brain, we performed co-immunoprecipitations from detergent extracts of purified synaptosomes using either anti-synapsin I or anti-amphiphysin I antibodies and analyzing the immunoprecipitates for the presence of synapsin I, amphiphysin I and dynamin I. As shown in Fig. 11, the Western blot analysis of the immunoprecipitates demonstrated that both synapsin I and amphiphysin I are present in the complexes and that the binding of dynamin I and the binding of synapsin I seem to be mutually exclusive, because no dynamin was found in association with amphiphysin I precipitated by anti-synapsin antibodies.

DISCUSSION

Synapsin I contains distinct structural and functional domains including a highly conserved central domain interacting with actin, membrane bilayers, and ATP and an elongated and proline-rich COOH-terminal domain which binds to SV-associ-

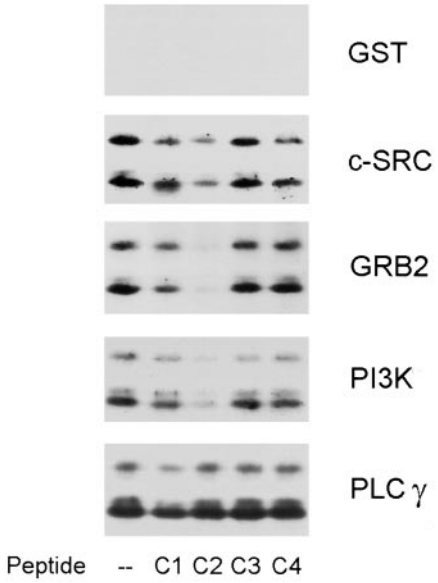

FIG. 7. **Effects of synapsin peptides on the binding of the COOH-terminal region of synapsin I to SH3 domains.** The purified COOH-terminal fragments of synapsins Ia/Ib (γ_a/γ_b fragments) were incubated in the presence or absence of peptides encompassing some of the *XPXXP* motifs of the COOH-terminal region of synapsin I (peptides C1-C4) with either GST alone or with the SH3 domains of c-Src, Grb2, PI3K, and PLCγ that had been coupled to glutathione-Sepharose as described under "Experimental Procedures." The γ_a/γ_b fragments recovered in the pellet were separated by SDS-PAGE and analyzed by immunoblotting assays using anti-synapsin I antibodies and the chemiluminescence detection system.

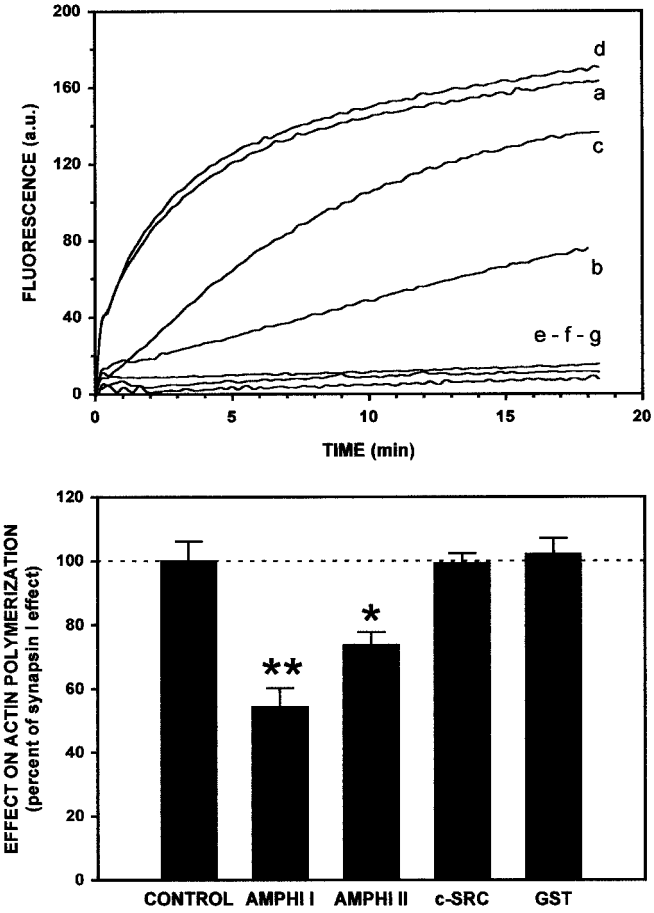

FIG. 8. **The binding of the SH3 domains of amphiphysins I and II decreases the ability of synapsin I to stimulate actin polymerization.** *Upper panel*, the polymerization of pyrenyl-G-actin (5 μM; 5% labeled) was triggered in the absence of KCl and $MgCl_2$ by the addition at time 0 of 300 nM dephosphorylated synapsin I preincubated for 10 min at 25 °C with buffer (*curve a*) or with 2 μM of either amphiphysin I SH3 domain (*curve b*), amphiphysin II SH3 domain (*curve c*), or c-Src SH3 domain (*curve d*). In control curves, G-actin was incubated with the SH3 domain of amphiphysin I, amphiphysin II, or c-Src in the absence of synapsin I (*curves e*, *f*, and *g*, respectively). Buffer conditions were 30 mM NaCl, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM β-mercaptoethanol, 0.5 mM NaN_3, 4.5 mM Tris-HCl, pH 7.6. *a.u.*, arbitrary units. *Lower panel*, the overall effects of the SH3 domains of amphiphysin I, amphiphysin II, c-Src, or of GST on the stimulation of actin polymerization by synapsin I were evaluated as average levels of fluorescence during the 20 min of the assay, corrected by the contribution of the SH3 domain added alone, and expressed as percentages of the effect of synapsin I (control). *Bars* in the plot are the means ± S.E. ($n = 5$). *, $p < 0.05$; **, $p < 0.01$, Dunnett's multiple comparison test.

ated CaMPKII and contains 11 minimal *XPXXP* motifs that can possibly interact with SH3 domains (6, 17, 25, 27, 39, 40). Indeed, we have previously reported that synapsin I is a good ligand for the SH3 domains of Grb2 and for the SH3 domain of c-Src (4, 14). The virtual absence of binding to the SH3 domain of the neuron-specific splice variant of Src, n-Src (Ref. 14; see also Ref. 15), suggested that the SH3 domain binding of synapsin I is specific and can select subsets of SH3 domains. Therefore, we have analyzed by a variety of techniques the interactions of synapsins I and II with an array of SH3 domains belonging to proteins implicated in signal transduction or cytoskeleton assembly. We have found that: (*a*) the binding of synapsin I to SH3 domains is highly specific; the SH3 domains of c-Src, PI3K, PLCγ, and N-Grb2 show the strongest interactions, and the SH3 domains of amphiphysins I and II, Crk, $p47^{phox}$, and α-spectrin exhibit intermediate to weak interactions, whereas the SH3 domains of RasGAP and C-Grb2 do not significantly bind to synapsin I; (*b*) the SH3 domains analyzed bind to distinct sites in the COOH-terminal proline-rich region of synapsin I; (*c*) phosphorylation of synapsin I on the COOH-terminal region by CaMPKII and, to a lesser extent, by MAPK decreases the weak interactions, without affecting the strong interactions with the SH3 domains of c-Src, PI3K, N-Grb2, and PLCγ; (*d*) synapsin II is also able to bind SH3 domains, although with a partly distinct binding pattern and a weaker binding than synapsin I; and (*e*) pools of synapsin I and amphiphysin I can be co-immunoprecipitated from intact nerve terminals and the binding of the SH3 domain of amphiphysin, but not of c-Src SH3 domain, to synapsin I significantly decreases its ability to interact with G- and F-actin and with SV.

In the affinity chromatography experiments, the binding patterns of dynamin I and synaptojanin I, two abundant SH3 domain-binding proteins enriched in nerve terminals, were in substantial agreement with previous reports (41–44). Synapsin I showed the following binding pattern partly distinct from dynamin I and synaptojanin I: all three proteins bound similarly well to the SH3 domains of c-Src, PI3K, and PLCγ; Grb2, dynamin I, and synapsin I bound to Crk SH3 domains, whereas synaptojanin I did not; and dynamin I and synaptojanin I bound with high affinity to both amphiphysins I and II, whereas synapsin I bound them with moderate affinity. As far as the high affinity interactions are concerned, synapsin I seems to be more selective that dynamin I or synaptojanin I; however, it exhibits a much broader spectrum of low affinity interactions that are not apparent with dynamin I or synaptojanin I. This apparent lack of selectivity in the low affinity interactions with SH3 domains is not likely to depend on nonspecific interactions of synapsin I, because the binding was absent with the SH3 domains of n-Src (14) and C-Grb2 and negligible with that of RasGAP. The multiplicity of SH3 domain binding partners shown for synapsin I may be explained either by the presence of one or multiple specific SH3-binding

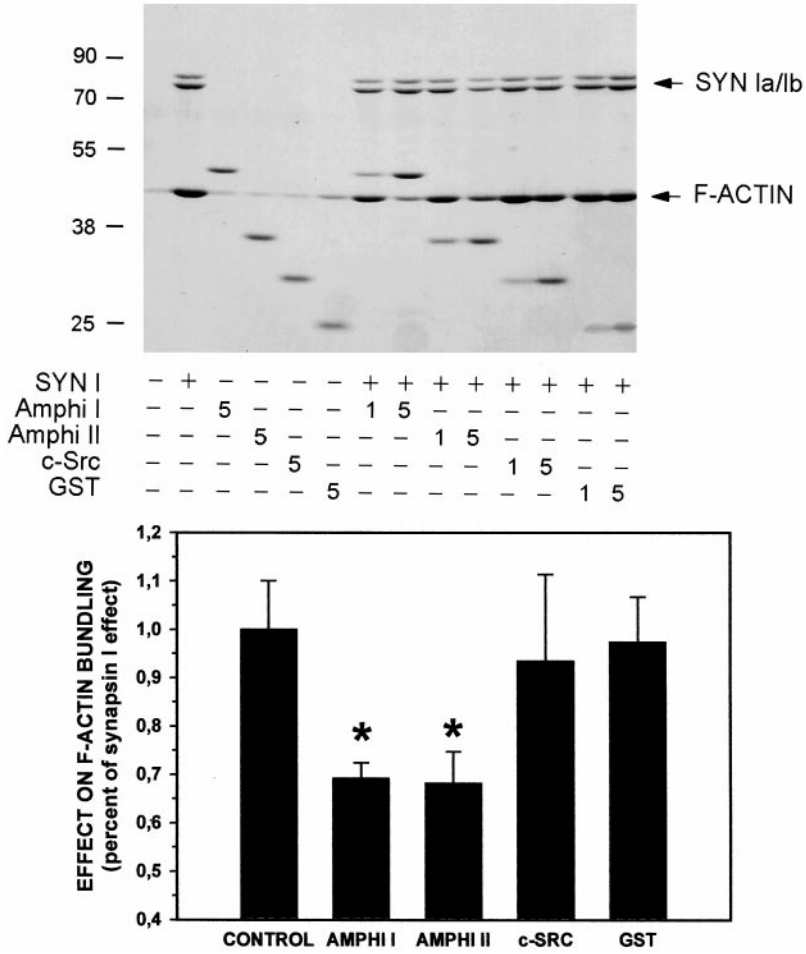

FIG. 9. **The binding of the SH3 domains of amphiphysins I and II decreases the ability of synapsin I to bundle actin filaments.** *Upper panel*, the F-actin bundling activity of synapsin I (*SYN Ia/Ib*; 0.5 μM) in the absence or presence of the SH3 domains of amphiphysin I, amphiphysin II, c-Src, or of GST at the indicated concentrations (in μM) were evaluated by using the low speed sedimentation assay. In the figure, the Coomassie-stained gel of the low speed pellets from a representative experiment is shown. In addition to the F-actin and synapsin I bands representing bundled filaments and synapsin I bound to actin bundles, small amounts of the total SH3 domains or GST added pelleted during the assay and are visible in the gel. *Lower panel*, the effects of the SH3 domains of amphiphysin I, amphiphysin II, c-Src, or of GST on the F-actin bundling activity of synapsin I were evaluated by measuring the light scattering of samples containing F-actin, synapsin I, and 3 μM of the respective SH3 domain or GST, corrected for the contribution of the SH3 domain added alone, and expressed as percentages of the light scattering of the samples containing F-actin and synapsin I (*CONTROL*). *Bars* in the plot are the means ± S.E. (n = 5). *, $p < 0.05$, Dunnett's multiple comparison test.

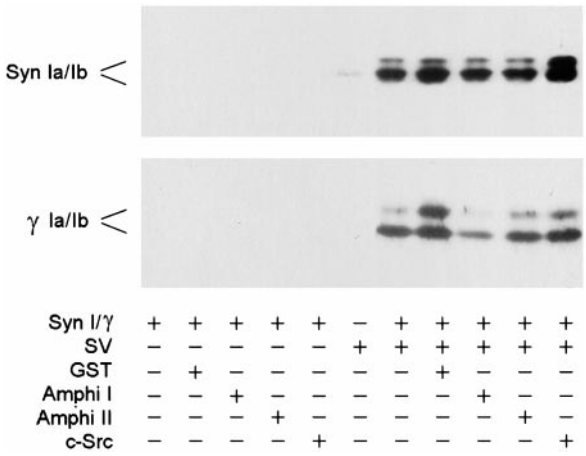

FIG. 10. **Effects of SH3 domains on the binding of synapsin I and of the COOH-terminal region of synapsin I to synaptic vesicles.** Purified synapsin depleted SV were incubated with either purified synapsin Ia/Ib (50 nM) or its COOH-terminal fragments obtained from cysteine-specific cleavage (fragments γ_a/γ_b; 100 nM) in standard assay buffer (300 mM glycine, 5 mM Hepes, pH 7.4, 40 mM NaCl equivalent) in the presence or absence of GST or of the SH3 domains of amphiphysin I, amphiphysin II or c-Src (2 μM). Bound synapsin I or synapsin fragments were separated from the free protein by ultracentrifugation through a 10% (w/v) sucrose cushion and analyzed by SDS/PAGE and immunoblotting. Synaptic vesicle recovery in the pellet, evaluated from synaptophysin immunoreactivity, ranged between 38 and 42%. Sedimentation of synapsin ligands in the absence of SV was negligible.

sites or by promiscuous interactions of various SH3 domains with numerous proline-rich sequences, given the high abundance of minimal *XPXXP* motifs in the synapsin molecule.

Fragment analysis has demonstrated that the portion of the synapsin I molecule engaged in the interaction with all the SH3 domains analyzed corresponds to its COOH-terminal region. The use of truncation mutants of this region, containing a progressively decreasing number of *XPXXP* motifs, and of synthetic peptides encompassing some of the putative synapsin I SH3-binding motifs revealed that distinct binding sites selective for the various SH3 domains are present. In this respect synapsin I resembles dynamin I, whose long proline-rich COOH-terminal region also binds multiple SH3 domains through distinct sites of interaction (41). This finding raises the possibility that synapsin I may recruit different SH3 domain containing proteins and trigger the formation of multimolecular complexes of signaling molecules at the SV membrane.

The putative SH3-binding sequences identified in the COOH-terminal region of synapsins do not conform completely to optimal ligand consensus motifs of the respective SH3 domains identified by screening libraries of phage-displayed or synthetic peptides (41, 45–49) and may therefore represent novel SH3-binding sequences. However, in many cases SH3 domains have been demonstrated to exhibit promiscuous behavior and to recognize proteins that do not contain the predicted optimal binding sequences, sometimes in the absence of either or both proline residues of the *XPXXP* motif (47, 50, 51).

The presence of CaMPKII and MAPK phosphorylation sites in the SH3 domain-binding region of synapsin I prompted us to study whether phosphorylation was an effective modulator of SH3 domain binding. Although phosphorylation was not able to regulate the high affinity interactions with c-Src, PI3K, PLCγ, full-length Grb2, and N-Grb2 SH3 domains, it effectively reduced the weaker interactions with the SH3 domains of am-

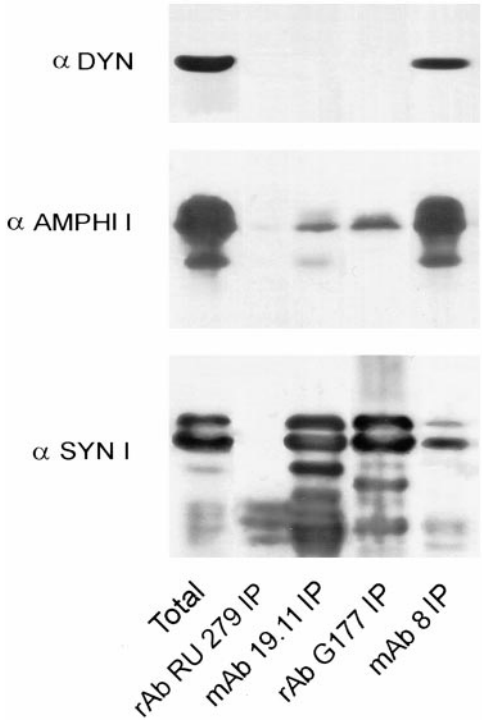

FIG. 11. **Pools of amphiphysin I and synapsin I co-immunoprecipitate from extracts of crude synaptosomal fractions.** Nonidet P-40 (2% v/v) extracts of rat forebrain purified synaptosomes (*Total*) were incubated with either anti-synapsin antibodies (mAb 19.11 or rabbit G177), anti-amphiphysin I antibodies (mAb 8), or a control antibody (rabbit RU279) (10 μg/sample). The immunocomplexes were sedimented with protein G-Sepharose, and the samples were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed with the primary antibodies indicated on the left (anti-dynamin I, α *DYN*; anti-amphiphysin I, α *AMPHI I*; anti-synapsin I, α *SYN I*) followed by a peroxidase-conjugated secondary antibody and chemiluminescence detection system.

phiphysins I/II, α-spectrin, Crk, and p47^{phox}. The effects were more marked for CaMPKII phosphorylation than for MAPK phosphorylation and were practically absent for PKA phosphorylation. The qualitatively similar effects of CaMPKII and MAPK phosphorylation on SH3 domain binding can be ascribed either to a direct electrostatic effect or, as shown for CaMPKII phosphorylation, to a conformational change (52).

Synapsin II also interacts with SH3 domains. Although the results of the affinity chromatography experiments cannot be considered conclusive because of synapsin self-association (53, 54), Far Western overlays with purified synapsin IIa demonstrated that synapsin II can bind SH3 domains directly. Synapsin IIa lacks the proline-rich domain D but exhibits the shorter proline-rich domains G and H, the former being shared by synapsin IIb. Analysis of synapsins IIa/IIb structure indeed revealed the presence of several minimal *XPXXP* consensus sequences in domains A/B, G, and H, including typical Class I motifs in domains A/B (QRPEPQQP; syn II $^{27-34}$), G (KTPPQRP; syn II $^{448-454}$), and H (RRLPSGP; syn IIa$^{477-483}$) (37).

The interactions with the c-Src, Grb2, PI3K, PLCγ, amphiphysin, and α-spectrin SH3 domains may participate in the functions of synapsins and represent an interplay between the regulation of SV trafficking and nerve terminal signaling pathways. The interaction of synapsin I with the SH3 domain of c-Src, followed by a potent stimulation of tyrosine kinase activity, may play a role in the regulation of nerve terminal tyrosine phosphorylation processes and of the cortical actin cytoskeleton (14). The similarity of the SH3 domain binding patterns of synapsin I and dynamin I suggests that both proteins are involved in pathways playing a role in SV trafficking and cytoskeleton assembly. The SH3 domains of amphiphysins exhibit a clear cut preference for dynamin I and synaptojanin I relative to synapsin I, suggesting that the binding sequences identified in dynamin I (PSRPNR; Ref. 43) and synaptojanin I (PIRPSR and PTIPPR; Ref. 52) represent better ligands than the synapsin I sequences. However, the synapsin I/amphiphysin interaction may represent one of the potential links by which amphiphysin may play a role in the physiology of the actin cytoskeleton as suggested by various studies (see Refs. 5 and 8 for review). Experimental evidence indicates that amphiphysin family members are implicated in the regulation of the dynamics of actin cytoskeleton at the nerve terminal: (*a*) amphiphysin mutants in yeast have defects in actin cytoskeleton organization (55, 56); (*b*) suppression of amphiphysin I expression in hippocampal neurons by antisense oligonucleotides results in actin cytoskeleton defects (57); and (*c*) pools of amphiphysin I and dynamin are colocalized with actin patches both in axonal growth cones and cotransfected fibroblasts (57, 58).

The ability of synapsin I to interact with actin and the binding of its COOH-terminal region to SV were decreased upon the interaction of the amphiphysin SH3 domains. The specificity of the effect confirmed that the amphiphysin SH3 domains bind to sequences distinct from those recognized by other SH3 domains. Although the COOH-terminal region of synapsin I is not primarily involved in the interactions with both actin monomers and filaments, it appears to contribute both to bundling of actin filaments (27) and to the clustering of SV at sites of actin filament assembly (59). These data may be of physiological importance because an interaction between synapsin I and amphiphysin I seems to occur *in vivo*, as demonstrated by co-immunoprecipitation experiments.

In conclusion, we have identified specific interactions of the synapsins with several SH3 domain-containing proteins. Most of these proteins are present in nerve terminals, suggesting that these interactions may play some role in the regulation of the exo-endocytotic cycle of SV. The synapsins may represent a template for the recruitment of different SH3 domain-containing proteins through specific interactions mediated by distinct binding sites and may be part of a complex molecular interaction network involving a variety of enzymes, signal transduction molecules and cytoskeletal proteins. Additional work will be required to further explore this possibility and to establish whether such potential protein-protein interactions may occur *in vivo* within nerve terminals.

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Specificity of the Binding of Synapsin I to Src Homology 3 Domains

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