

Synapsin Phosphorylation by Src Tyrosine Kinase Enhances Src Activity in Synaptic Vesicles*[§]

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Synapsins are synaptic vesicle-associated phosphoproteins implicated in the regulation of neurotransmitter release. Synapsin I is the major binding protein for the SH3 domain of the kinase c-Src in synaptic vesicles. Its binding leads to stimulation of synaptic vesicle-associated c-Src activity. We investigated the mechanism and role of Src activation by synapsins on synaptic vesicles. We found that synapsin is tyrosine phosphorylated by c-Src *in vitro* and on intact synaptic vesicles independently of its phosphorylation state on serine. Mass spectrometry revealed a single major phosphorylation site at Tyr³⁰¹, which is highly conserved in all synapsin isoforms and orthologues. Synapsin tyrosine phosphorylation triggered its binding to the SH2 domains of Src or Fyn. However, synapsin selectively activated and was phosphorylated by Src, consistent with the specific enrichment of c-Src in synaptic vesicles over Fyn or n-Src. The activity of Src on synaptic vesicles was controlled by the amount of vesicle-associated synapsin, which is in turn dependent on synapsin serine phosphorylation. Synaptic vesicles depleted of synapsin *in vitro* or derived from synapsin null mice exhibited greatly reduced Src activity and tyrosine phosphorylation of other synaptic vesicle proteins. Disruption of the Src-synapsin interaction by internalization of either the Src SH3 or SH2 domains into synaptosomes decreased synapsin tyrosine phosphorylation and concomitantly increased neurotransmitter release in response to Ca²⁺-ionophores. We conclude that synapsin is an endogenous substrate and activator of synaptic vesicle-associated c-Src and that regulation of Src activity on synaptic vesicles participates in the regulation of neurotransmitter release by synapsin.

The elucidation of signal transduction pathways involved in the actions of neurotransmitters and growth factors in the reg-

ulation of neuronal development, synaptic plasticity, and neuronal survival is of central importance in cellular and molecular neuroscience. A family of synaptic vesicle (SV)²-associated phosphoproteins, the synapsins (synapsins I, II, and III), appear to play a pivotal role both in the regulation of neurotransmitter release and synaptic plasticity from mature neurons and in the development of synaptic connections (1, 2). The synapsins start to be expressed during synaptogenesis and are selectively concentrated in presynaptic terminals where they associate with the cytoplasmic surface of small SVs. The synapsins also interact with both actin filaments and actin monomers, stimulating actin monomer polymerization into filaments and mediating clustering of SVs and their reversible attachment to the actin cytoskeleton (3–5).

The synapsins are at the convergence of multiple signal transduction pathways originating from neurotransmitter receptors, ion channels, and growth factor receptors. They are phosphorylated by cAMP-dependent, Ca²⁺/calmodulin-dependent and mitogen-activated protein kinases (PKA, CaMKI, CaMKII, and MAPK/ERK, respectively) at distinct serine residues. Whereas all synapsins are phosphorylated by PKA and CaMKI in the highly conserved NH₂-terminal domain A, synapsin I is further phosphorylated by CaMKII and MAPK/ERK (1). Serine phosphorylation of synapsins by the various kinases decreases their interactions with SVs and/or actin and promotes their dissociation from the SV clusters and cytoskeletal meshwork of the nerve terminal (6–9). This is accompanied by the diffusion of synapsins toward the preterminal regions of the axon and the transition of SVs from the reserve to the releasable pool (10–13).

The proline-rich COOH-terminal region of synapsin I was shown to interact specifically with an array of SH3 domains including that of c-Src (14, 15). Synapsin I was found to be the major binding protein for the SH3 domain of c-Src (but not of n-Src) in highly purified SVs (16). The interaction of c-Src with synapsin I results in a severalfold stimulation of the tyrosine kinase activity of c-Src that can be antagonized by the purified

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² The abbreviations used are: SV, synaptic vesicle; CaMK, Ca²⁺/calmodulin-dependent kinase; GST, glutathione S-transferase; KO, knockout; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MAPK, mitogen-associated protein kinase; ERK, extracellular signal-regulated kinase; MS/MS, tandem mass spectrometry; PKA, protein kinase A; SH2, Src homology 2; SH3, Src homology 3; WT, wild type; SSV, salt-treated synapsin vesicle; USV, untreated synaptic vesicle; MS, mass spectrometry.

c-Src SH3 domain (17). The stimulatory effect of synapsin I on c-Src activity was ascribed to a competition for the intramolecular interaction between the c-Src SH3 domain and a peptide linking SH2 and catalytic domains that make the catalytic site inaccessible (17). These data suggest that synapsin I may potentially act as an endogenous regulator of c-Src activity in neurons and that the interactions between synapsin I and Src and the synapsin I-induced stimulation of tyrosine kinase activity may be physiologically important in nerve terminal signal transduction.

Neurons express both c-Src and at least two neuron-specific splice variants known as n-Src (18). The very high concentrations of Src family kinases in post-mitotic neurons as compared with other tissues further suggest that pathways involving Src may be implicated not only in neuronal development and differentiation, but also in the response of mature neurons to extracellular stimuli and in synaptic transmission (19, 20). It has been reported that Src and other non-receptor tyrosine kinases such as Pyk2 are necessary and sufficient for the expression of long term potentiation, but that their actions are exerted exclusively or predominantly at the postsynaptic level through phosphorylation and modulation of glutamate and nicotinic receptors (21–24).

Despite the relative abundance of Src kinases in nerve terminals and the identification of Src-phosphorylated SV substrates (16, 25, 26), little is known on the functional role and regulation of Src kinases at the presynaptic level. However, an array of recent studies performed in PC12 cells, primary neurons, and brain synaptosomes using specific Src inhibitors indicates that Src family kinases modulate neurotransmitter release by interfering with activity-dependent Ca^{2+} entry and SV exocytosis (27–30). Src is enriched in purified SVs 4–5-fold as compared with brain homogenate, and accounts for over 70% of the tyrosine kinase activity of these organelles (16). An array of SV proteins can be phosphorylated on tyrosine including 90–110-kDa glycoproteins, synaptophysin, synaptogyrin, and cellugyrin (25, 26, 31–33).

In this paper, we have studied in more detail the regulation of SV-associated Src kinases and the functional role of their interactions with synapsins. We have found that the association/dissociation cycle of synapsin I regulates SV-associated Src activity and tyrosine phosphorylation of SV substrates and that the stimulation of c-Src kinase activity by synapsin I is followed by phosphorylation of synapsin I on tyrosine residues and by its subsequent interaction with the SH2 domains of Src and Fyn.

EXPERIMENTAL PROCEDURES

Materials— $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ ($>3,000$ Ci/mmol) was obtained from Amersham Biosciences. Antibodies were obtained from the following sources: monoclonal anti-Src antibody (clone 327), Calbiochem-Oncogene Research Products (Cambridge, MA); free and agarose-conjugated monoclonal anti-phosphotyrosine antibody (clone 4G10), Upstate Biotechnology (Lake Placid, NY); rabbit anti-phospho-(Tyr⁴¹⁶)Src antibody, Cell Signaling Technology (Boston, MA); peroxidase-conjugated goat anti-rabbit and anti-mouse antibodies, Bio-Rad. Isoform- and domain-specific antibodies against synapsin I raised in our laboratory have been previously described (34). The polyclonal

antibody specific for n-Src, kindly provided by Dr. T. Hunter (The Salk Institute, San Diego, CA), was previously characterized (35). The SH3 and SH2 domain constructs used in this study were generously provided by Drs. Superti-Furga (EMBL, Heidelberg, Germany) and J. S. Brugge (Ariad Pharmaceuticals, Cambridge, MA). Human recombinant c-Src (specific activity 3.0 units/ μl) and Fyn (specific activity 2.2 units/ μl) kinases were purchased from Upstate Biotechnology. Protein G-Sepharose, glutathione-Sepharose, pGEX-2T, and anti-glutathione S-transferase (GST) antibodies were from GE Healthcare. The tyrosine kinase substrate peptide poly(Glu⁸⁰, Tyr²⁰), 2-nitro-5-thiocyanobenzoic acid, N-chlorosuccinimide, ionomycin, and bovine serum albumin were from Sigma. The Src kinase inhibitors geldanamycin, lavendustin C, PP2, and the inactive analogue PP3 were purchased from Calbiochem (Cambridge, MA) and dissolved in dimethyl sulfoxide. The Src inhibitory peptide (PASADGHRGPSAAFVPPAA, corresponding to residues 40–58 of mouse c-Src) and its scrambled (sc) version (AGS HAPFPSPARAGVAPDA) (36) were obtained from Primm (Milano, Italy). Sprague-Dawley rats were from Harlan (S. Pietro al Natisone, Italy). Synapsin I, synapsin II, and synapsin I/II/III knock-out (KO) mice and the corresponding wild-type (WT) littermates were generated in the laboratory of Dr. Greengard (The Rockefeller University, New York) as described (37, 38) and bred in our animal facility. All animal procedures were approved by the Animal Care Committee of the University of Genova and the Italian Ministry of Health. All other materials were obtained from standard commercial suppliers.

Protein Purification and Subcellular Fractionation—Bacterial cells were transformed to ampicillin resistance with pGEX-2T alone or pGEX-2T constructs containing SH3 or SH2 domains by electroporation. Large scale cultures of LB containing ampicillin (100 mg/ml) were inoculated with small overnight cultures, grown at 37 °C, and induced with isopropyl 1-thio- β -D-galactopyranoside (100 mM) for 3.5 h. Glutathione S-transferase and GST fusion proteins were extracted from bacterial lysates and purified to homogeneity by affinity chromatography on glutathione-Sepharose as described (15). Synapsin I was purified from bovine brain (3). Purified synapsin I was stoichiometrically phosphorylated at specific sites *in vitro* using the purified catalytic subunit of PKA, CaMKII, and activated MAPK/ERK as previously described (4, 7). Recombinant rat synapsin II was expressed in insect cells infected with the recombinant baculovirus transfer vector pVLSynIIa and purified to homogeneity (39, 40). Subcellular fractions were prepared from rat forebrain as described (41). Synaptosomes were purified from P2 fractions by centrifugation on discontinuous Percoll gradients as previously described (42). Purification of SVs was performed through the step of either sucrose density gradient centrifugation (mouse) or controlled-pore glass chromatography (rat) (41, 43). Untreated SVs (USV) were quantitatively depleted of synapsin I by dilution (10 μg of protein/ml) in 0.15 M glycine, 0.2 M NaCl, 2.5 mM Hepes (pH 7.4) immediately after elution from the column and incubation for 2 h in ice. After incubation, salt-treated SVs (SSV) were recovered by high speed centrifugation and resuspended in 0.3 M glycine, 5 mM Hepes (pH 7.4) at a protein concentration of 1 mg/ml.

Tyrosine Phosphorylation of Synapsin by Src

SH3 and SH2 Domain Binding Assays—The binding of GST or GST-SH3 domains to purified synapsin I was assessed by co-precipitation experiments using glutathione-Sepharose as previously described (14, 15). Affinity resins for the isolation of SH3/SH2 domain-binding proteins were prepared by immobilizing either GST alone or GST-SH3/SH2 domain fusion proteins on glutathione-Sepharose (0.1 nmol of fusion protein per μ l of settled Sepharose beads) by an overnight incubation at 4 °C in binding buffer (10 mM Hepes, 150 mM NaCl, 1% v/v, Triton X-100, pH 7.4) under gentle rotation in small columns. After extensive wash, 15–20 μ g of fusion protein-coupled beads (15–30 μ l/sample) were incubated with purified dephosphorylated or phosphorylated synapsin I (200 ng) or with SV extracts (100 μ g protein/sample) in 500 μ l 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. The eluted proteins were then separated by SDS-PAGE and analyzed by Coomassie Blue staining of the gels or by immunoblot assay.

Phosphorylation Assays—For the determination of kinase activity, purified recombinant c-Src or Fyn (3 Units/sample) was incubated for 15 min at 30 °C in phosphorylation buffer (20 mM Hepes, pH 7.4, 10 mM MnCl₂, 2 mM EGTA, 0.4 mM Na₃VO₄, 10 mM magnesium acetate, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 50 μ M [γ -³²P]ATP, 1.5 μ Ci/sample) with the substrate peptide poly(Glu⁸⁰, Tyr²⁰) (10 mg/sample) in the absence or presence of increasing concentrations of synapsin I (20–400 nM) and/or the c-Src SH3 domain (0.2–4 mM). Radioactive phosphate incorporation into the substrate peptide was quantified by Cherenkov counting of the lanes excised from SDS-PAGE gels. Synapsin phosphorylation by either Src or Fyn was assessed by incubating various concentrations of purified synapsin I for various times at 30 °C under the same conditions, except that poly(Glu⁸⁰, Tyr²⁰) was omitted. Endogenous SV-associated tyrosine kinase activity and tyrosine phosphorylation of SV proteins was assessed by incubating 30 μ g of protein/sample of either USV or synapsin I-depleted SVs in phosphorylation buffer (20 mM Hepes, pH 7.4, 10 mM MnCl₂, 2 mM EGTA, 0.4 mM Na₃VO₄, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 50 μ M [γ -³²P]ATP, 1.5 μ Ci/sample) in the absence (phosphorylation of SV substrates) or presence (endogenous tyrosine kinase activity) of the substrate peptide. Src activity in the presence of the substrate peptide was evaluated as described above. Tyrosine phosphorylation of SV proteins was assessed by either autoradiography of the SDS-PAGE gels after treatment with alkali (1 M KOH, 1% methanol for 2 h at 60 °C) as previously described (25) or by immunoblotting with anti-phosphotyrosine antibodies.

Immunoprecipitation—Nonidet P-40 (2% v/v) extracts of highly purified SVs or synaptosomes were incubated for 3 h at 4 °C with either anti-Src monoclonal antibody 327 or anti-synapsin I polyclonal antibodies (10 μ g/sample) coupled with protein G-Sepharose (25 μ l of settled prewashed beads) or with anti-phosphotyrosine antibody-coated agarose beads. After extensive washing of the beads, the immunocomplexes were

subjected to phosphorylation assays and/or to SDS-PAGE and immunoblotting assay.

MALDI-TOF and Tandem MS Analysis—Synapsin Ia/Ib purified from bovine brain was phosphorylated with cold ATP, enriched by immunoprecipitation with anti-phosphotyrosine beads, and purified on SDS-polyacrylamide gels. The excised phosphosynapsin I band was then reduced, alkylated, and digested overnight with bovine trypsin as described elsewhere (44). Proteins were unambiguously identified by MALDI-TOF peptide mass mapping. One μ l of the supernatant of the digestion was loaded onto the MALDI target using the dried droplet technique and α -cyano-4-hydroxycinnamic acid or dihydroxybenzoic acid as matrix. MALDI-MS measurements were performed on a Voyager-DE STR time of flight (TOF) mass spectrometer and processed via the Data Explorer software (Applied Biosystems, Foster City, CA). Phosphopeptides were purified with immobilized metal affinity chromatography. Immobilized metal affinity chromatography was performed using the Phosphopeptide Isolation Kit (Pierce Biotechnology) according to the manufacturer's instructions. Phosphopeptides were confirmed by alkaline phosphatase treatment as described previously (45, 46). Phosphorylated peptides were analyzed by tandem MS experiments performed on a Q-Star pulsar (PE SCIEX Instrument, Toronto, Canada). The phosphopeptides purified on immobilized metal affinity chromatography columns were concentrated and desalted over a capillary column manually packed with 200 nl of POROS R3 material, conditioned with 5% formic acid. The peptide mixture was eluted using 1 μ l of 50% methanol, 5% formic acid directly into the nanoelectrospray needle (Protana, Denmark). Multiply charged peptides were fragmented to assign the phosphorylated site.

Protein Entrapping into Synaptosomes and Neurotransmitter Release Assays—For protein entrapment, tissue was homogenized in 2 ml of phosphate-buffered, 0.32 M sucrose (pH 7.4) in the absence or presence of either GST or GST fused with the c-Src SH3 or SH2 domains (6.7 and 20 μ M) and subjected to the standard synaptosome purification procedure (42). The efficiency of protein entrapment was assessed by immunoblotting and found to range between 5 and 10% of the peptide concentration in the homogenization medium (47). Synaptosomes were incubated at 37 °C for 15 min in the presence of 0.04 μ M D-[³H]aspartate (D-[³H]ASP). After labeling, aliquots of the suspensions (<100 μ g of protein/filter) were stratified onto microporous filters at the bottom of parallel superfusion chambers maintained at 37 °C and superfused with standard medium at 0.5 ml/min (47). Under these conditions, synaptosomes constituted a non-confluent monolayer, avoiding indirect effects mediated by compounds released by neighboring particles. After a 36-min equilibration period, four 3-min fractions were collected. Synaptosomes were exposed to a 90-s ionomycin (0.5 μ M) pulse at the end of the first collected fraction ($t = 39$ min). Collected samples and filters were counted for radioactivity. The amount of radioactivity present in each sample was calculated as fractional rate and the stimulus-evoked overflow was estimated by subtracting the appropriate basal release from the radioactivity measured in the samples collected during and after the stimulation pulse. The effects of the internalized SH3

or SH2 domains were then evaluated as the ratio between the evoked overflow calculated in the presence of the proteins and that calculated under control or internalized GST conditions.

Miscellaneous Procedures—SDS-PAGE was performed according to Laemmli (48). Immunoblotting was performed using peroxidase-conjugated secondary antibodies coupled with the chemiluminescence detection system as previously described, except for immunoblotting with anti-phosphotyrosine antibodies in which bovine serum albumin (50 mg/ml) was used as a blocking agent. Immunoblots were quantified by densitometric scanning of the fluorograms obtained in the linear range of the emulsion response or by direct radioactivity counting, followed by interpolation of the values into a suitable standard curve. Statistical analysis was carried out by one-way analysis of variance followed by the Student's *t* test or the Dunnett's multiple comparison test. Dose-response curves were fitted using the Sigmaplot 8.0 program (SPSS Inc., Chicago, IL).

RESULTS

Src Phosphorylates Synapsin I on Tyrosine Residues—We have previously shown that synapsin I interacts with the SH3 domain of c-Src and activates its kinase activity (15, 16). In view of this interaction, we explored the possibility that synapsin I could act as a substrate for c-Src and be phosphorylated on tyrosine residues. *In vitro* experiments, in which purified c-Src was incubated with increasing concentrations of purified synapsin I in the presence of Mn^{2+} /ATP, revealed that synapsin I is indeed phosphorylated on tyrosine residues as shown by both anti-phosphotyrosine immunoblots (Fig. 1A) and residual ^{32}P incorporation after alkali treatment of the gels (not shown). Phosphate incorporation increased with the synapsin I concentration and was inhibited by the c-Src SH3 domain (Fig. 1A). To better characterize the substrate properties of synapsin I for c-Src, ^{32}P incorporation was analyzed as a function of incubation time and substrate concentration. Tyrosine phosphorylation of synapsin I was relatively slow, as compared with serine phosphorylation, increased progressively with time at 30 °C with a time constant (τ_{on}) of 85 ± 6 min (Fig. 1B) and reached a stoichiometry of $\sim 0.52 \pm 0.02$ mol of phosphate/mol of synapsin I. Tyrosine phosphorylation of synapsin I followed a simple Michaelis-Menten kinetics characterized by a K_m of $0.67 \pm 0.14 \mu M$ (Fig. 1C).

We also tested the possibility that serine phosphorylation of synapsin I at distinct sites could alter its ability to catalytically activate c-Src and/or to be phosphorylated by Src. We have previously shown that both dephosphorylated and site-specific phosphorylated synapsin I bind equally well to the SH3 domain of c-Src, but not of n-Src (15–17). As expected, dephosphorylated synapsin I and synapsin I stoichiometrically phosphorylated by PKA, CaMKII, or MAPK/ERK were equally effective in stimulating the activity of purified c-Src with ED_{50} of 50–80 nM (Fig. 2A). Similar to what was found for the catalytic activation of c-Src, phosphorylation of synapsin I by PKA, CaMKII, or MAPK/ERK did not significantly affect ^{32}P incorporation into synapsin I tyrosine residues at any of the concentrations tested (Fig. 2B). The absence of tyrosine residues in the COOH-terminal region of synapsin I restricts the substrate region to the NH_2 -terminal portion of the molecule that is shared, to a large

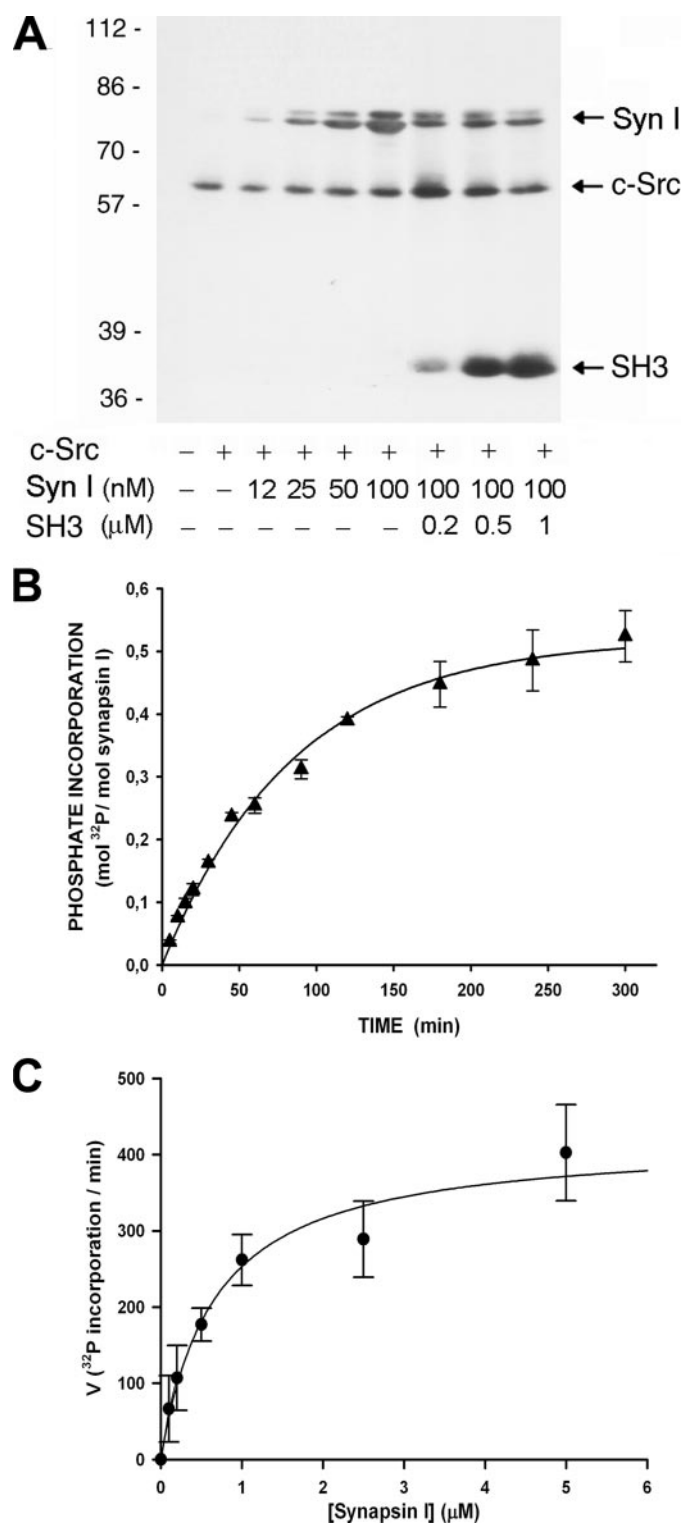


FIGURE 1. Purified synapsin I is phosphorylated by c-Src on tyrosine residues. A, the phosphorylation of synapsin I (12–100 nM) following incubation with c-Src in the presence of Mn^{2+} /ATP was revealed by immunoblotting with anti-phosphotyrosine antibodies. The phosphate incorporation in synapsin I tyrosine residues was inhibited in the presence of increasing concentrations of the c-Src SH3 domain (0.2–1.0 μM). B, ^{32}P incorporation into tyrosine residues of synapsin I (50 nM) in the presence of Na_3VO_4 and c-Src (3 units/sample) increased with the incubation time at 30 °C to reach an average stoichiometry of 0.52 ± 0.02 mol of phosphate/mol of synapsin I with a time constant of 85 ± 6 min. C, the synapsin I phosphorylation rate analyzed after a 15-min incubation at 30 °C followed a Michaelis-Menten kinetics with a K_m of $0.67 \pm 0.14 \mu M$.

Tyrosine Phosphorylation of Synapsin by Src

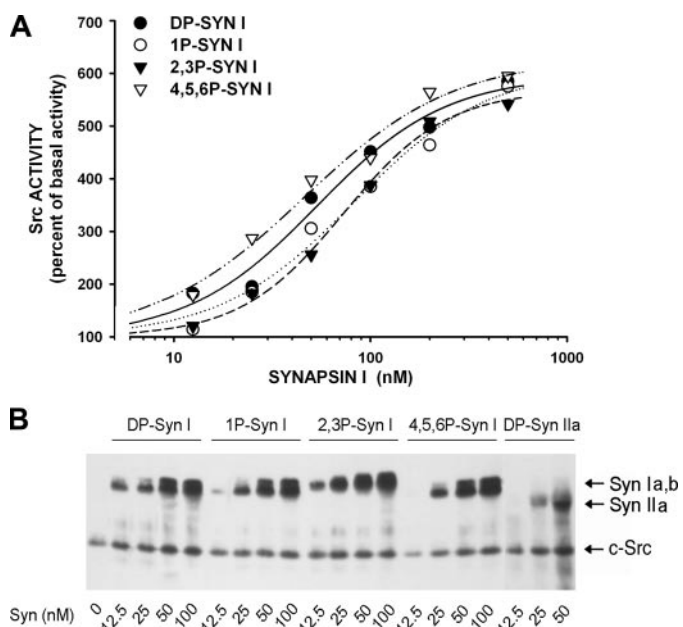


FIGURE 2. The stimulation of c-Src activity and synapsin tyrosine phosphorylation are not affected by the phosphorylation state of synapsin I on serine residues. *A*, dose-response curves for the stimulation of c-Src activity by dephosphorylated synapsin I (DP-Syn I) or synapsin I phosphorylated by either PKA on site 1 (1P-Syn I), CaMKII on sites 2 and 3 (2,3P-Syn I), or MAPK/ERK on sites 4, 5, and 6 (4,5,6P-Syn I). Src activity, evaluated as ^{32}P incorporation into the synthetic peptide poly(Glu⁸⁰, Tyr²⁰), is reported on the y axis in percent of basal activity in the absence of synapsin I. Data reported in the plot are means of three independent experiments and were fitted using a three-parameter logistic function yielding mean (\pm S.E.) plateau values of 596 ± 40 , 609 ± 50 , 569 ± 25 , and $629 \pm 45\%$ and ED_{50} values of 53 ± 10 , 81 ± 17 , 73 ± 9 , and 46 ± 10 nm for DP-Syn I, 1P-Syn I, 2,3P-Syn I, and 4,5,6P-Syn I, respectively. *B*, the phosphorylation on serine residues by PKA (1P-Syn I), CaMKII (2,3P-Syn I), or MAPK/ERK (4,5,6P-Syn I) did not significantly affect the ability of synapsin I (12.5–100 nm) to be phosphorylated by Src. Purified recombinant synapsin IIa (DP-Syn IIa) was also phosphorylated on tyrosine by c-Src.

extent, with synapsin II. As predicted, purified recombinant synapsin IIa was also phosphorylated by Src *in vitro* (Fig. 2B).

Src Phosphorylates Synapsin I at a Single Major Site—To obtain information on the location of the phosphorylation site(s), tyrosine-phosphorylated synapsin I was enriched by immunoprecipitation with anti-phosphotyrosine beads and subjected to MALDI-TOF MS and nanospray MS/MS analysis for the identification of the phosphorylated residues. By MALDI-TOF MS we identified a peptide at $m/z = 1436.7$ that corresponds to phosphorylated Syn^{300–311} and that was shifted to $m/z = 1356.7$ after alkaline phosphatase treatment (data not shown). After immobilized metal affinity chromatography purification the peptide was sequenced by nanospray MS/MS to unambiguously assign the phosphorylation on tyrosine 301 (Fig. 3A). As MS analysis could not exclude the existence of additional sites, we restricted the region containing the major phosphorylation site(s) by subjecting ^{32}P -phosphorylated synapsin I to chemical cleavage at either cysteine (Cys²²³, Cys³⁶⁰, Cys³⁷⁰) or tryptophan (Trp¹²⁶, Trp²²⁷, Trp³³⁵, Trp³⁵⁶) residues (see supplementary Fig. 1). The bulk of phosphate incorporation was found in a central tryptophan-generated peptide (Syn^{227–335/356}) and in the overlapping central cysteine-generated peptide (Syn^{223–360/370}) (Fig. 3B). This region, containing seven tyrosine residues, was fully covered in the MALDI-TOF and MS/MS experiments, supporting the possibility that Tyr³⁰¹

is the major, if not the only, phosphorylation site. This site belongs to one of the most evolutionary conserved synapsin regions and the phosphorylated tyrosine is present in all the analyzed synapsin isoforms and orthologues from *Drosophila* to human (Fig. 3C; Ref. 49).

Synapsin Phosphorylation and Kinase Activation Are Specific for c-Src—To elucidate the specificity of synapsin binding to other major neuronal Src family kinases, we compared the synapsin-induced kinase activation and synapsin tyrosine phosphorylation using purified c-Src and Fyn. Despite a similar interaction with the isolated SH3 domains of both c-Src and Fyn, the stimulation of kinase activity was specific for c-Src (about 7-fold stimulation), whereas it was virtually absent in the case of Fyn (Fig. 4A), suggesting that the SH3 domain-mediated interaction is not the only determinant of the stimulation of kinase activity. This was also suggested by the much less intense Src stimulation brought about by the COOH-terminal region of synapsin I containing the SH3 binding site ($\text{ED}_{50} > 10 \mu\text{M}$; supplementary Fig. S2). Consistent with these data, when synapsin I was incubated under phosphorylation conditions with the same kinase activity of c-Src and Fyn (determined on the basis of the ability to phosphorylate a synthetic substrate peptide) and subsequently analyzed by anti-phosphotyrosine immunoblotting, it was only poorly phosphorylated by Fyn with respect to the strong and dose-dependent phosphorylation induced by c-Src (Fig. 4, B and C).

c-Src Is the Most Enriched Src Family Kinase in Synaptic Vesicles—It was previously shown that an endogenous tyrosine kinase activity, predominantly represented by Src, is associated with highly purified SVs and that multiple integral SV proteins are phosphorylated on tyrosine residues (25, 26). We have also shown that Src is enriched in nerve terminals with respect to homogenate and is associated with the SV membrane from which it can be dissociated only after membrane solubilization (16). In addition to c-Src, neurons express high levels of Fyn and alternative forms of Src in which one or more short exons are inserted into the SH3 domain (n-Src). To get insights into the functional role of the interactions between synapsin I and c-Src, we analyzed the distributions of c-Src, n-Src, and Fyn in subcellular fractions obtained from rat cerebrocortical synaptosomes (P2 fraction) by using antibodies specific for total Src (c-Src + n-Src), n-Src, and Fyn and compared them with the distribution of SV markers synapsin I and synaptophysin. The specific enrichment of total Src, n-Src, and Fyn, expressed in percent of the respective enrichment factors found in synaptosomes revealed that all kinases are enriched in nerve terminal membrane fractions (LP2, SG2/SG4, FT, and SV) and virtually absent from the synaptosol (LS2). Interestingly, n-Src and Fyn appear to be preferentially associated with presynaptic membrane fractions (SG4 and FT), whereas total Src (*i.e.* c-Src + n-Src) exhibits the highest enrichment in highly purified SV, indicating a predominant association of c-Src with SVs (Fig. 5A). This distribution is compatible with a potential functional role of the synapsin I/c-Src interactions in SVs.

The Association of Synapsin I with Synaptic Vesicles Regulates Tyrosine Kinase Activity and Phosphorylation of Vesicle Substrates—Using highly purified SVs, we analyzed whether the SV-associated tyrosine kinase activity and tyrosine phos-

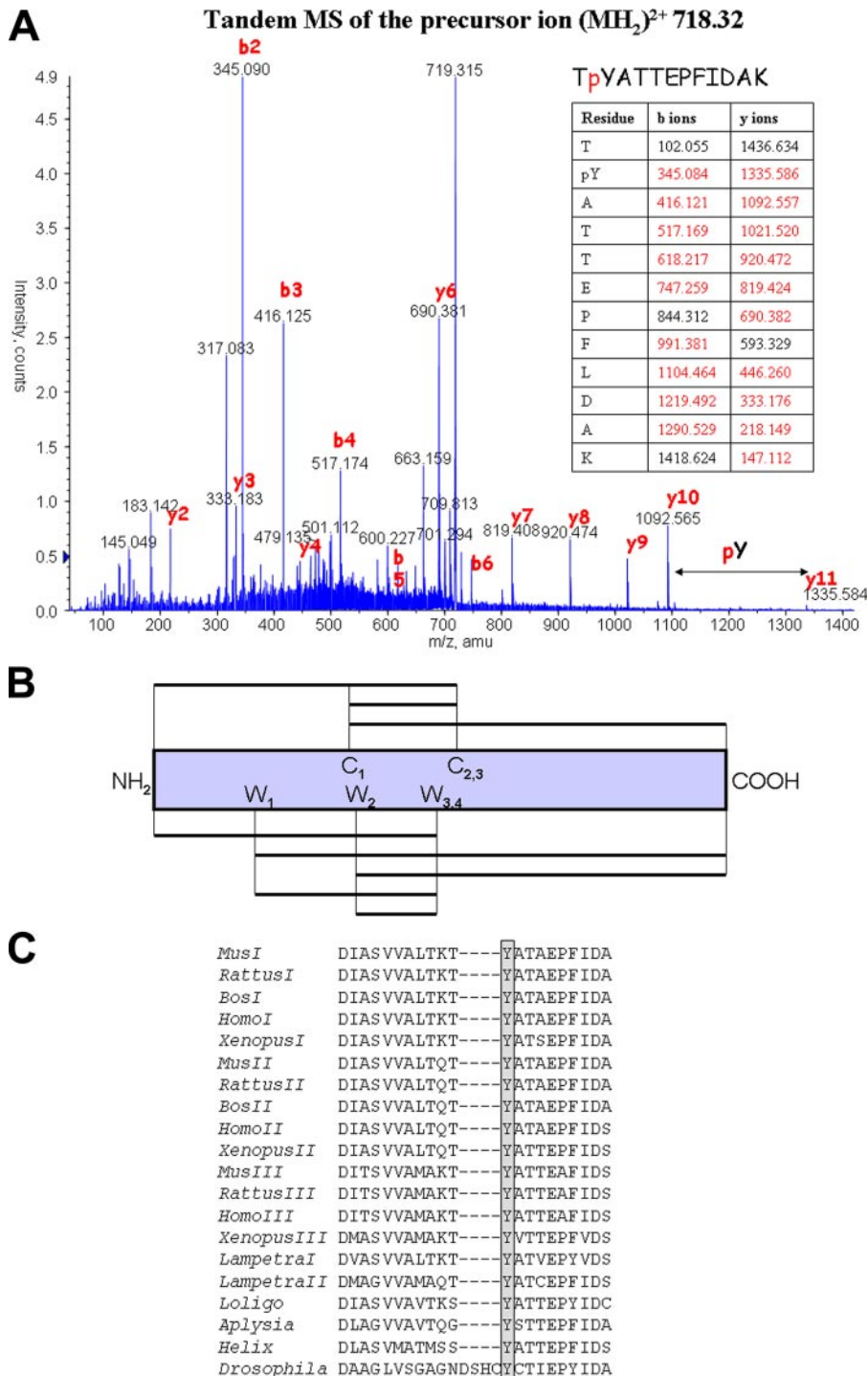


FIGURE 3. Tyr³⁰¹ is the major Src phosphorylation site in synapsin I. *A*, the tyrosine-phosphorylated synapsin I peptide Syn^{300–311} was identified by nanospray MS/MS analysis. The tandem MS spectrum of the phosphopeptide TpYATTEPFIDAK (precursor ion $(MH_2)^{2+}$ 748.3), obtained as described under "Experimental Procedures," is shown. Peaks corresponding to the ions of the series *b* and *y* are labeled and indicated in red in the table. *B*, results of the fragment analysis (see supplementary Fig. 1) showing the location of the cleavage sites within the synapsin molecule and the fragments that displayed significant phosphate incorporation in tyrosine residues after either cysteine- (*C*₁, Cys²²³; *C*_{2,3}, Cys^{360,370}) or tryptophan- (*W*₁, Trp¹²⁶; *W*₂, Trp²²⁷; *W*_{3,4}, Trp^{335,356}) specific cleavage. The fragments that resulted phosphorylated are shown as thick lines. The results restrict a major phosphorylation site between Trp²²⁷ and Trp³³⁵. *C*, the comparison of the primary structure of synapsins across species from human to *Drosophila* demonstrates that the Src-phosphorylated tyrosine residue (Tyr³⁰¹) is evolutionarily highly conserved (49).

phorylation of SV proteins are endogenously activated by the presence of synapsins. When untreated SVs were either incubated with anti-synapsin I (but not with non-related) antibodies

or depleted of endogenous synapsin I by dilution under mild salt conditions (a treatment that does not affect the association of c-Src with SVs; Ref. 16), both the total SV-associated tyrosine kinase activity and the tyrosine phosphorylation of SV proteins were markedly depressed (Fig. 5*B*), suggesting that synapsin I could act as an endogenous activator of SV-associated c-Src. To ascertain that the mild salt treatment used to dissociate synapsin from SVs did not alter Src activity, we examined tyrosine kinase activity and phosphorylation of SV proteins in the absence or presence of the non-ionic detergent Nonidet P-40. The tyrosine kinase activity, depressed in synapsin-depleted SVs, was greatly increased by Nonidet P-40 to levels comparable with those observed with untreated SVs in the presence of detergent, indicating that the endogenous tyrosine kinase activity of SV was not affected by the salt treatment (Fig. 5*C*). At variance with these results, tyrosine phosphorylation of SV substrates was markedly depressed by both synapsin dissociation and detergent-induced solubilization of SVs, supporting the idea that tyrosine phosphorylation of SV substrates is strictly dependent on the integrity of the SV membrane and on the existence of functional interactions between activator, enzyme, and substrates (Fig. 5*C*).

Endogenous Src Is Responsible for Synapsin Phosphorylation in Purified Synaptic Vesicles—We then studied whether endogenous synapsin I could be tyrosine phosphorylated by Src kinases in untreated SV preparations. Highly purified SVs were incubated in the presence or absence of ATP and subjected to immunoprecipitation with anti-phosphotyrosine beads to enrich SV tyrosine phosphoproteins. Synapsin I could be effectively precipitated by the anti-phosphotyrosine beads together with previously described SV-associated phosphoproteins,

including 90–110-kDa glycoproteins, synaptophysin, synaptogyrin (25, 26, 31) and an additional protein of 25 kDa. Interestingly, whereas synaptophysin and synaptogyrin were not

Tyrosine Phosphorylation of Synapsin by Src

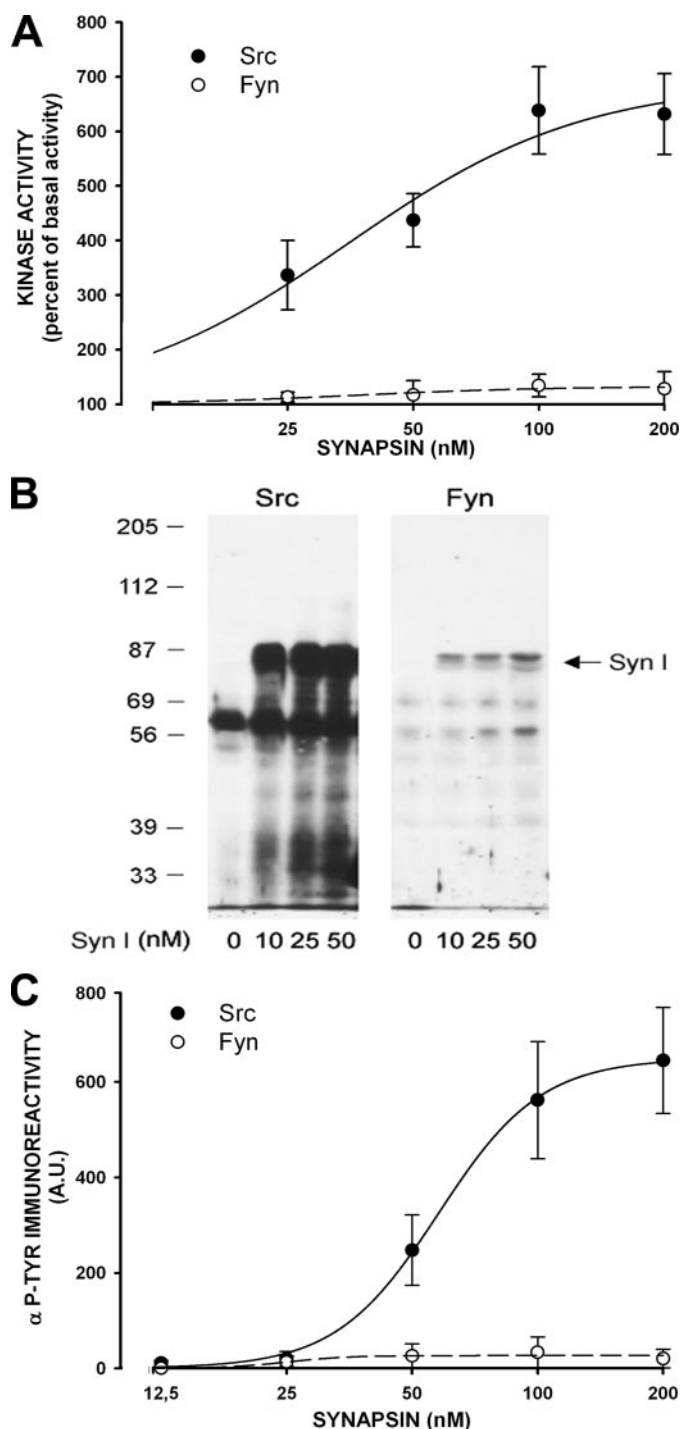


FIGURE 4. Synapsin I preferentially activates and is phosphorylated by Src. *A*, preferential stimulation of c-Src activity by synapsin I with respect to Fyn. Increasing concentrations of dephosphorylated synapsin I (25–200 nM) were incubated with equivalent activities of either purified c-Src (closed symbols) or purified Fyn (open symbols). The resulting kinase activity, evaluated as the ^{32}P incorporation into the substrate peptide poly(Glu⁸⁰,Tyr²⁰), is expressed in percent of the activity of the respective kinase in the absence of synapsin I (mean \pm S.E. of five independent experiments run in triplicate). Data were fitted using a three-parameter logistic function yielding mean (\pm S.E.) plateau values of 691 ± 95 and $133 \pm 9\%$ and ED_{50} values of 35 ± 9 and 36 ± 16 nM for c-Src and Fyn, respectively. *B* and *C*, synapsin I is preferentially phosphorylated by Src with respect to Fyn. *B*, tyrosine phosphorylation of synapsin I (10–50 nM) after 15 min incubation at 30 °C with either c-Src or Fyn was analyzed by immunoblotting with anti-phosphotyrosine antibodies. *C*, increasing concentrations of synapsin I (12.5–200 nM) were incubated with equivalent activities of either purified c-Src (closed symbols) or purified Fyn

immunoprecipitated under basal conditions, synapsin I showed a detectable, albeit low, degree of constitutive phosphorylation (Fig. 6A).

To demonstrate that the SV-associated Src kinase is necessary and sufficient to bring about synapsin I phosphorylation, Src was immunoprecipitated from SV extracts and incubated in the presence of ATP and synapsin I under phosphorylation conditions in the absence or presence of specific inhibitors (Fig. 6, *B* and *C*). In this on-bead phosphorylation assay, endogenous Src immunoprecipitated from SVs effectively phosphorylated purified synapsin I. The Src specificity of synapsin phosphorylation was demonstrated by its sensitivity to Src inhibitors. In fact, the Src-specific inhibitors geldanamycin and lavendustin C significantly inhibited, and the Src family kinase inhibitor PP2 (but not its inactive analogue PP3) virtually abolished, synapsin phosphorylation (Fig. 6*B*). Synapsin I phosphorylation by immunisolated SV Src was also inhibited in a dose-dependent fashion by micromolar concentrations of the specific Src inhibitory peptide Src^{40–58} ($\text{ED}_{50} \approx 70 \mu\text{M}$), whereas the corresponding scrambled peptide was completely ineffective (Fig. 6*C*).

Phosphorylation of Synapsin I by Src Recruits SH2 Domains of Src and Fyn—Src kinases are regulated by a dual mechanism of intramolecular interactions involving both SH3 and SH2 domains. The latter domains are known to bind to sequences in target proteins that contain phosphorylated tyrosines (18). Because synapsin I, as well as several other SV proteins, were found to be phosphorylated on tyrosine, we investigated if any of these substrates, once phosphorylated, were able to recruit the SH2 domain of Src kinases. Isolated SVs or purified synapsin I were preincubated under phosphorylation conditions in the presence of ATP/Src, solubilized, and incubated with either GST or with the GST-SH2 domains of Abl, Fyn, and Src. Tyrosine-phosphorylated synapsin I and the 90–110-kDa glycoproteins effectively co-precipitated from SV extracts with the SH2 domains of Fyn and Src, whereas the interaction with the Abl SH2 domain was weaker. In contrast, synaptophysin and synaptogyrin, which are among the most prominent SV tyrosine phosphoproteins, did not bind to any of the SH2 domains tested (Fig. 7A).

Whereas the 90–110-kDa tyrosine-phosphorylated glycoproteins had a strong preference for the Fyn SH2 domain (Fig. 7A), tyrosine-phosphorylated synapsin I exhibited a similar binding to the SH2 domains of Src and Fyn, with a pull-down efficiency of about 60% of the total phosphorylated synapsin I (Fig. 7, *A* and *B*). As expected, the binding of synapsin I to the SH2 domains of Src/Fyn was strictly phosphorylation-dependent, in contrast with that to the Src/Fyn SH3 domains that was not significantly affected by the synapsin tyrosine phosphorylation state (as previously reported in the case of the serine phosphorylation of synapsin I; Ref. 15), being almost quantitative in the case of both dephosphorylated and phosphorylated synap-

(open symbols). The resulting synapsin phosphorylation, evaluated by quantitative anti-phosphotyrosine immunoblotting, is expressed in arbitrary densitometric units (a.u.) as mean \pm S.E. of five independent experiments run in triplicate. Data were fitted using a three-parameter logistic function yielding mean (\pm S.E.) plateau values of 649 ± 14 and 27 ± 4 and ED_{50} values of 57 ± 2 and 25 ± 4 nM for c-Src and Fyn, respectively.

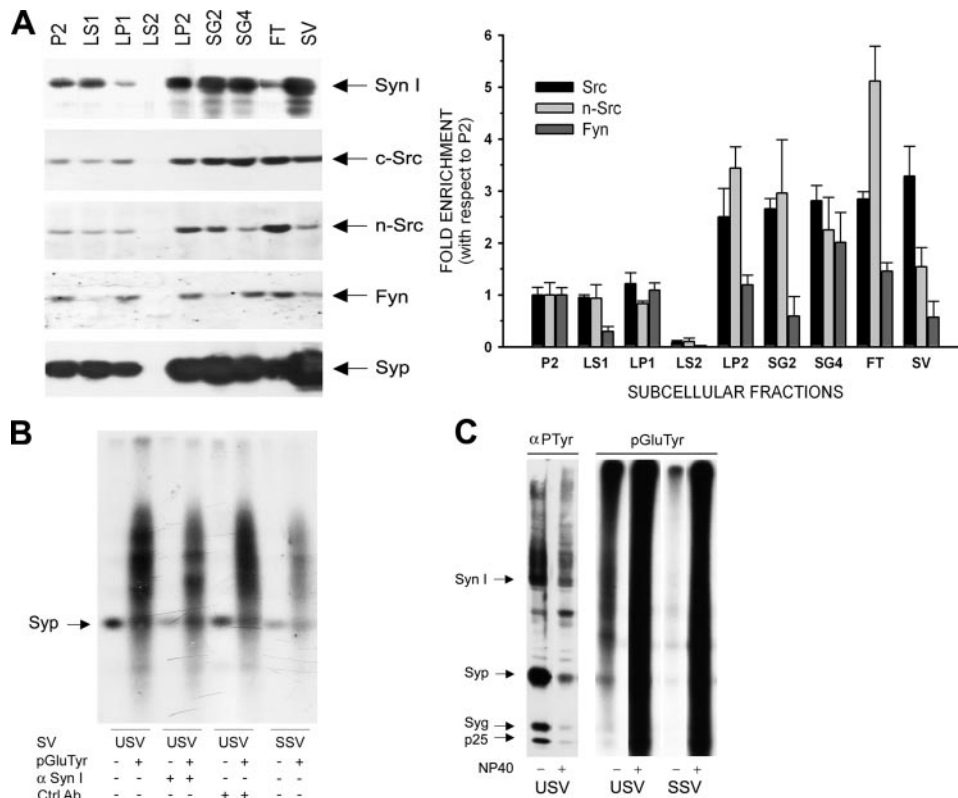


FIGURE 5. c-Src is the major Src family kinase associated with synaptic vesicles, and its activity is stimulated by synapsin I in intact vesicles. *A*, the distribution of Src, n-Src, and Fyn in subcellular fractions of rat forebrain (P2, synaptosomes; LS1, supernatant of LP1; LP1, crude synaptic membranes; LS2, synaptosol; LP2, crude synaptic vesicles; SG2, sucrose gradient-purified synaptic vesicles; SG4 and FT, small synaptic membranes; SV, highly purified synaptic vesicles) was analyzed by quantitative immunoblotting using antibodies against pan-Src (Src), n-Src, and Fyn. The distribution of SV markers synapsin I (Syn I) and synaptophysin (Syp) in the same fractions is also shown for comparison (left panel). The enrichment factors of Src (black bars), n-Src (light gray bars), and Fyn (dark gray bars) in subsynaptosomal fractions are reported as mean \pm S.E. of four independent experiments (right panel). *B* and *C*, depletion of synapsin I results in a marked reduction of SV-associated tyrosine kinase activity and phosphorylation of SV substrates. *B*, USV or SSV (40 μ g/sample) were incubated with [32 P]ATP under phosphorylation conditions in the absence or presence of anti-synapsin I (α Syn I) or control (Ctrl Ab) antibodies. Tyrosine kinase activity was evaluated in the absence or presence of the substrate peptide poly(Glu 80 ,Tyr 20) (pGluTyr) by autoradiography of alkali-treated gels. *C*, the total tyrosine kinase activity of SVs is not affected by synapsin depletion in the presence of non-ionic detergents, whereas phosphorylation of endogenous SV substrates requires the integrity of the SV membrane. USV or SSV (40 μ g/sample) were incubated at 30 $^{\circ}$ C for 15 min under phosphorylation conditions in the absence or presence of 1% Nonidet P-40. The SV tyrosine kinase activity was evaluated both as phosphorylation of SV proteins by immunoblotting with anti-phosphotyrosine antibodies (α PTyr, left) and as 32 P incorporation into the substrate peptide poly(Glu 80 ,Tyr 20) (pGluTyr) by autoradiography of alkali-treated gels (right) and subsequent Cherenkov counting of the excised lanes. No differences in the total kinase activities of USV and SSV were found in the presence of Nonidet P-40. Syn I, synapsin I; Syp, synaptophysin; Syg, synaptogyrin; p25, 25-kDa tyrosine phosphoprotein.

sin I (Fig. 7B). To ascertain whether the interaction of phosphorylated synapsin with the Src SH2 domain is physiologically important in regulating Src activity, we incubated synapsin I with Src in the absence or presence of the SH3, SH2, SH3 plus SH2, or the SH2–SH3 tandem domain of c-Src. Either domain inhibited synapsin-stimulated Src and synapsin tyrosine phosphorylation. When both domains were present (as individual molecules or as tandem domain), the inhibition was more intense, indicating an additive effect of the interactions mediated by the two domains (Fig. 7C).

Src Activity and Tyrosine Phosphorylation of SV Proteins Are Depressed in Synapsin Knock-out Mice—As synapsin I acts as an endogenous activator of c-Src on SVs, it was of interest to analyze whether the levels of Src kinases and/or the tyrosine kinase activity associated with SVs were altered in genetically

altered mice lacking synapsin I, synapsin II, or synapsin I/II/III KO mice. Although the immunoreactive levels of SV-associated Src and Fyn were unaffected in SVs purified from either KO strain as compared with WT littermates (Fig. 8A), the tyrosine kinase activity associated with purified SVs was significantly decreased in the synapsin I KO mice, slightly and not significantly decreased in synapsin II KO and strongly inhibited in triple synapsin KO mice (Fig. 8B). Consistently, a marked inhibition of tyrosine phosphorylation of the SV substrates synaptophysin, synaptogyrin, and p25 was observed in SVs purified from triple synapsin KO mice (Fig. 8, C and D). The latter changes were accompanied by a clear-cut decrease in Src auto-phosphorylation at Tyr 416 , a phosphorylation site linked to Src activation (Fig. 8C).

Inhibition of SH3- or SH2-mediated Interactions in Nerve Terminals Potentiates Ionomycin-induced Neurotransmitter Release—Tyrosine phosphorylation of synapsin I by SV-associated c-Src is believed to follow Src activation by synapsin I through SH3- and SH2-mediated interactions. To evaluate the functional role of these interactions in the control of SV trafficking and neurotransmitter release, purified recombinant SH3 or SH2 domains of Src were entrapped into synaptosomes during the homogenization procedure (Fig. 9A) (47). Synaptosomes were then prelabeled with D-[3 H]aspartate and subjected to

release experiments using ionomycin (0.5 μ M) stimulation, known to diffusely increase intrasynaptosomal Ca $^{2+}$ concentration and induce SV exocytosis with the predominant contribution of the SV reserve pool (50, 51). Whereas basal aspartate release was not affected by any of the entrapped proteins (not shown), the ionomycin-stimulated aspartate release was significantly higher in the presence of the Src SH3 or SH2 domains at both 6.7 and 20 μ M (Fig. 9B). The effects were specific for the internalized peptides, because addition of the same domains after the homogenization procedure (*i.e.* after resealing of the synaptosomes) was totally ineffective (Fig. 9B). The effects of the disruption of Src interactions by entrapping SH2 or SH3 domains indicate a limiting effect of Src activation and tyrosine phosphorylation of Src substrates on neurotransmitter release. One such substrate could be synapsin I, whose tyrosine phos-

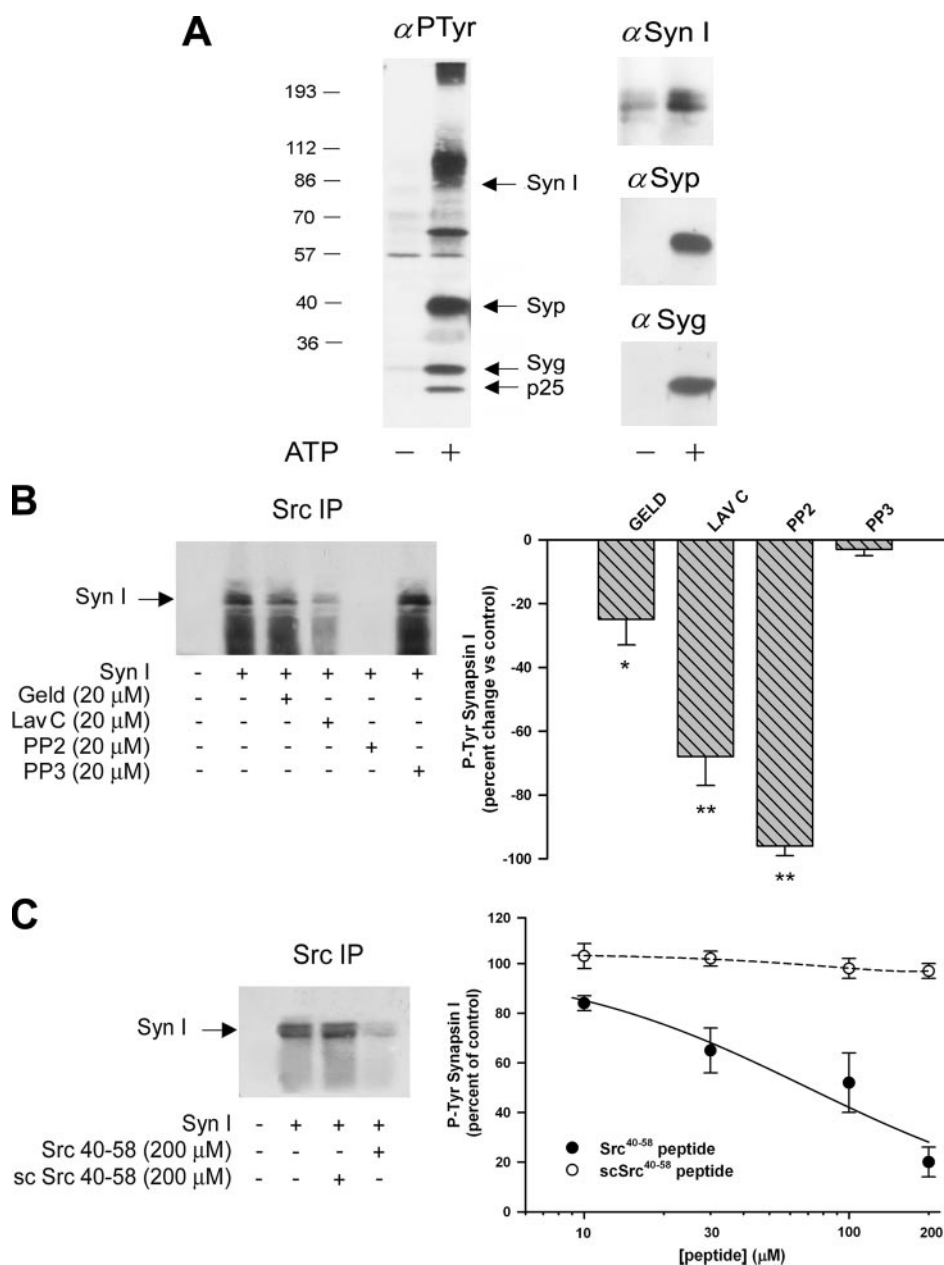


FIGURE 6. Endogenous SV-associated Src is responsible for synapsin I tyrosine phosphorylation in intact synaptic vesicles. *A*, tyrosine-phosphorylated synapsin I can be immunoprecipitated with anti-phosphotyrosine antibodies from SVs. Native SVs (50 μ g/sample) were incubated in the absence or presence of ATP and *c*-Src at 30 $^{\circ}$ C for 15 min, solubilized, and subjected to immunoprecipitation with anti-phosphotyrosine antibodies linked to agarose beads. The immunoprecipitated sample was assayed by immunoblotting with anti-phosphotyrosine antibodies (α PTyr, left) or with antibodies directed against synapsin I (α Syn I), synaptophysin (α Syp), and synaptogyrin (α Syg) (right). *B*, Src was immunoprecipitated from extracts of highly purified SVs using protein G-coupled anti-Src monoclonal antibodies (clone 327; Src IP). Purified synapsin I (50 nM) was then added to Src-containing beads and the samples incubated under phosphorylation conditions in the absence or presence of the Src kinase inhibitors geldanamycin (*Geld*), lavendustin C (*Lav C*), PP2, or its inactive analogue PP3. Tyrosine phosphorylation was detected by quantitative immunoblotting with anti-phosphotyrosine antibodies. The levels of phosphotyrosine immunoreactivity associated with synapsin I, determined by densitometric scanning of the blots, are reported in the right panel in percent of the immunoreactivity of control samples that received the same volume of vehicle (mean \pm S.E. of four independent experiments). *, $p < 0.05$; **, $p < 0.01$, Dunnett's multiple comparison test versus control. *C*, Src was immunoprecipitated and incubated with synapsin I as described above in the absence or presence of increasing concentrations of the Src inhibitory peptide Src⁴⁰⁻⁵⁸ or its scrambled version (scSrc⁴⁰⁻⁵⁸). The levels of phosphotyrosine immunoreactivity associated with synapsin I, determined by densitometric scanning of the blots, are reported in the right panel in percent of the immunoreactivity of control peptide-free samples (mean \pm S.E. of four independent experiments). Data were fitted using a three-parameter logistic function yielding an ED₅₀ value of 70 μ M.

phorylation, evaluated in parallel stimulated samples by immunoprecipitation and anti-phosphotyrosine immunoblotting was significantly decreased by either SH2 or SH3 domain entrapment (Fig. 9C).

DISCUSSION

The understanding of the detailed molecular mechanisms by which non-receptor tyrosine kinases are regulated in neurons and affect neuronal function and development is an important goal in neuronal physiology. It was previously found that the tyrosine kinase *c*-Src is associated with SVs, where it interacts with the proline-rich domain of synapsin I. Upon engagement of its SH3 domain, *c*-Src is activated and phosphorylates a multiplicity of SV proteins including synaptophysins and synaptogyrins (25, 26, 31). As both synapsins and Src have been implicated in the regulation of neurotransmitter release, we have investigated in detail the mechanism of Src activation by synapsins and the possibility that synapsins act as Src substrates on SVs. We have found that: (i) synapsin I is phosphorylated by *c*-Src both *in vitro* and in intact SVs at a single major site conserved in synapsin isoforms and orthologues; (ii) SV-associated Src is regulated by the extent of association of synapsin with SVs and is depressed in synapsin-depleted SVs and synapsin KO mice; (iii) tyrosine-phosphorylated synapsin I interacts with the Src SH2 domain and this association, together with the previously described interaction with the *c*-Src SH3 domain, contributes to Src activation; (iv) internalization of the Src SH3 or SH2 domains into synaptosomes is accompanied by decreased synapsin tyrosine phosphorylation and by a significant increase in neurotransmitter release in response to Ca²⁺ ionophores.

Src SH3 and SH2 domains play important functional roles: they represent an intra/intermolecular mechanism regulating catalytic activity and contribute to the sub-cellular compartmentalization of

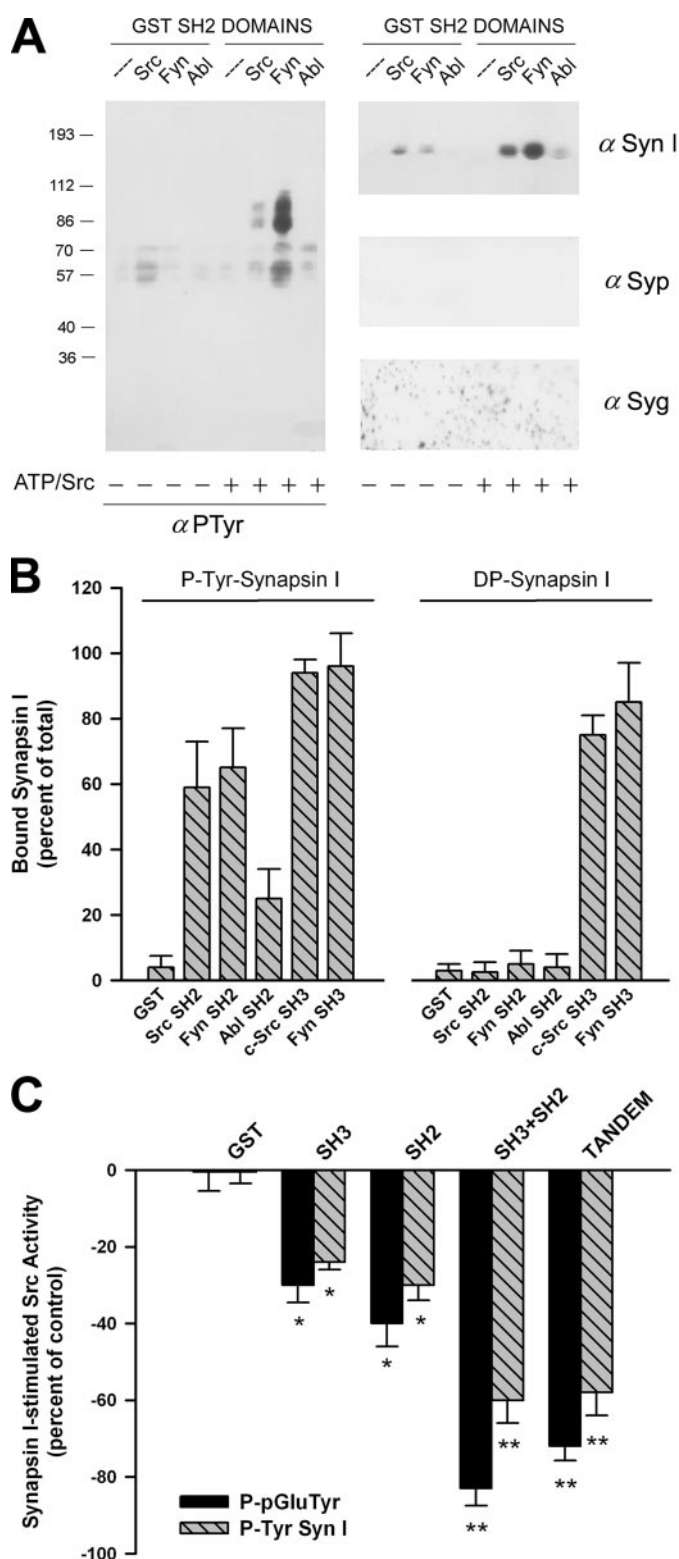


FIGURE 7. Tyrosine-phosphorylated synapsin I binds to the SH2 domains of Src and Fyn. *A*, purified SVs were incubated under tyrosine phosphorylation conditions in the absence or presence of ATP/Src. Sample extracts were subsequently incubated with the indicated GST-SH2 domains immobilized onto glutathione-agarose beads and the specifically bound proteins revealed by immunoblotting with antibodies against either phosphotyrosine (α P-Tyr, left panel) or synapsin I (α Syn I), synaptophysin (α Syp), and synaptogyrin (α Syg) (right panels). Synapsin I and the 90–110-kDa glycoproteins bound to Fyn and Src SH2 domains in a tyrosine phosphorylation-dependent fashion. *B*, synapsin I (200 nM) was incubated in the presence or absence of ATP/Src under tyrosine phosphorylation conditions before the addition of either GST

the kinase and the recruitment of Src interactors and substrates. Src possesses complex built-in regulatory mechanisms that ultimately affect the opening of the cleft between the two lobes of the catalytic domain (18, 52). The apparatus that keeps the kinases in the closed, inactive state has two components, namely an interaction between the SH3 domain and a cryptic SH3 ligand present in the SH2 kinase-linker region (clamp) and an interaction between the SH2 domain and a COOH-terminal phosphotyrosine (Tyr⁵²⁷; latch). As these interactions are relatively weak, exogenous ligands can efficiently compete with them, leading to unclamping (by extrinsic SH3 ligands) and/or unlatching (by extrinsic SH2 ligands) and promoting kinase activation. One such ligand in nerve terminals is synapsin I, which was previously reported to bind the c-Src SH3 domain through its proline-rich Syn^{585–595} sequence present in domain D (15).

Synapsin I is phosphorylated by Src both *in vitro* and in intact SVs. MALDI-TOF MS analysis identified a major phosphorylation site (Tyr³⁰¹) that is highly conserved across evolution and is located in the central domain C. Although MS analysis can theoretically miss additional phosphorylation sites, the results of cysteine- and tryptophan-specific fragment analysis of phosphorylated synapsins support the possibility that Tyr³⁰¹ is the major (or only) Src phosphorylation site in synapsin I. Although the sequence encompassing this site (TKTpYATAE) does not fully conform to the putative Src phosphorylation consensus sequence (EEIpYGEF), the ideal consensus is not present at other potential phosphorylation sites and, furthermore, a large array of proteins that are good substrates for Src kinases do not contain such consensus sequence (18, 53). In contrast to most of serine/threonine kinases that show high specificity for short linear sequences that are also phosphorylated *in vivo*, Src kinases display a significant disparity between ideal peptide consensus sequences and actual sites phosphorylated in proteins and their substrate preferences are not very stringent (54).

It has been shown that the substrate specificity of some tyrosine kinases and the binding specificity of their SH2 domains often overlap, in such a way that some Src kinases can bind their substrates upon phosphorylation (55). The interaction between Src and synapsin I may follow such a model. SH2 domains have a positively charged binding cavity that coordinates binding of the Tyr(P) in the target and provides the majority of the binding energy, whereas the specificity of binding is due to relatively weak interactions with the adjacent residues (18, 53). Studies

or the indicated SH2 or SH3 domains. After pull down with glutathione beads, the binding of tyrosine-phosphorylated (P-Tyr) or dephosphorylated (DP) synapsin I to the domains was assessed by quantitative immunoblotting and expressed in percent of the total synapsin added to the samples (mean \pm S.E. of five independent experiments). *C*, synapsin I (200 nM) was incubated with [³²P]ATP/Src under tyrosine phosphorylation conditions in the presence or absence of the synthetic peptide poly(Glu⁸⁰Tyr²⁰) to evaluate total Src activity (black bars) and synapsin tyrosine phosphorylation (dashed bars), respectively. Incubations were performed in the presence of GST, c-Src SH3 domain (2 μ M), Src SH2 domain (2 μ M), c-Src SH3 domain (2 μ M) plus Src SH2 domain (2 μ M), or c-Src SH2-SH3 domain tandem (2 μ M). Total Src activity and synapsin tyrosine phosphorylation, determined by ³²P counting and anti-phosphotyrosine immunoreactivity, respectively, are expressed in percent of the control samples incubated with GST alone (mean \pm S.E. of five independent experiments). *, $p < 0.05$; **, $p < 0.01$, Dunnett's multiple comparison test versus control.

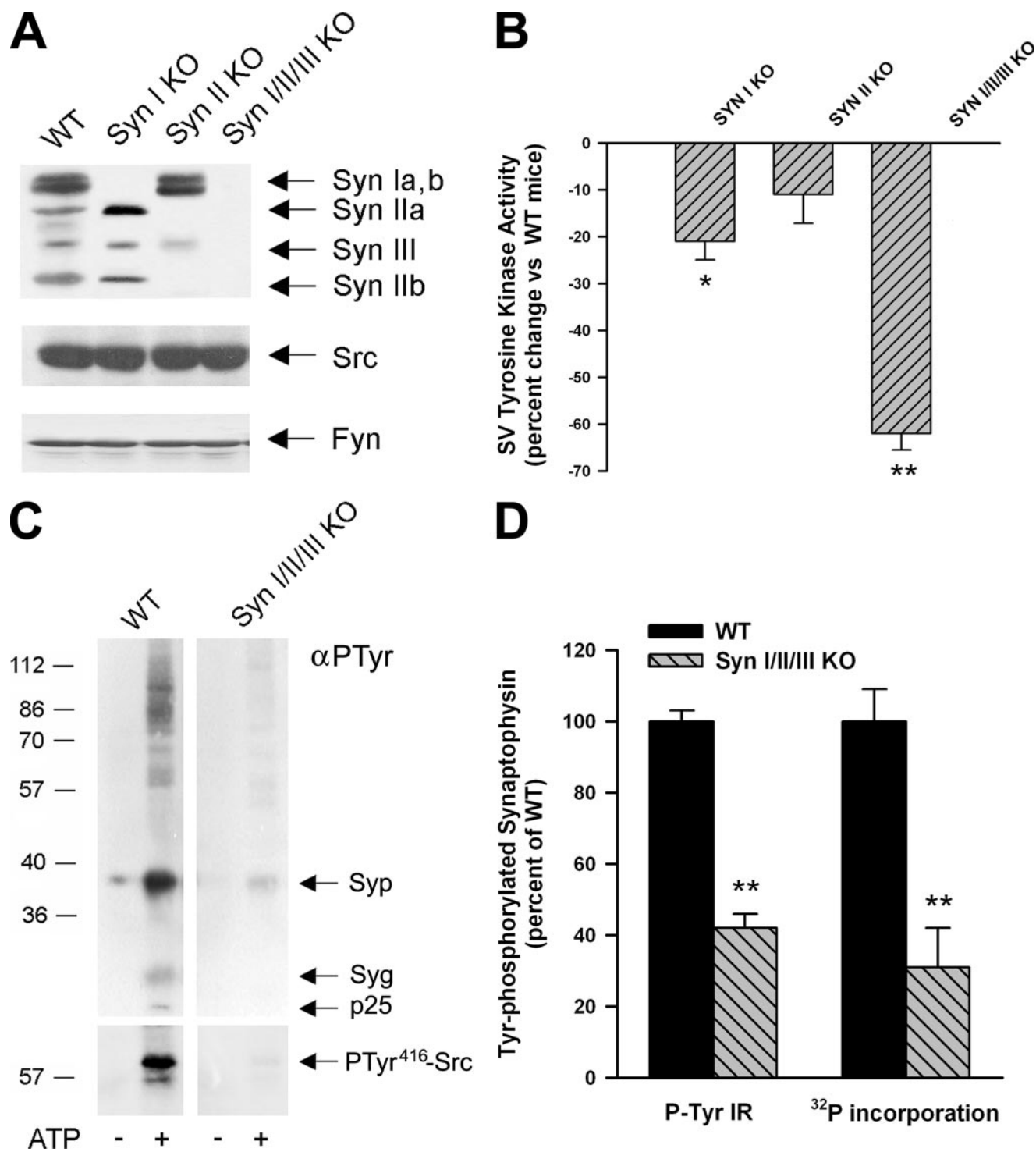


FIGURE 8. Synapsin I knock-out mice exhibit a decreased SV-associated tyrosine kinase activity. *A*, synapsin KO mice have a normal SV complement of Src and Fyn. Native SVs (SG2 fraction) were purified from WT, synapsin I (*Syn I*), synapsin II (*Syn II*), or triple synapsin I/II/III (*Syn I/II/III*) KO mice. *A*, SV samples (5–10 μ g/sample) were analyzed by immunoblotting with antibodies against synapsin I/II/III, Src, and Fyn as described in the legend to Fig. 5. *B*, synapsin KO mice display a decreased tyrosine kinase activity associated with SVs. SVs (25 μ g/sample) were incubated under tyrosine phosphorylation conditions in the presence of poly(Glu⁸⁰,Tyr²⁰). The SV-associated tyrosine kinase activity in synapsin KO mice was quantitatively evaluated by phosphate incorporation into the substrate peptide and expressed as percent changes (mean \pm S.E.; $n = 5$) with respect to WT littermates. *, $p < 0.05$; **, $p < 0.01$; Dunnett's multiple comparison test versus control. *C* and *D*, synapsin I/II/III KO mice exhibit a decreased Src activation and tyrosine phosphorylation of SV substrates. *C*, SVs (25 μ g/sample), incubated as described above in the absence of poly(Glu⁸⁰,Tyr²⁰), were subjected to immunoblotting with either anti-phosphotyrosine antibodies (α PTyr) or anti-Src-P-Tyr⁴¹⁶ phosphospecific antibodies (PTyr⁴¹⁶-Src). *D*, phosphate incorporation into synaptophysin was evaluated in WT and synapsin I/II/III KO mice both as anti-phosphotyrosine immunoreactivity (PTyr-IR) and as ³²P incorporation in the synaptophysin band excised from alkali-treated gels. Data are expressed in percent of the respective WT values (mean \pm S.E. of five independent experiments). **, $p < 0.01$; Dunnett's multiple comparison test versus WT.

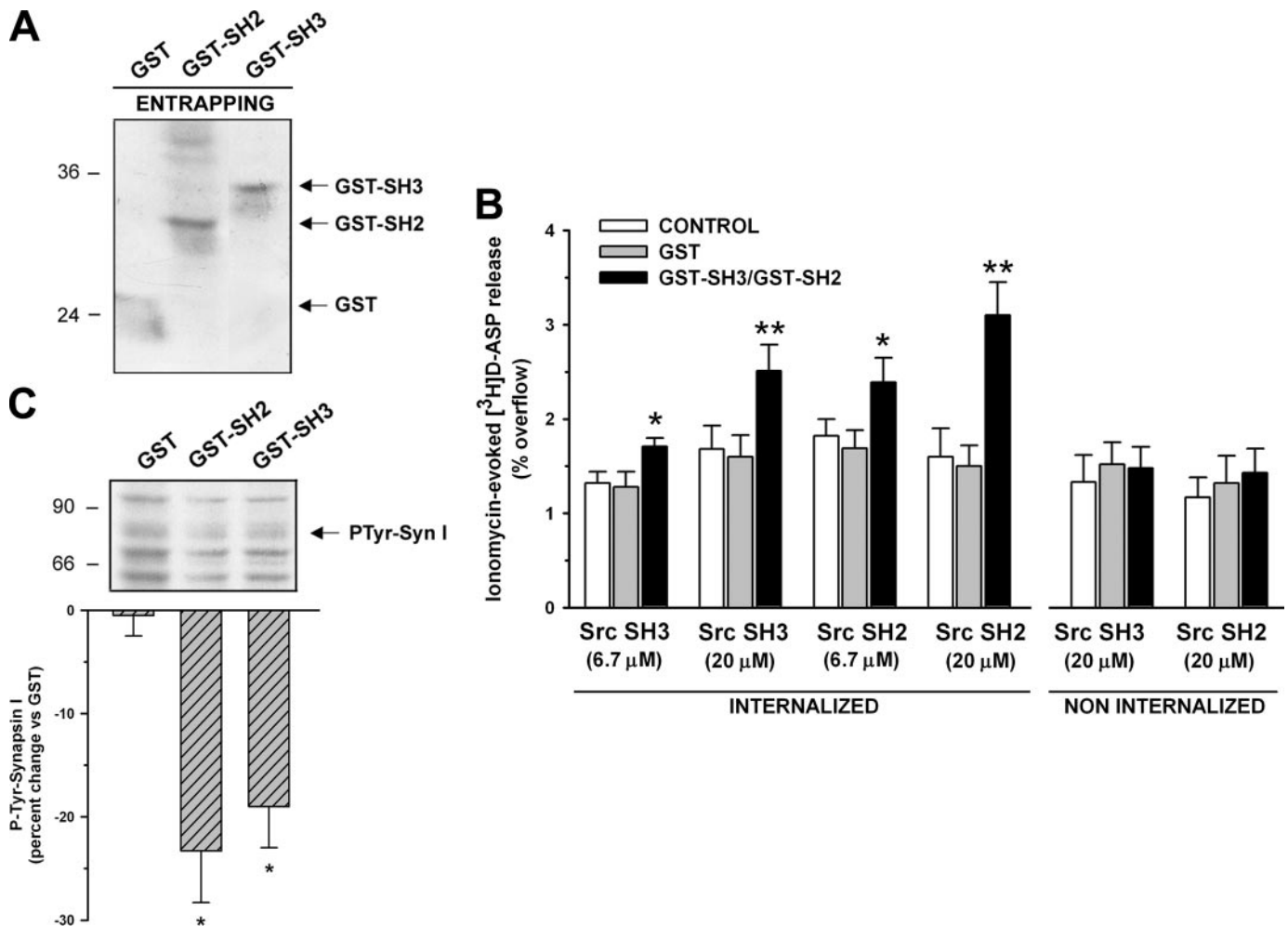


FIGURE 9. Internalization of Src SH3 and SH2 domains increases ionomycin-evoked [³H]aspartate release. Rat brain cortex was homogenized in the absence (control) or presence of GST, GST-c-Src SH3 domain, or GST-Src SH2 domain (6.7 and 20 μM) before synaptosome purification through the Percoll gradient procedure. *A*, before the release experiments, samples of the synaptosomal preparations were subjected to SDS-PAGE and immunoblotting with anti-GST antibodies to check the entrapment of the respective peptides. *B*, control synaptosomes (white bars) or synaptosomes containing either GST (gray bars) or the respective Src domain (black bars) were pre-labeled with D-[³H]aspartate ([³H]D-ASP) and exposed in superfusion to 0.5 μM ionomycin for 90 s. Results are expressed as stimulus-evoked overflow, estimated by subtracting the amount of radioactivity of the appropriate basal release from the radioactivity collected during and after the stimulation pulse. The ³H overflow in the 3-min fraction collected before the onset of stimulation was 0.67 ± 0.03% (*n* = 14). As a negative control for internalization, SH3 or SH2 domains were added after homogenization (see "non-internalized" samples on the right). Bars are mean ± S.E. of 5–10 independent experiments. *, *p* < 0.05; **, *p* < 0.01; Dunnett's multiple comparison test versus control or GST alone. *C*, parallel samples of ionomycin-stimulated synaptosomes were subjected to synapsin I immunoprecipitation with polyclonal antibodies (G177) followed by immunoblotting with monoclonal anti-phosphotyrosine antibodies. In the lower panel, synapsin I tyrosine phosphorylation, analyzed by densitometric scanning of the fluorograms, is expressed as percent changes with respect to the samples in which GST alone was entrapped. *, *p* < 0.05; Dunnett's multiple comparison test versus GST.

with degenerate phosphopeptide libraries have suggested the preference of the SH2 domains of Src, Fyn, and Lck for the sequence pYEEI (55, 56). With respect to this ideal consensus, the sequence encompassing Tyr³⁰¹ in synapsin I contains two hydrophobic alanine residues at pY+1/+3 positions and a glutamate residue at the pY+4 position. Moreover, it has been shown that phosphoproteins that deviate from the ideal consensus may, nonetheless, act as SH2 ligands. The Src SH2 domain has been reported to bind to the phosphorylated sequence pYQPGEN in its COOH-terminal regulatory tail or to the pYYYYV sequence of the β-platelet-derived growth factor receptor and the SH2 domain of the homologous kinase Hck binds to the respective COOH-terminal regulatory sequence pYQQQ, all sequences that are quite different from the preferential binding consensus (57–59). In addition, SH2 peptide studies revealed that the phosphorylated tyrosine can be followed by three residues at which virtually any amino

acid, except for tryptophan and cysteine, can be present (55), indicating that residues beyond the pY+3 position, as well as long range interactions, can participate in SH2 binding by affecting affinity and/or specificity.

The experimental data are compatible with a model in which dephosphorylated synapsin I recruits c-Src through an SH3-mediated interaction and induces an initial activation of the kinase, which is followed by synapsin phosphorylation at Tyr³⁰¹, recruitment of the SH2 domain, and further activation of the kinase. Several protein-protein interactions are mediated by tandems of SH3 and/or SH2 domains. For example, the stimulation of SH-PTP2 (protein-tyrosine phosphatase 2) activity or the activation of RasGAP through the formation of a complex with RhoGAP involve a tandem of SH2 domains (60, 61). An SH3/SH2 tandem interaction has been reported to occur in the case of Src and Fyn activation by the focal adhesion kinase in

Tyrosine Phosphorylation of Synapsin by Src

which integrin engagement or activation of G protein-coupled receptors promotes autophosphorylation of focal adhesion kinase, followed by recruitment and activation of Src and Fyn through a tandem of SH2 and SH3 domain-mediated interactions (62, 63). Here we have shown that tyrosine-phosphorylated synapsin I effectively binds to both Src SH2 and SH3 domains, and that addition of either or both domains inhibits both the synapsin-induced Src activation and synapsin phosphorylation on tyrosine. Moreover, it is likely that the interaction between synapsin I and c-Src involves multiple sites in addition to those binding to the SH3 and SH2 domains. This possibility could also explain the specificity for Src activation and phosphorylation with respect to Fyn, notwithstanding the comparable binding specificity of synapsin I for the SH3 and SH2 domains of both kinases.

Studies in synapsin-depleted SVs and synapsin KO mice suggest that the synapsin-induced activation of Src is responsible for tyrosine phosphorylation of SV proteins and is therefore dependent on the extent of association of synapsin I with SVs. Synapsin I is known to cycle between a cytosolic and a SV-bound form based on its serine phosphorylation by an array of extracellular signal-regulated kinases including PKA, CaMKs, and MAPK/ERK (10–13). Thus, serine phosphorylation/dephosphorylation of synapsin I, by controlling synapsin I dissociation from/association with SVs (6, 64), may indirectly regulate SV-associated Src activity and tyrosine phosphorylation of SV substrates such as synaptophysins, synaptogyrins, and synapsins. Thus, cycles of synapsin dissociation from/association with SVs under the control of serine kinases may be nested with parallel cycles of Src inactivation-activation by synapsin that in turn affect the phosphorylation state of synapsin at tyrosine residues. Because Src has been recently demonstrated to negatively modulate neurotransmitter release (27, 30), the reduction of Src activity brought about by the stimulus- and serine phosphorylation-dependent dissociation of synapsin from SVs may participate in the facilitation of neurotransmitter release.

The Src phosphorylation site is located in synapsin domain C involved in SV and actin binding as well as in synapsin dimerization (64–68). Thus, the possibility exists that tyrosine phosphorylation of synapsin I modulates these interactions and thereby participates in the regulation of SV trafficking and availability for exocytosis. Unfortunately, a squid synapsin peptide encompassing the Src phosphorylation site (s-PepC3) could not be tested physiologically owing its poor solubility in aqueous medium (69).

The results obtained in synaptosomes in which the Src SH2 and SH3 domains were entrapped are consistent with previously reported data showing an increase in depolarization- or ionomycin-evoked glutamate release from cerebellar granule cells (27) and rat brain synaptosomes (30) following Src family kinase inhibition with PP1 or PP2. Whereas our data support the previously reported inhibitory role of Src family kinases in the modulation of Ca^{2+} -dependent glutamate release, they directly implicate Src and tyrosine phosphorylation of synapsins and/or other SV-associated Src substrates in the regulation of SV trafficking and neurotransmitter release. Because the Ca^{2+} ionophore-induced release reflects the major contribution of reserve SVs (50, 51), the enhancement of neurotransmit-

ter release induced by ionomycin observed after disruption of Src SH3/SH2-mediated interactions is consistent with an effect of Src activation and synapsin tyrosine phosphorylation on neurotransmitter release that occurs downstream of Ca^{2+} entry and mainly involves the reserve pool of SVs.

In conclusion, the present data further emphasize the central role of synapsin I in the convergence and cross-talk of distinct signal transduction pathways activated by extracellular messengers and electrical activity and in the intracellular integration of these signals. It will be of key interest to establish the effects of tyrosine phosphorylation on the molecular interactions of synapsin I in nerve terminals and whether synapsin phosphorylation by Src is physiologically regulated by extracellular messengers.

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