

# Characterization of Synapsin I Fragments Produced by Cysteine-specific Cleavage: a Study of Their Interactions with F-actin

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**Abstract.** Synapsin I is a neuron-specific phosphoprotein that is concentrated in the presynaptic nerve terminal in association with the cytoplasmic surface of synaptic vesicles. It has been demonstrated to bundle F-actin in a phosphorylation-dependent manner *in vitro*, a property consistent with its proposed role in linking synaptic vesicles to the cytoskeleton and its involvement in the regulation of neurotransmitter release. Synapsin I is composed of two distinct domains, a COOH terminal, collagenase-sensitive, hydrophilic, and strongly basic tail region, and an NH<sub>2</sub> terminal, collagenase-resistant head region relatively rich in hydrophobic amino acids. To elucidate the structural basis for the interactions between synapsin I and F-actin and how it relates to other characteristics of synapsin I, we have performed a structure-function analysis of fragments of synapsin I produced by cysteine-specific

cleavage with 2-nitro-5-thiocyanobenzoic acid. The fragments were identified and aligned with the parent molecule using the deduced primary structure of synapsin I and the known phosphorylation sites as markers. We have purified these fragments and examined their interactions with F-actin. Two distinct fragments, a 29-kD NH<sub>2</sub>-terminal fragment and a 15-kD middle fragment, were shown to contain F-actin binding sites. A 51/54-kD middle/tail fragment retained the F-actin binding and bundling activity of synapsin I, but the isolated tail fragment did not retain either activity. In contrast to phosphorylation of sites two and three in intact synapsin I, which abolishes F-actin bundling activity, phosphorylation of these sites in the middle/tail fragment failed to abolish this activity. In conclusion, three domains of synapsin I appear to be involved in F-actin binding and bundling.

**S**YNAPSIN I is a neuronal phosphoprotein implicated in regulation of neurotransmitter release (for review, see reference 9). It consists of two closely related proteins, termed synapsin Ia and synapsin Ib. Synapsin I is concentrated in presynaptic nerve terminals where it is associated with small synaptic vesicles (10, 11, 16, 29). It is a major substrate for cAMP-dependent protein kinase and for calcium/calmodulin-dependent protein kinase I, which phosphorylate it on site 1, and for calcium/calmodulin-dependent protein kinase II, which phosphorylates it on sites 2 and 3 (8, 15, 27, 28). A variety of physiological and pharmacological stimuli that affect synaptic function have been demonstrated to modify the phosphorylation state of synapsin I (9, 12).

Synapsin I binds with high affinity to purified synaptic vesicles (32), exhibits a tendency to self-associate (35) and is highly surfactant (M. Ho et al., manuscript in preparation). Injection of dephosphorylated synapsin I into the presynaptic terminal of the squid giant synapse inhibits neurotransmitter

release. This effect is prevented by phosphorylation of sites 2 and 3 within the tail region of synapsin I (20). In addition, synapsin I has been found to interact *in vitro* with several cytoskeletal proteins (2-4, 13, 30). Synapsin I binds to the sides of actin filaments and bundles them (2, 30). This bundling activity is diminished by phosphorylation of site 1 in the head region, and is virtually abolished by phosphorylation of sites 2 and 3 in the tail region of synapsin I (2).

To elucidate the structural basis for the interactions between synapsin I and actin and how this may relate to other characteristics of synapsin I, we have characterized the binding of various fragments of synapsin I to F-actin. Synapsin I has a globular, collagenase-resistant NH<sub>2</sub>-terminal head region that contains a high proportion of the hydrophobic amino acids found in the molecule, and an elongated, collagenase-sensitive, COOH-terminal tail region that is hydrophilic and strongly basic (8, 26, 35). Fragments of synapsin I which contain these domains have been produced by cysteine-specific cleavage of the molecule with 2-nitro-5-thiocyanobenzoic acid (NTCB).<sup>1</sup> The resultant fragments, designated NH<sub>2</sub> terminal, middle, tail, NH<sub>2</sub> terminal/middle, and

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1. *Abbreviation used in this paper:* NTCB, 2-nitro-5-thiocyanobenzoic acid.

middle/tail, were identified and aligned using the known phosphorylation sites as markers. Furthermore, the fragments were purified and their abilities to bind to actin filaments and to bundle them were assessed by high speed sedimentation, light scattering, and EM. We present evidence that F-actin binding domains are located within the 29-kD NH<sub>2</sub>-terminal fragment and the 15-kD middle fragment of synapsin I and that the tail fragment is necessary for the actin bundling activity of synapsin I.

## Materials and Methods

### Purification of Proteins

Actin was prepared from an acetone powder of rabbit skeletal muscle (33) and gel filtered on Sephadex G-150 (22). Synapsin I was purified from bovine brain as described by Schiebler et al. (32) and modified by Bähler and Greengard (2). The catalytic subunit of cAMP-dependent protein kinase, calmodulin and calcium/calmodulin-dependent protein kinase II were purified as described (18, 27, 36), and were gifts of A. Horiuchi, A. C. Nairn, F. Gorelick, and G. Thiel of our laboratory. Protein content was determined by a modification (23) of the method of Lowry et al. (21).

### NTCB Cleavage of Synapsin I and Cleavage Fragment Mapping

Purified bovine brain synapsin I was reduced and cleaved with NTCB (Sigma Chemical Co., St. Louis, MO) as described by Matsudaira et al. (25). The NTCB cleavage fragments of synapsin I generated during an 80-h period were electrophoresed on 7.5–15% SDS polyacrylamide gradient gels and either stained with Coomassie blue or immunoblotted with rabbit antisera raised against synthetic peptides containing the sequence surrounding phosphorylation sites 1 or 3 of bovine synapsin I (8) followed by <sup>125</sup>I-protein A overlay. The apparent molecular masses of the generated peptides were calculated using standards of 66, 45, 36, 29, 24, 20, and 14.2 kD.

### Purification of NTCB Fragments of Synapsin I

Preparative NTCB cleavage of synapsin I was performed by dialyzing synapsin I against a buffer containing 200 mM NaCl, 100 mM Tris/Cl, pH 8.0, 0.1 mM EDTA, 1 mM DTT, and then against 6 M guanidine hydrochloride, 200 mM Tris/Cl, pH 8.0, 0.1 mM DTT, and 0.1 mM EDTA. The protein concentration of synapsin I was 3–4 mg/ml for cleavage. NTCB was added to 2–5 mM, and the sample was incubated in the dark at 37°C for 65–75 h. The reaction was stopped by adding 2-mercaptoethanol to 10 mM. The reaction mixture was applied to a Sephadex G-150 column (2.4 × 80 cm) that had been preequilibrated with 6 M guanidine hydrochloride, 0.1 mM EDTA, 0.1 mM DTT, 50 mM Tris/Cl, pH 8.0. The elution of the fragments from the column was monitored by absorbance at 280 nm. The eluted peptides were pooled into four separate fractions (see Fig. 2 a), put into dialysis bags, and concentrated by covering the bags with dry Ficol 400. After gel filtration, a pool (lane 4, Fig. 2 a) was usually obtained that contained purified 15/16-kD fragment. From a pool containing 29-kD fragment and a minor amount of 15/16-kD fragment (lane 3, Fig. 2 a), the latter was removed quantitatively by ultracentrifugation. Alternative purification of the 29-kD fragment was carried out by dialyzing pool III (lane 3, Fig. 2 a) against 4 M urea, 10 mM NaPO<sub>4</sub>, pH 6.0, followed by chromatography with a Mono-S cation-exchange column (Pharmacia Fine Chemicals, Piscataway, NJ) using a linear NaCl gradient (0–0.4 M) for elution. The 29-kD fragment eluted at 100–150 mM NaCl. A gel filtration pool (lane 2, Fig. 2 a) containing 35/36- and 39/40-kD fragments subsequently shown to be four COOH-terminal fragments (see Results), and a 40-kD fragment subsequently shown to be the NH<sub>2</sub>-terminal/middle fragment (see Results), was dialyzed against 10 mM KPO<sub>4</sub>, pH 6.0, 0.1 mM EGTA, and loaded onto a Mono-S column followed by elution with a linear NaCl (0–1 M) gradient. Fractions containing pure 35/36–39/40-kD fragments eluted at ~250 mM NaCl. All other fragments present eluted from the FPLC column above 400 mM NaCl as a single peak, with a substantial amount of protein being retained on the column. The Mono-S column was then reequilibrated in 4 M urea, 10 mM K-phosphate, pH 6.0, and the protein fraction, which initially eluted above 400 mM NaCl, was reloaded after dialysis against 4 M urea, 10 mM K-phosphate, pH 6.0. Applying a linear NaCl gradient (0–0.4 M), a 40-kD

fragment eluted at ~120 mM NaCl. The pool that had the highest amount of the 51/54-kD fragments (lane 1, Fig. 2 a) was dialyzed against 4 M urea, 10 mM K-phosphate, pH 6.0. The dialyzed pool was loaded onto a Mono-S cation-exchange column and the proteins were eluted with a linear NaCl gradient (0–0.5 M). Fractions enriched in the 51/54-kD fragments were pooled and diluted threefold with 4 M urea, 10 mM K-phosphate, pH 6.0. To obtain homogeneous preparations of the two fragments, the chromatography was repeated twice.

### Phosphorylation and Repurification of NTCB Fragments of Synapsin I

The 35/36–39/40-kD tail fragments and the 51/54-kD middle/tail fragments were phosphorylated with calcium/calmodulin-dependent protein kinase II as described for intact synapsin I (32). Phosphorylated tail fragments were repurified by dilution with 10 mM KPO<sub>4</sub>, pH 6.0, followed by batch adsorption to carboxymethylcellulose (Whatman Inc., Clifton, NJ). The resin was washed with a buffer of 10 mM KPO<sub>4</sub>, pH 6.0, 0.1 mM EGTA, and the tail fragments were eluted as described for synapsin I (32). Purified 29-kD fragment was phosphorylated with the catalytic subunit of cAMP-dependent protein kinase as described for synapsin I (32). The phosphorylated fragment was exhaustively dialyzed to remove [ $\gamma$ -<sup>32</sup>P]ATP. Alternatively, the fragment was batch adsorbed to carboxymethylcellulose at pH 6.0 and repurified as described above for the tail fragments. This method, however, resulted in a very poor recovery of the 29-kD fragment.

### Actin-binding Assay

Binding of synapsin I and fragments of synapsin I to F-actin was performed as described (2) with some modifications. Samples (50  $\mu$ l) containing 4  $\mu$ M actin and variable amounts of a given fragment were incubated in 110 mM KCl, 30 mM NaCl, 3 mM MgCl<sub>2</sub>, 0.5 mM ATP, 25 mM Hepes, pH 7.4, 1 mM Na<sub>2</sub>S<sub>2</sub>O<sub>8</sub> for 1 h at room temperature, and then centrifuged in a rotor (Ti 42.2; Beckman Instruments, Inc., Palo Alto, CA) at 42,000 rpm for 20 min. Pellets were resuspended either in 4.7 M NH<sub>4</sub>Cl or 100 mM NaCl and 10 mM Tris/Cl, pH 8.0. The recovery of actin and the amount of dephosphorylated and phosphorylated 51/54-kD fragments bound were quantified by densitometry of Coomassie blue-stained bands after SDS-PAGE. Standard curves of the fragment and actin were included on each gel. Phosphorylated 51/54-kD fragment was also quantified by directly counting <sup>32</sup>P. The two procedures yielded comparable results. Intact synapsin I, 29-kD fragment, 40-kD fragment, and tail fragment were quantified by means of a dot-immunobinding assay (17). *K<sub>d</sub>* and *B<sub>max</sub>* values were determined by nonlinear regression analysis with the computer program Recept (5). Since the Hill coefficient was sometimes observed to be >1, we tried to fit the curves following both the simple one-site model and the bivalent cooperativity model, testing for the goodness of fit by means of the F-test and the runs test. The more complex cooperativity model produced a significant improvement of the goodness of fit to the experimental points only in the case of the data obtained for the 29-kD fragment (see Results section).

### EM

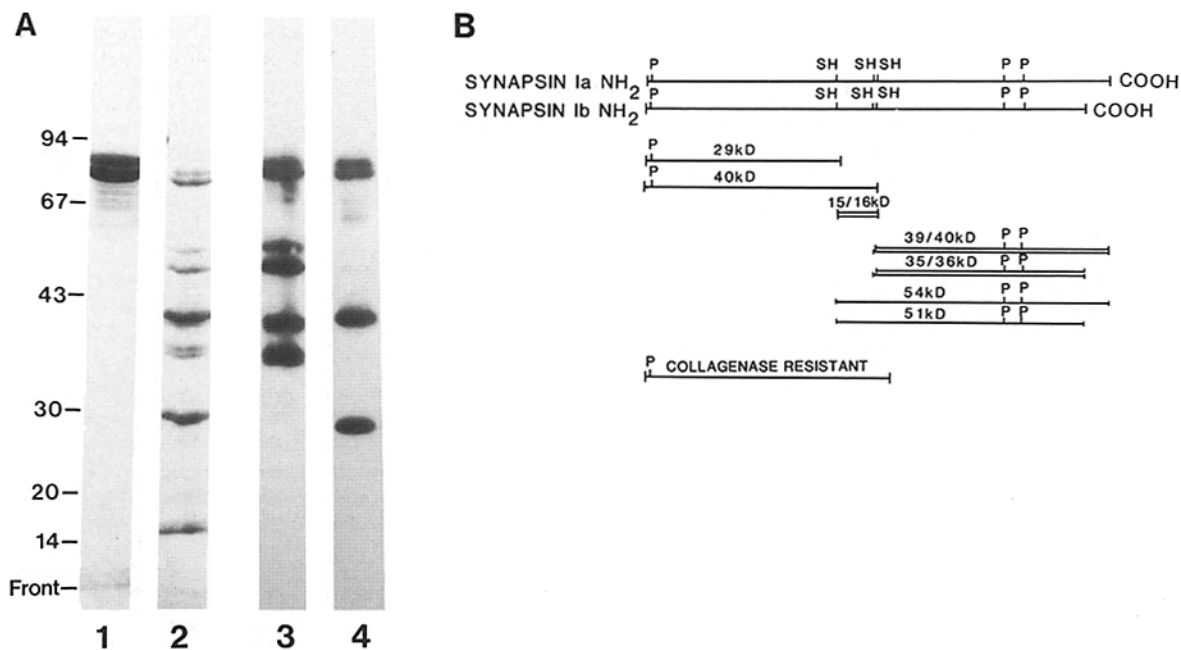
Samples in the same buffer as that described for the binding assays, containing 6  $\mu$ M actin and 1.5  $\mu$ M of the various fragments, were adsorbed onto glow-discharged carbon-coated grids, rinsed with a few drops of 0.1 M ammonium acetate, and negatively stained with 1% uranylacetate. The stained samples were examined in a Jeol 100 CX electron microscope.

### Light Scattering

Various amounts of synapsin I or NTCB fragments in 30 mM NaCl, 10 mM Tris/Cl (pH 8.0), 0.1 mM EDTA, 0.5 mM 2-mercaptoethanol, and 2 mM Na<sub>2</sub>S<sub>2</sub>O<sub>8</sub>, precleared by centrifugation at 200,000 g for 15 min before use, were added to aliquots of F-actin (0.1 mg/ml) in 100 mM KCl, 2 mM MgCl<sub>2</sub>, 1 mM ATP, 10 mM Hepes (pH 7.4), 0.5 mM 2-mercaptoethanol, and 2 mM Na<sub>2</sub>S<sub>2</sub>O<sub>8</sub>. Light scattering was measured as described (1, 37) at an angle of 90° in a fluorescence spectrophotometer (model 650-40; Perkin-Elmer Corp., Norwalk, CT) with excitation and emission wavelengths set at 400 nm. The excitation and emission slit widths were set at 2 nm and 3 nm, respectively. An increase in scattering of one unit represents a 20-fold increase over that observed with F-actin alone.

### Miscellaneous

Gel electrophoresis was performed according to either Laemmli (19) or



**Figure 1.** Identification of the cysteine-specific cleavage fragments of synapsin I. (A) Synapsin I was partially cleaved with NTCB for 0 h (lane 1) or 31 h (lanes 2–4). The resultant cleavage products were separated by SDS-PAGE (7.5–15%) followed either by Coomassie blue stain (lanes 1 and 2) or by electrophoretic transfer to nitrocellulose paper (lanes 3 and 4). The blotted fragments were mapped using antisera directed against phosphorylation site 3 (lane 3) or phosphorylation site 1 (lane 4) followed by <sup>125</sup>I-protein A overlay and autoradiography. (B) Structural model of synapsin I indicating the locations of the phosphorylation sites and the cysteine residues. The fragments produced by cysteine-specific cleavage of synapsin I with NTCB are aligned as mapped by their phosphorylation sites. The position of the collagenase resistant head domain is also indicated.

Matsudaira and Burgess (24). Immunoblotting was performed as described by Towbin et al. (34). Autoradiography of dried gels was performed at  $-70^{\circ}\text{C}$  using Kodak XAR-5 x-ray film and Cronex Lightning-Plus intensifying screens (DuPont, Wilmington, DE). Two-dimensional tryptic/chymotryptic phosphopeptide mapping was performed as described (15).

## Results

### Cysteine-specific Cleavage of Synapsin I

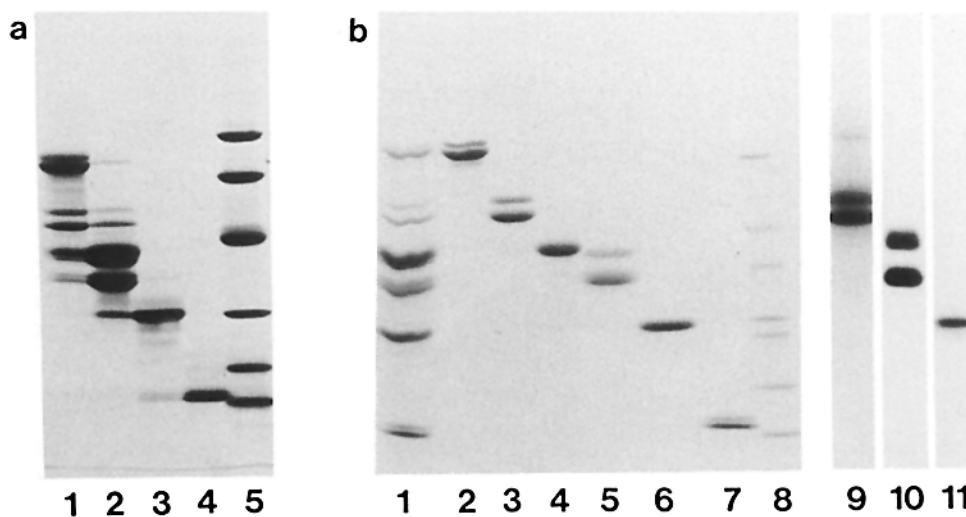
Purified bovine synapsin I is composed of two species, synapsins Ia and Ib, with apparent molecular masses of 84 and 80 kD, respectively, in SDS-PAGE. Cysteine-specific cleavage of synapsin I with NTCB yielded a defined number of fragments (Fig. 1 A) whose relative amounts increased with time. The fragments exhibited apparent molecular masses of 54, 51, 40, 39, 36, 35, 29, 16 and 15 kD. To assist in deducing the origin of the fragments, the NTCB cleavage fragments of synapsin I were immunoblotted with antisera raised against synthetic peptides comprising phosphorylation site 1, located near the NH<sub>2</sub> terminal of the molecule, or phosphorylation site 3, located within the COOH terminal region of the molecule (Fig. 1 A, lanes 4 and 3). The fragments containing the COOH-terminal region migrated as doublets that differed in molecular mass increments of 3–4 kD, with the faster migrating band being more prominent than the slower migrating one. This was reminiscent of the molar ratio observed with purified synapsins Ia and Ib and agreed with earlier findings (15) that the difference in primary structure of synapsins Ia and Ib is located in the COOH-terminal region of the molecule. Three doublets were observed after cleavage with NTCB, exhibiting apparent molecular masses of 54/51,

40/36, and 39/35 kD, indicating that synapsin I contains three cysteine residues. The location of the three cysteine residues and the alignment of the fragments are depicted in the structural model of synapsin I shown in Fig. 1 B, using the previously determined location of the phosphorylation sites (8) to align the fragments. Note that two of the three cysteine residues are separated by only  $\sim 1$  kD.

Two fragments with apparent molecular masses of 40 kD and 29 kD were detected by the NH<sub>2</sub>-terminal-specific antibody (Fig. 1 A, lane 4). The 40-kD NH<sub>2</sub>-terminal fragment co-migrated with a COOH-terminal 40-kD fragment. The failure to detect two NH<sub>2</sub>-terminal fragments of similar size ( $\sim 40$  kD) was most likely because of the inability to separate the two peptides on SDS-PAGE. This explanation was supported by the observation that quantitatively more 40-kD fragment was produced than 29-kD fragment, consistent with random cleavage at two closely spaced residues rather than at a single residue.

The 15/16-kD fragments generated by NTCB cleavage were not recognized by either of the two antibodies. However, their apparent molecular masses agreed well with the expected values for fragments arising from simultaneous cleavage at cysteine residues one and two for the 15-kD fragment and one and three for the 16-kD fragment. Therefore, they were aligned to the middle region of the molecule as shown in Fig. 1 B.

Identical alignments were obtained when purified NTCB cleavage fragments were phosphorylated with [ $\gamma$ -<sup>32</sup>P]ATP by either the catalytic subunit of cAMP-dependent protein kinase or calcium/calmodulin-dependent protein kinase II, followed by tryptic/chymotryptic two-dimensional phospho-



(lane 5); 29 kD NH<sub>2</sub>-terminal fragment, 10 µg (lane 6); 15/16-kD fragment, 10 µg (lane 7). Molecular mass standards (lane 8) included BSA, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase, trypsinogen, trypsin inhibitor, and α-lactalbumin. Synapsin I fragments were purified as described in Materials and Methods. Lanes 9–11 show autoradiograms of [<sup>32</sup>P]phosphate incorporation: purified 51/54-kD middle/tail fragment phosphorylated by calcium/calmodulin-dependent protein kinase II (lane 9), purified tail phosphorylated by calcium/calmodulin-dependent protein kinase II (lane 10), and purified 29-kD fragment phosphorylated by cAMP-dependent protein kinase (lane 11).

peptide mapping; the fragment-derived phosphopeptide maps were identical to those obtained with holo-synapsin I after phosphorylation by the corresponding kinase (data not shown). Furthermore, the number and location of the cysteine residues were in agreement with the amino acid sequence as derived from cDNA cloning (T. Südhof et al., manuscript in preparation) and from direct protein sequencing (8).

#### **Purification of the Fragments Which Arise by Cysteine-specific Cleavage of Synapsin I**

The purification protocols are described in Materials and Methods. The gel filtration pools used for the further purification and the purified fragments are shown in Fig. 2, *a* and *b*, respectively. To simplify terminology, we refer to the four COOH-terminal fragments, 35/36 and 39/40 kD, as “tail” fragment, since these four fragments contain the collagenase-sensitive tail domain of synapsin I. The tail fragment was easily purified from the mixture of peptides generated by NTCB cleavage because it was the only fragment that remained completely soluble in nondenaturing buffers, and showed no tendency to associate with any other fragment (see below). The purified tail fragment was readily phosphorylated stoichiometrically by calcium/calmodulin-dependent protein kinase II (Fig. 2 *b*, lane 10) at the same sites as in intact synapsin I (data not shown). In fact, it was a better substrate for the kinase than intact synapsin I (M. Bähler and F. Valtorta, unpublished observations).

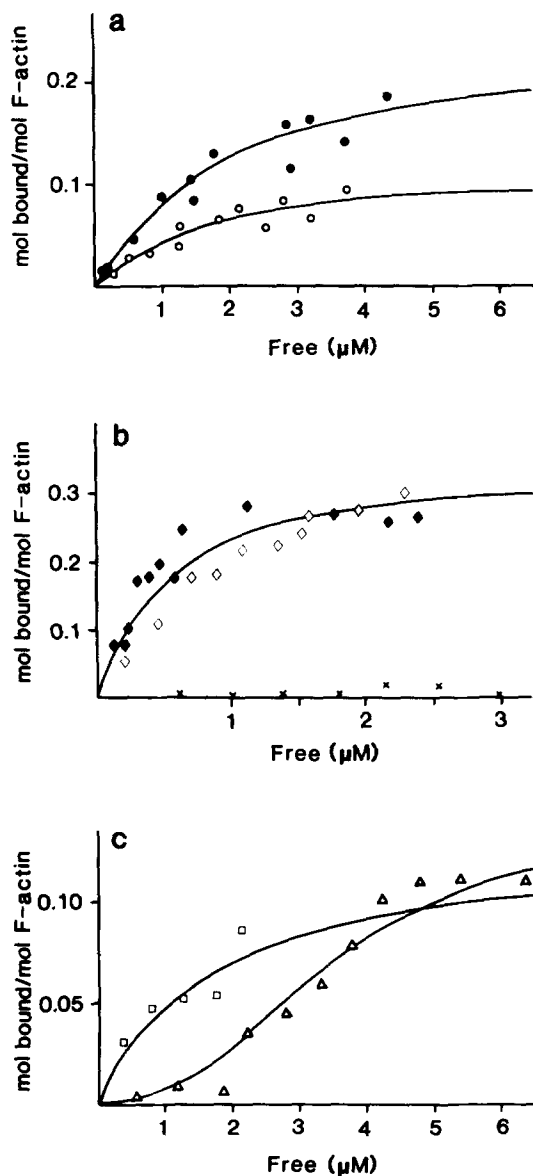
With the exception of the tail fragment, the various fragments could be separated from each other only under denaturing conditions, including the 51/54-kD middle/tail fragment. However, once purified, the 51/54-kD middle/tail fragment was readily soluble in nondenaturing conditions and was also readily phosphorylated stoichiometrically by calcium/calmodulin-dependent protein kinase II (Fig. 2 *b*, lane 9).

The purified NH<sub>2</sub>-terminal 29-kD fragment exhibited a strong tendency to self-associate in biological buffers so that only small and variable amounts could be resolubilized under these conditions, diminishing the yield of the purified fragment considerably. It was phosphorylated by cAMP-dependent protein kinase (Fig. 2 *b*, lane 11) at site 1 (data not shown), although to a lower stoichiometry (0.4 mol/mol). The purified 40-kD NH<sub>2</sub>-terminal/middle fragment was even less soluble upon dialysis against nondenaturing buffers than was the NH<sub>2</sub>-terminal 29-kD fragment. Therefore, we were able to perform only a very limited number of experiments with this fragment.

The purified 15/16-kD fragment, obtained by gel filtration in 6 M guanidine hydrochloride, was virtually insoluble in nondenaturing buffers. For this reason, it was not possible to include this fragment in our actin-binding studies. Head-domain fragments derived from digestion of synapsin I under nondenaturing conditions with collagenase or trypsin also exhibited low solubility and a tendency to aggregate, indicating that these properties were not the result of the exposure to denaturing conditions. Furthermore, primary sequence analysis revealed that the head domain contained ~80% of the hydrophobic residues present in the whole molecule (T. Südhof et al., manuscript in preparation).

#### **Ability of Purified NTCB Fragments to Bind F-actin**

The ability of synapsin I and purified NTCB fragments of synapsin I to interact with actin filaments was tested using a high speed sedimentation assay (Fig. 3; Table I). The ability of synapsin I to bind F-actin has already been described (2, 30). Binding data for dephospho synapsin I and synapsin I phosphorylated on all three sites are shown in Fig. 3 *a*. Nonlinear regression analysis of the binding data, assuming a one binding site model, yielded a dissociation constant ( $K_d$ ) of 2 µM for dephosphorylated synapsin I and 1.8 µM for synapsin I phosphorylated on all three sites. The major



**Figure 3.** Binding of synapsin I and synapsin I fragments to F-actin. F-actin (4  $\mu\text{M}$ ) was incubated with various amounts of (a) dephosphorylated synapsin I ( $\bullet$ ) and synapsin I phosphorylated on all three sites (2.6 mol phosphate/mol synapsin I) ( $\circ$ ); (b) dephosphorylated 51/54-kD middle/tail fragment ( $\blacklozenge$ ), phosphorylated 51/54-kD middle/tail fragment (2.75 mol phosphate/mol fragment) ( $\lozenge$ ), and tail fragment ( $\times$ ); and (c) 29-kD  $\text{NH}_2$ -terminal fragment ( $\Delta$ ) and 40-kD  $\text{NH}_2$ -terminal/middle fragment ( $\square$ ). Samples were centrifuged at 220,000  $g$  for 20 min, and the amount of protein pelleted was determined as described in Materials and Methods. Free synapsin I or synapsin I fragment was calculated by subtracting bound protein from the total amount of protein added to each sample. The data were fitted using a nonlinear regression analysis following the simple model of one ligand and one noncooperative binding site, with the exception of the data obtained for the 29-kD  $\text{NH}_2$ -terminal fragment, which were fitted according to a bivalent cooperativity model.  $K_d$  and  $B_{\text{max}}$  values are given in the text and in Table I.

difference in binding between the two forms of synapsin I was found in the ratio of synapsin I bound to F-actin at saturation; this was 0.25 mol and 0.14 mol of synapsin I/mol of actin monomer for dephosphorylated synapsin I and for synapsin

I phosphorylated on all three sites, respectively. When the data were plotted according to the Hill equation, the Hill coefficient was  $1.27 \pm 0.11$  for dephospho synapsin I and  $1.10 \pm 0.19$  for phospho synapsin I, indicating a small degree of positive cooperativity in the binding of the dephosphorylated form to F-actin.

To address the questions of how many and which region(s) of synapsin I are involved in binding to F-actin, the purified NTCB fragments were tested for F-actin binding activity. The 51/54-kD middle/tail fragment, missing the 29-kD  $\text{NH}_2$ -terminal region of synapsin I, bound F-actin in a saturable manner (Fig. 3 b). Analysis of the binding data of dephosphorylated 51/54-kD middle/tail fragment revealed a  $K_d$  of 0.6  $\mu\text{M}$ , a  $B_{\text{max}}$  of 0.37 mol/mol actin monomer, and a Hill coefficient of  $1.37 \pm 0.21$ . The effect of phosphorylation was also investigated (Fig. 3 b). Phosphorylated 51/54-kD middle/tail fragment (2.75 mol phosphate/mol fragment) also bound F-actin; in contrast to intact synapsin I, no reduction in the  $B_{\text{max}}$  (0.35 mol/mol actin) was observed upon phosphorylation. However, a lower affinity for F-actin ( $K_d = 1.7 \mu\text{M}$ ) and a Hill coefficient of  $1.07 \pm 0.05$  were found.

The tail fragment behaved very differently from the middle/tail fragment with regard to F-actin binding. The tail fragment, which is the most hydrophilic and basic fragment of synapsin I, did not exhibit significant binding to F-actin under the conditions employed (Fig. 3 b). Identical results were obtained with phosphorylated tail fragment (data not shown). These results indicate that the 15-kD middle fragment of synapsin I is involved in the F-actin binding activity of the middle/tail fragment. The results also rule out the possibility that synapsin I binding to F-actin is attributable solely to its high basicity (see Discussion).

The 40-kD  $\text{NH}_2$ -terminal/middle fragment exhibited F-actin binding activity (Fig. 3 c). A  $K_d$  value of 1.3  $\mu\text{M}$  and a  $B_{\text{max}}$  value of 0.12 were determined. The Hill coefficient for the binding of this fragment was calculated to be  $0.89 \pm 0.12$ . Finally, we measured the binding of the 29-kD  $\text{NH}_2$ -terminal fragment to F-actin (Fig. 3 c). This fragment was also found to bind F-actin. However, analysis of the data revealed a more complex binding than that observed for the other fragments. The value of the Hill coefficient was determined to be  $3.18 \pm 0.32$ , indicating that the 29-kD  $\text{NH}_2$ -terminal fragment binds to F-actin with an apparent positive cooperativity. Indeed, the experimental points could be fitted only using a bivalent cooperativity model (see Materials and Methods), yielding an initial  $K_d = 168 \mu\text{M}$  and a final  $K_d = 0.12 \mu\text{M}$  with a  $B_{\text{max}}$  of 0.19 (mol/mol actin). These binding results indicate that there is at least one F-actin binding site located in the 29-kD  $\text{NH}_2$ -terminal fragment. The stoichiometry of phosphorylation of the 29-kD  $\text{NH}_2$ -terminal fragment was lower (0.3–0.4 mol phosphate/mol fragment) than that observed for synapsin I. The phosphorylated and the nonphosphorylated 29-kD  $\text{NH}_2$ -terminal fragment behaved similarly with respect to actin binding (data not shown). A summary of the binding data is given in Table I.

#### Ability of Purified NTCB Fragments to Bundle F-actin

Synapsin I is an F-actin bundling protein *in vitro* (2, 30). It was therefore of interest to test NTCB fragments for F-actin bundling activity. We used a light scattering assay as a measure of F-actin bundle formation (Fig. 4). Dephosphorylated

Table I. Binding of Synapsin I and NTCB Fragments of Synapsin I to F-actin

|   | $K_d$     | $B_{max}$  | Hill coefficient |
|---|-----------|------------|------------------|
| Dephosphorylated synapsin I                     | 2         | 0.25       | 1.27 ± 0.11      |
| Phosphorylated synapsin I                       | 1.8       | 0.14       | 1.10 ± 0.19      |
| 29-kD NH <sub>2</sub> -terminal fragment        | 168, 0.12 | 0.19       | 3.18 ± 0.32      |
| 40-kD NH <sub>2</sub> -terminal/middle fragment | 1.3       | 0.12       | 0.89 ± 0.12      |
| Dephosphorylated 51/54-kD middle/tail fragment  | 0.6       | 0.37       | 1.37 ± 0.21      |
| Phosphorylated 51/54-kD middle/tail fragment    | 1.7       | 0.35       | 1.07 ± 0.05      |
| Tail fragment                                   |           | no binding |                  |

Binding was carried out as described in Materials and Methods.  $K_d$  and  $B_{max}$  values of a representative set of experiments are given in micromoles and moles of synapsin I or synapsin I fragment/mol actin monomer, respectively. The two  $K_d$  values given for the 29-kD NH<sub>2</sub>-terminal fragment represent initial and final values, respectively. The Hill coefficients (±SEM) were calculated as described in reference 5.

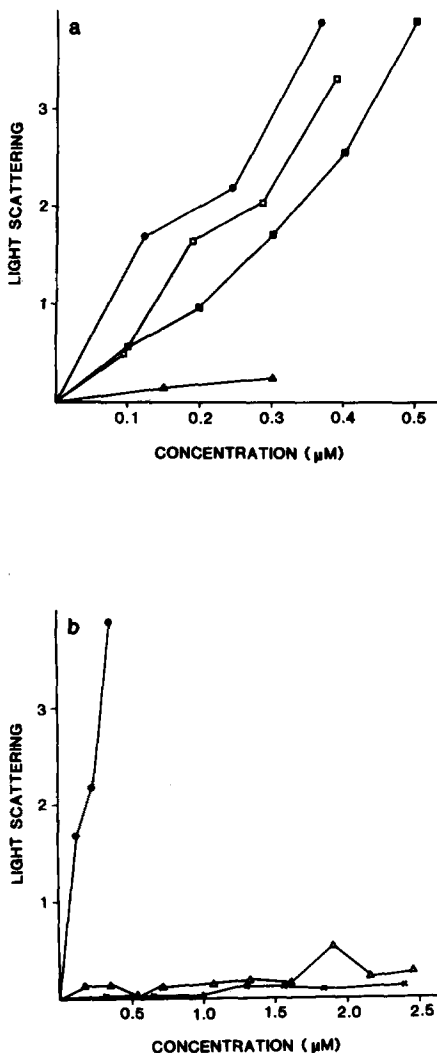


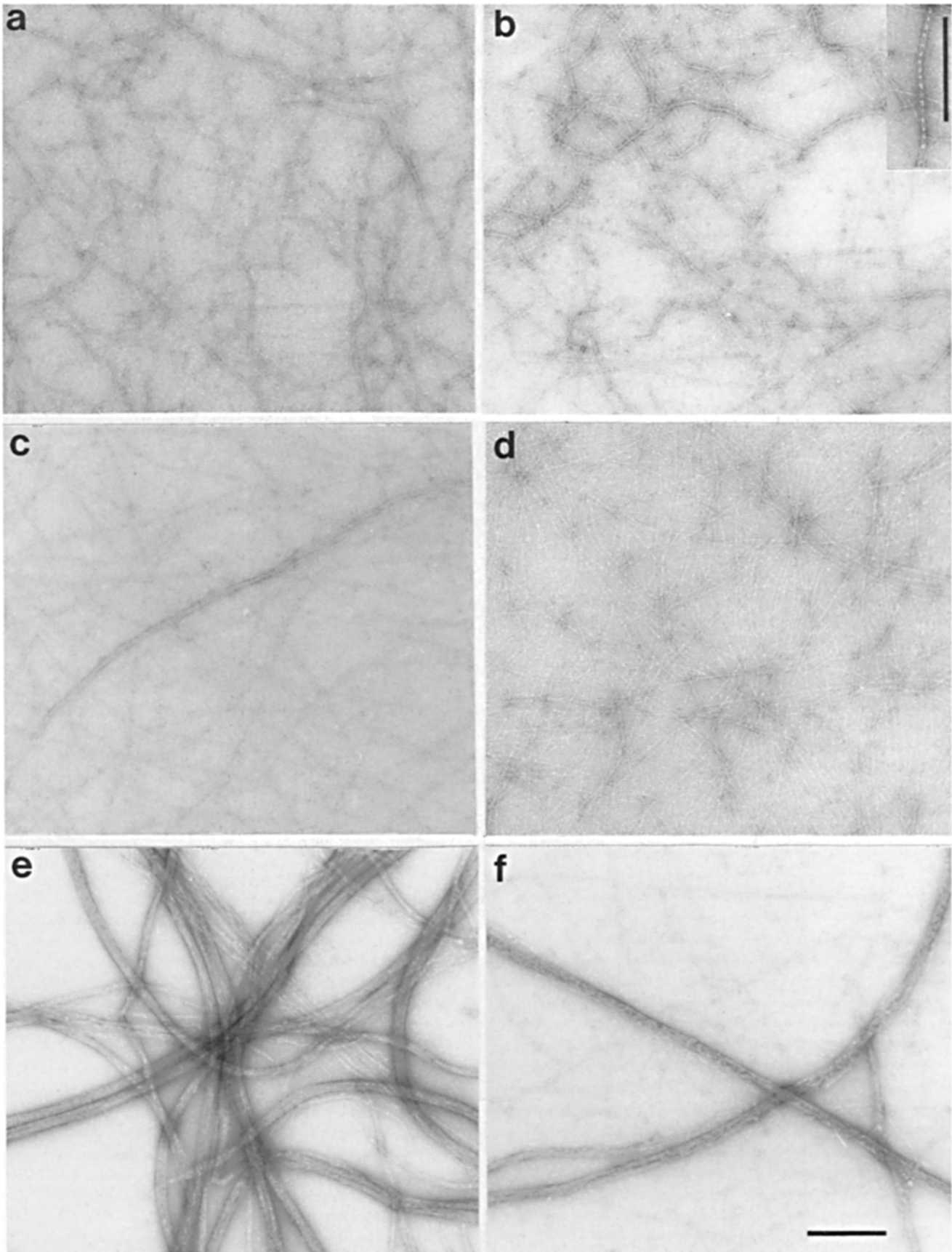
Figure 4. Effect of synapsin I and various fragments of synapsin I on actin bundle formation as measured by a light scattering assay. Various amounts of synapsin I or synapsin I fragment were added to separate samples of actin (0.1 mg/ml) in 100 mM KCl, 2 mM MgCl<sub>2</sub>, 1 mM ATP, 10 mM HEPES, pH 7.4, 0.5 mM 2-mercaptoethanol, and 2 mM Na<sub>3</sub>N. Light scattering was measured as described in Materials and Methods. (a) Dephosphorylated synapsin I (●), dephosphorylated 51/54-kD middle/tail fragment (□), phosphorylated 51/54-kD fragment (2.75 mol phosphate/mol fragment) (■), and 40-kD NH<sub>2</sub>-terminal/middle fragment (▲). (b) Dephosphorylated synapsin I (●), 29-kD NH<sub>2</sub>-terminal fragment (△), and tail fragment (×). Note the difference in the scale of the abscissa between a and b.

synapsin I was very effective in bundling F-actin, as reported previously (2, 30). The tail fragment, although highly basic, did not exhibit significant F-actin bundling activity at physiological pH and ionic strength (Fig. 4 b), in agreement with the binding data. In contrast, the 51/54-kD middle/tail fragment retained the F-actin bundling activity of dephosphorylated synapsin I (Fig. 4 a). In contrast to synapsin I, however, phosphorylation of the 51/54-kD middle/tail fragment only slightly reduced its ability to bundle F-actin (Fig. 4 a). Therefore, although the 51/54-kD fragment is capable of achieving actin filament bundle formation, it has lost the property of being regulated by phosphorylation. The 40-kD NH<sub>2</sub>-terminal/middle fragment exhibited only minor F-actin bundling activity compared to dephosphorylated synapsin I and the 51/54-kD middle/tail fragment (Fig. 4 a). These results indicate that the tail fragment is involved in the bundling activity of the 51/54-kD middle/tail fragment, although it did not by itself bind to or bundle F-actin. The low bundling activity of the 40-kD NH<sub>2</sub>-terminal/middle fragment can be explained by the binding data that assigned one binding site to the 15-kD middle fragment and a second binding site, albeit of lower affinity, to the 29-kD NH<sub>2</sub>-terminal fragment. The purified 29-kD NH<sub>2</sub>-terminal fragment did not exhibit any significant F-actin bundling activity (Fig. 4 b).

Negative stain EM, used as an alternative way to examine the effects of NTCB fragments on F-actin, confirmed the results of the light scattering assay. The purified 29-kD NH<sub>2</sub>-terminal fragment (Fig. 5 b) and the purified tail fragment (Fig. 5 d) had no noticeable effects on the appearance of actin filaments. On rare occasions, a few actin bundles were observed in both cases, but this was likely to be because of the self-association of the 29-kD NH<sub>2</sub>-terminal fragment in the former case and to a nonspecific effect resulting from the basic nature of the tail fragment in the latter case. When F-actin was incubated with the 40-kD NH<sub>2</sub>-terminal/middle fragment, some small, thin bundles of actin filaments were observed (Fig. 5 c). In confirmation of the light scattering data, addition of the dephosphorylated 51/54-kD middle/tail fragment to actin resulted in the formation of F-actin bundles of relatively uniform diameter (Fig. 5 e). Incubation of actin with phosphorylated 51/54-kD middle/tail fragment also resulted in the formation of actin filament bundles (Fig. 5 f); however, they appeared somewhat less abundant than in the former case.

## Discussion

In the present study, we obtained evidence that F-actin bind-



**Figure 5.** Electron micrographs of negatively stained F-actin ( $6\ \mu\text{M}$ ) in the absence or presence of synapsin I fragments ( $1.5\ \mu\text{M}$ ). (a) F-actin alone; (b) F-actin plus 29-kD  $\text{NH}_2$ -terminal fragment, the inset shows two actin filaments held together by aggregated 29-kD  $\text{NH}_2$ -terminal fragments bound to F-actin; (c) F-actin plus 40-kD  $\text{NH}_2$ -terminal/middle fragment; (d) F-actin plus tail fragment; (e) F-actin plus dephosphorylated 51/54-kD middle/tail fragment; and (f) F-actin plus phosphorylated 51/54-kD middle/tail fragment (2.75 mol phosphate/mol fragment). Bars,  $0.5\ \mu\text{m}$ .



ing activity is located within two fragments of synapsin I, a 29-kD NH<sub>2</sub>-terminal fragment and a 15/16-kD middle fragment, and we determined that the tail fragment of synapsin I is necessary for F-actin bundling. Cysteine-specific cleavage of synapsin I was employed. Maps constructed from the cleavage pattern using the phosphorylation sites as markers indicate that synapsin I contains three cysteine residues located in the middle region of the molecule. This number is in agreement with the primary sequence information deduced from cDNA cloning and direct protein sequencing (T. Südhof et al., manuscript in preparation; reference 8). Furthermore, we report the purification of these fragments and describe some properties of the purified fragments.

Fragments derived from the hydrophobic collagenase-resistant head domain (NH<sub>2</sub>-terminal-, middle-, NH<sub>2</sub>-terminal/middle fragments) exhibit a tendency to self-associate in physiological buffer solutions. It seems likely that this domain is responsible for the observed self-association properties of intact synapsin I. In particular, the 15/16-kD fragment located in the middle region of the molecule proved to be virtually insoluble in nondenaturing buffers. In contrast, fragments containing the hydrophilic tail region were fully soluble in biological buffers, as expected from the amino acid sequence (reference 8; T. Südhof et al., manuscript in preparation).

Previously, we (2) and Petrucci and Morrow (30) reported that synapsin I bundles F-actin in a phosphorylation-dependent manner. Dephosphorylated synapsin I was very effective in bundling F-actin; the bundling activity was reduced by phosphorylation of synapsin I in the head region at site 1, and was virtually abolished by phosphorylation of synapsin I in the tail region at sites 2 and 3. The bundling of F-actin by dephosphorylated synapsin I implies either that synapsin I has two or more sites that can bind F-actin, or that it has a single binding site for F-actin and achieves bundling by self-association. Hence, the phosphorylation of synapsin I could affect bundling by regulating either the direct binding of synapsin I to F-actin or its self-association. In the present study, we addressed the question as to the number of domains in the synapsin I molecule involved in binding to F-actin. An NH<sub>2</sub>-terminal 29-kD fragment bound F-actin in a manner showing apparent positive cooperativity, exhibiting an initial  $K_d$  two orders of magnitude higher and a final  $K_d$  one order of magnitude lower than the  $K_d$  found for synapsin I, but reaching a similar  $B_{max}$  value. The tendency of this fragment to self-associate may contribute to its observed binding characteristics; this fragment failed to show bundling activity, and its exact contribution to the F-actin binding characteristics of intact synapsin I remains to be clarified.

It has recently been reported that the 29-kD NH<sub>2</sub>-terminal fragment shows cross-reactivity with antibodies against villin, another actin binding protein (31), raising the possibility that this region of synapsin I and villin may share some structural motifs involved in actin binding. Based on a limited sequence homology among regions located in villin and profilin, two actin-binding proteins and a region located in the tail fragment of synapsin I, it was also proposed that the tail fragment contains an actin-binding site (26). However, the isolated tail fragment did not exhibit any significant binding (or bundling activity) under conditions of physiological ionic strength and pH. When the ionic strength was reduced, an interaction of this fragment with actin was ob-

served (data not shown). However, this interaction is likely not to have physiological significance, since similar findings have been reported for other basic macromolecules (14).

The  $K_d$  and  $B_{max}$  values for the binding of the 40-kD NH<sub>2</sub>-terminal/middle fragment to F-actin were similar to those for phosphorylated synapsin I. Unfortunately, only very small amounts of this fragment were soluble in biological buffers after purification, prohibiting a more extensive analysis. The purified 15/16-kD middle fragment proved to be virtually insoluble and therefore was not amenable to testing.

The 51/54-kD middle/tail fragment, which comprises the 15/16-kD fragment plus the hydrophilic tail fragment, was readily soluble. The dephosphorylated form of this fragment exhibited a threefold higher affinity for F-actin than did synapsin I and bundled F-actin with a potency similar to that of synapsin I. Phosphorylation of this fragment reduced the affinity to that of synapsin I, without any detectable effect on the  $B_{max}$  value. In contrast, the 40-kD NH<sub>2</sub>-terminal/middle fragment exhibited only minor bundling activity, indicating that F-actin bundling cannot be ascribed to the 15/16-kD fragment alone and strongly suggesting that the tail fragment is involved in F-actin bundling, although this fragment is not able to bundle F-actin by itself.

When the tail fragment is connected with the middle fragment, which appears to contain a binding site for F-actin, it becomes capable of bringing about the observed bundling, possibly by interacting with F-actin. This notion is consistent with the fact that synapsin I and the 51/54-kD middle/tail fragment exhibit an approximately twofold higher  $B_{max}$  than the 40-kD NH<sub>2</sub>-terminal/middle fragment, suggestive of an additional binding site either in the tail fragment itself, or induced in the middle fragment by the presence of the tail. Some caution, however, needs to be applied concerning the interpretation of the various  $B_{max}$  and  $K_d$  values, since we do not know to what extent different states of self-association of the various fragments might play a role.

One unexpected result of the present study is that, in contrast to phosphorylation of intact synapsin I, phosphorylation of the middle/tail fragment only slightly reduced the bundling activity and had no effect on the  $B_{max}$  value. These results indicate that phosphorylation of holosynapsin I on the tail sites abolishes bundling by an action involving the head domain. The data further suggest that, for the tail phosphorylation to achieve a conformational change in the head domain, a more extended part of the head domain is needed than the 15-kD middle region.

The results of the present study suggest that synapsin I bundles F-actin by a mechanism other than self-association. The NH<sub>2</sub>-terminal fragment, the middle fragment, and the NH<sub>2</sub>-terminal/middle fragment all were found to self-associate, but little or no bundling activity was observed. Moreover, the tail region of synapsin I, which is essential for bundling activity, is hydrophilic and possesses a high net positive charge, making it unlikely that this fragment would self-associate, although we cannot exclude this possibility.

For an understanding of the molecular basis of synaptic transmission, it is of considerable importance to determine the structural basis for the interaction of synapsin I with synaptic vesicles and other cytoskeletal elements, as well as the structural basis for its high surface activity. The use of the purified synapsin I fragments described in the present report should be of considerable help in the attainment of these



goals. Their use in studying the binding of synapsin I to phospholipid bilayers and to small synaptic vesicles is described by Benfenati et al. (6, 7).

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