Synapsin I: An Actin-bundling Protein under Phosphorylation Control

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Abstract. Synapsin I is a neuronal phosphoprotein comprised of two closely related polypeptides with apparent molecular weights of 78,000 and 76,000. It is found in association with the small vesicles clustered at the presynaptic junction. Its precise role is unknown, although it probably participates in vesicle clustering and/or release. Synapsin I is known to associate with vesicle membranes, microtubules, and neurofilaments. We have examined the interaction of purified phosphorylated and unphosphorylated bovine and human synapsin I with tubulin and actin filaments, using cosedimentation, viscometric, electrophoretic, and morphologic assays. As purified from brain homogenates, synapsin I decreases the steady-state viscosity of solutions containing F-actin, enhances the sedimentation of actin, and bundles actin filaments. Phosphorylation by cAMP-dependent kinase has minimal effect on this interaction, while phosphorylation by brain extracts or by purified calcium- and calmodulin-dependent kinase II reduces its actin-bundling and -binding activity. Synapsin's microtubule-binding activity, conversely, is stimulated after phosphorylation by the brain extract. Two complementary peptide fragments of synapsin generated by 2-nitro-5-thiocyanobenzoic cleavage and which map to opposite ends of the molecule participate in the bundling process, either by binding directly to actin or by binding to other synapsin I molecules. 2-Nitro-5-thiocyanobenzoic peptides arising from the central portion of the molecule demonstrate neither activity. In vivo, synapsin I may link small synaptic vesicles to the actin-based cortical cytoskeleton, and coordinate their availability for release in a Ca^{++}-dependent fashion.

SYNAPSIN I is a neuronal phosphoprotein associated with small synaptic vesicles (for review see DeCamilli and Greengard, 1986). As routinely purified from brain homogenates, it is composed of two closely related polypeptides with apparent molecular weights of 78,000 and 76,000, termed synapsin Ia and Ib, respectively. These two peptides arise from separate mRNA transcripts (Kilimann and DeGennaro, 1985). Because of its concentration on synaptic vesicles, it has generally been assumed that synapsin I must play a role in mediating synaptic transmission, possibly by regulating vesicle release (Llinas et al., 1985; Goldenring et al., 1986). Only recently, however, have specific functions been ascribed to this molecule.

Recent studies have suggested that synapsin I interacts with both small synaptic vesicle membranes (Navone et al., 1984; Schiebler et al., 1986; Ueda, 1981) and several cytoskeletal elements. Synapsin I copurifies with both neuronal microtubules and neurofilaments, and can be detected on the filaments using immunocytochemical methods (Goldenring et al., 1986). Baines and Bennett (1985) have suggested that synapsin I may be related to erythrocyte protein 4.1, and have also detected an interaction between synapsin I and spectrin (fodrin) (1985) and with microtubules (Baines and Bennett, 1986). Interactions between synapsin I and actin have not previously been reported.

We now present evidence that synapsin I bundles actin filaments in vitro under physiologic conditions. This activity is not related simply to the basic nature of the protein (pI = 11.5), since it is subject to phosphorylation control, and since actin-binding activity can be recovered in specific 2-nitro-5-thiocyanobenzoic (NTCB)[1] cleavage fragments, while other similarly sized and more basic fragments do not bind. The inhibition of actin bundling by phosphorylation is specific for those sites regulated by calcium- and calmodulin-dependent kinases. These results suggest that one role of synapsin I may be to stabilize and organize small vesicle clusters by linking them to the actin-based cortical cytoskeleton. A preliminary account of these results has been reported in abstract form (Petrucci and Morrow, 1986).

Materials and Methods

Protein Purification

Synapsin I was purified after acidic extraction from frozen bovine brain by chromatography on carboxymethol-cellulose and hydroxylapatite exactly as described (Ueda and Greengard, 1977). Alternatively, for the phosphorylation experiments, synapsin I was purified under nondenaturing conditions

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1. *Abbreviations used in this paper*: MAP, microtubule-associated protein; NTCB, 2-nitro-5-thiocyanobenzoic acid.

by solubilization of synapsin I from crude synaptosomes with 3-(3-cholamidopropyl)-dimethylammoni)-1-propanesulfonic acid (CHAPS) (Schiebler et al., 1986).

Rabbit skeletal muscle actin was prepared from acetone powder extracts by standard methods (Spudich and Watt, 1971). Freshly prepared actin was stored at 4°C under conditions of continuous dialysis versus G buffer (2 mM Tris-HCl, 0.2 mM ATP, 0.2 mM calcium chloride, 0.5 mM dithiothreitol [DTT], 0.2% sodium azide, pH 8.0), and then polymerized by the addition of the appropriate amount of potassium chloride immediately before use. The final buffer conditions for each experiment were as specified in the figure legends.

Bovine brain microtubule protein was purified from brain homogenates by three cycles of temperature-dependent assembly and dissassembly in 0.1 M Pipes, 1 mM EGTA, 0.1 mM magnesium chloride, 1 mM GTP, pH 6.62, according to the method of Murphy (1982).

Actin Studies

Cosedimentation assays were performed using 150-μl aliquots of 7–8 μM actin and various concentrations of synapsin I (0.0–5.0 μM) in G buffer containing 0.1 M KCl, 2 mM magnesium chloride. After a 30-min incubation at room temperature, the samples were spun at either 100,000 *g* (high speed) or at 10,000 *g* (low speed) for 1 h at 4°C in a 42.2 Ti rotor (Beckman Instruments, Inc., Palo Alto, CA). The pellets were resuspended in the original volume of incubation buffer, and equal aliquots of supernatant and pellet were analyzed by SDS-PAGE (Laemmli, 1970) and stained with Coomassie Brillant Blue. The relative amounts of synapsin I and actin in each sample were determined by quantitative densitometry using a model No. 1650 Scanning Densitometer (Bio-Rad Laboratories, Richmond, CA) or by the OD at 595 nm of the Coomassie Blue dye after elution with 25% pyridine.

High-shear viscometry was used to measure the effect of synapsin I on the steady-state viscosity of F-actin solutions. These assays were performed using Ostwald high-shear viscometry tubes at 25°C with flow rates of ~60 s (Cannon Instruments, State College, PA).

Electron Microscopy

Aliquots of the samples used for the cosedimentation assays (100 μl) containing synapsin I and/or actin were visualized by electron microscopy after negative staining with 1% uranyl acetate on carbon-parlodian–coated grids, using a Phillips EM300 electron microscope.

Microtubule–Synapsin I Binding

Synapsin I (1.9 μM) and the microtubule preparation (4 μM) were subjected to two cycles of warm polymerization and cold depolymerization, followed by sedimentation at 100,000 *g* for 45 min. The resulting pellet and supernatant were analyzed by SDS-PAGE. The presence of synapsin I in the supernatant and pellet was determined by Western immunoblotting (Towbin et al., 1979), using affinity-purified rabbit antibodies against bovine synapsin I.

Phosphorylation of Synapsin I

Purified synapsin I (1 mg) was phosphorylated as described (Schiebler et al., 1986) using either the catalytic subunit of cAMP-dependent kinase (Sigma Chemical Co., St. Louis, MO) or Ca^{++}/calmodulin-dependent kinase II purified from bovine brain (McGuinness et al., 1985). The calmodulin kinase was a kind gift of Sheenah Mische, Yale University. Calmodulin was purified as previously described (Anderson and Morrow, 1987). Alternatively, synapsin I was phosphorylated by endogenous kinases according to Kennedy and Greengard (1981).

To repurify the synapsin I after phosphorylation and to remove the added kinases, the protein mixture was diluted with 10 vol cold water and loaded on 1 × 1-cm column of hydroxylapaptite preequilibrated with elution buffer (25 mM Tris-HCl, 0.1 mM EDTA, 0.1 mM 2-mercaptoethanol, 100 μM phenylmethylsulfonyl fluoride [PMSF], pH 8.0). Bound protein was eluted by the addition of 0.5 M ammonium chloride to the buffer. Fractions containing phosphorylated synapsin I were dialyzed into G-buffer containing 2 mM magnesium chloride and 0.1 M KCl, and used immediately for the functional assays.

Determination of the native phosphorylation state of synapsin I was done by inorganic phosphate analysis of the purified protein after exhaustive dialysis against phosphate-free buffers. All glassware used was detergent free and acid washed. Water was HPLC grade and prepared immediately before use by a Milli Q water purification system (Millipore Corp., Bedford, MA). Phosphate analysis was done in triplicate, according to established methods (Duck-Chang, 1979).

Estimates of ^{32}P incorporated into the protein (mol/mol) were made by liquid scintillation counting (model No. LS-230 counter; Beckman Instruments, Inc.) of the purified protein after phosphorylation with [^{32}P]-γ-ATP of known specific activity (New England Nuclear, Boston, MA). Standard corrections for decay, quench, and counting efficiency were taken.

Cysteine-specific Chemical Cleavage of Synapsin I by S-Cyanylation

Purified synapsin I was cleaved with NTCB by the method of Jacobson et al. (1973), using the modifications described for the cleavage of protein 4.1 (Leto and Marchesi, 1984). The protein was prepared for hydrolysis by dialysis against 7.5 M guanidine hydrochloride, 0.2 M Tris-HCl, 0.1 mM EDTA, pH 8.0. After dialysis, NTCB was added to 2 mM and the sample was incubated for 1 h at room temperature. The pH was then adjusted to 9.0 with an equal-parts mixture of 1 M Tris base and 1 M NaOH, and the sample incubated overnight at 37°C. The reaction was terminated by the addition of 2-mercaptoethanol to 10 mM, followed by dialysis against 10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0.

Two-dimensional Cellulose Peptide Mapping

Cellulose chymotryptic iodopeptide maps were prepared by the method of Elder (1977), as modified (Speicher et al., 1982). An additional modification was the use of 20 × 20-cm "Baker-flex" microcrystalline cellulose plates for the mapping (J. T. Baker Chemical Co., Phillipsburg, NJ), which gave better resolved maps. The peptides used for mapping were separated by SDS-PAGE, excised, and iodinated while in the gel slice using [^{125}I]sodium iodine (Amersham Corp., Arlington Heights, IL) and chloramine T (Sigma Chemical Co., St. Louis, MO).

Protein Iodination

Purified synapsin I was reacted at 2 mg/ml (250 μl) with 1 mCi ^{125}I (Amersham Corp.) in 5 mM sodium phosphate, 0.15 M NaCl, pH 7.4, with 50 μl of freshly hydrated beads containing immobilized lactoperoxidase and glucose oxidase (Enzymobeads; Bio-Rad Laboratories) and 50 μl of 2 M glucose. After incubation of the reaction mixture at 0°C for 1 h, the unreacted iodine and the beads were removed by desalting on a 1 × 12-cm column of G-25 Sephadex (Pharmacia Fine Chemicals, Piscataway, NJ) in PBS.

Other Methods

Western immunoblots were prepared after electrophoretic transfer of SDS gels to nitrocellulose paper (Schleicher & Schuell, Inc., Keene, NH) (Towbin et al., 1979). Protein determinations were by the method of Lowry (1951). Radioactive iodine was determined by gamma counting (Beckman Instruments, Inc.); ^{32}P was determined by scintillation counting using Optiflor (Hewlett-Packard Co., Palo Alto, CA) or by autoradiography using Kodak XAR film and fluorescent intensifying screens at −60°C.

Results

Synapsin I Binds Actin and Reduces the Viscosity of F-actin Solutions

The ability of synapsin I to bind actin was investigated by measuring the ability of purified solutions of synapsin I to cosediment with F-actin. Under the conditions chosen for these experiments, actin exists primarily as intermediate length filaments which sediment readily at 100,000 *g*, but not at 10,000 *g*. Experiments were performed using both unlabeled synapsin I and with two different preparations of synapsin I labeled with ^{125}I. These results are presented in Fig. 1. While a systematic variability was observed between different preparations of synapsin I and actin (Fig. 1), it is clear that synapsin I binds actin in a saturable and inhibitable manner. At saturation, approximately one synapsin I molecule is

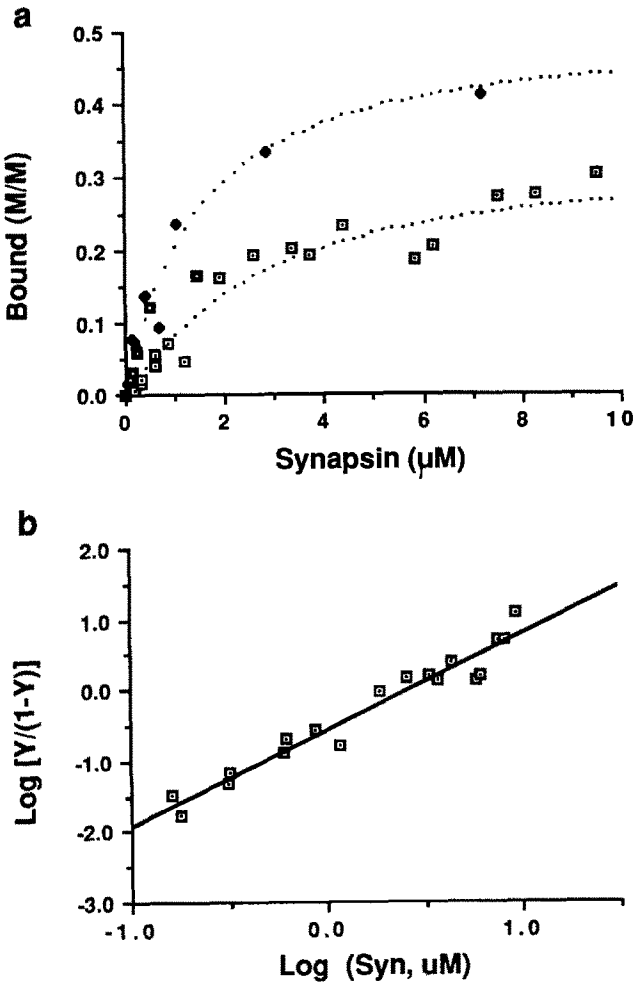

Figure 1. Synapsin I binds to F-actin. Various amounts of unlabeled (*solid symbols*) or ^{125}I-labeled synapsin I (*open symbols*) were co-sedimented at 100,000 *g* with F-actin for 1 h, and the amount of protein precipitated was measured by elution of the dye from stained SDS gels or by gamma counting. (*a*) The results from three preparations of synapsin I and actin are shown. The binding of synapsin to F-actin saturates near a synapsin/actin ratio of ~1:3–1:2. There is a slight systematic variation in the binding isotherms from preparation to preparation, and generally the iodinated preparations gave slightly weaker binding. Nonlinear regression analysis of one of the experiments with unlabeled synapsin I yielded a dominant K_d of 1.24 μM (variance = 0.0008) with a binding stoichiometry of 1:2 (synapsin/actin) (*top dotted curve*); a similar analysis of one of the experiments with the ^{125}I-labeled synapsin I yielded an estimated K_d of 1.91 μM (variance of fit = 0.0006) with a binding stoichiometry of 1:3 (*bottom dotted curve*). (*b*) The binding data from one of the experiments shown above (same symbols) replotted according to the Hill equation. Regression analysis of this data yields a slope of 1.35 ± 0.15 (2 SD) (r = 0.974).

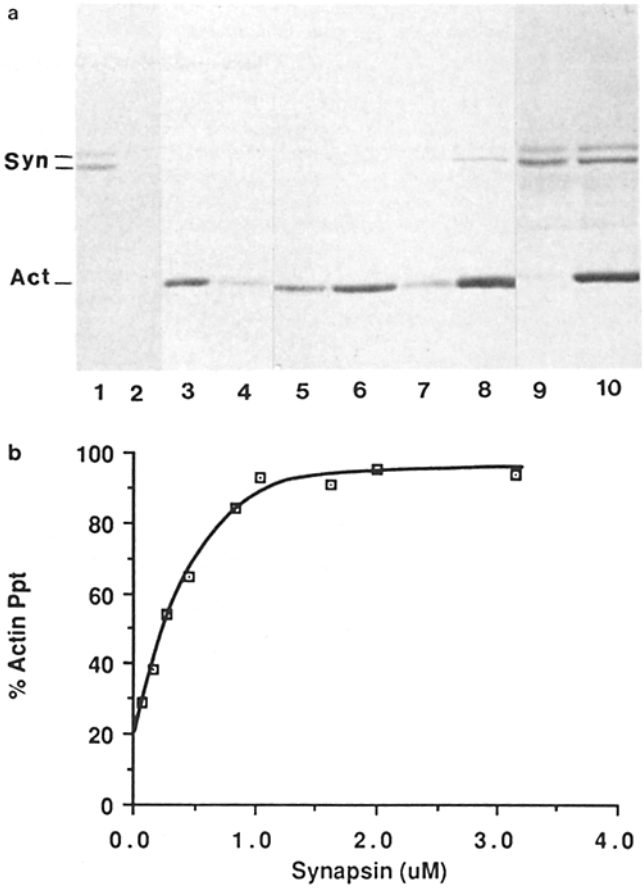

Figure 2. Synapsin I enhances F-actin sedimentation. Various concentrations of synapsin were incubated with F-actin for 30 min at 25°C, and then sedimented at 10,000 *g*. The amount of actin and synapsin in the supernatant and pellet for each assay was determined by densitometry of Coomassie Blue–stained SDS gels. (*a*) SDS-PAGE of mixtures of synapsin I and F-actin. Samples from the supernatants (lanes *1*, *3*, *5*, *7*, and *9*) and pellets (lanes *2*, *4*, *6*, *8*, and *10*) after low speed sedimentation are shown. Lanes *1* and *3* display the supernatants of synapsin I and F-actin alone. Lanes *2* and *4* are from the corresponding pellets of the isolated proteins. Lanes *5–10* demonstrate the effects of increasing amounts of synapsin on the sedimentation of F-actin. Lanes *5*, *7*, and *9* are the supernatants, and lanes *6*, *8*, and *10* are their corresponding pellets for an experiment with 0.38, 0.77, and 3.0 μM total synapsin, respectively. Each assay (except for lanes *1* and *2*) contained 7.0 μM actin. (*b*) Quantitation of the amount of actin sedimenting at 10,000 *g* indicates that even in the presence of low concentrations of synapsin I, all of the actin is recovered in the pellet. The abscissa represents the total synapsin concentration.

bound for every 2–3 actin monomers, a value typical of other actin-binding proteins (Stossel et al., 1985; Pollard and Cooper, 1986). The apparent K_d for synapsin I binding to actin determined by nonlinear regression analysis of several experiments ranged from 1.2 to 1.9 μM (Fig. 1). The source of the slight systematic variation between the experiments shown in Fig. 1 is not known with certainty, although it correlates best with specific preparations of either synapsin I or actin, and in general the preparations which have been iodinated show slightly weaker binding. When the data is plotted according to Hill (Fig. 1 *b*), the slope of the regression line is 1.35 ± 0.15 (2 SD), suggesting that synapsin I may bind to actin with a small degree of positive cooperativity. Separate experiments examining the inhibition by ^{125}I synapsin I binding by native unlabeled synapsin I demonstrate a simple competitive inhibition between the labeled and unlabeled material, with a K_i of ~2.0 μM (data not shown), similar to the K_ds estimated above. The ability of synapsin I to bind to F-actin requires a native conformation,

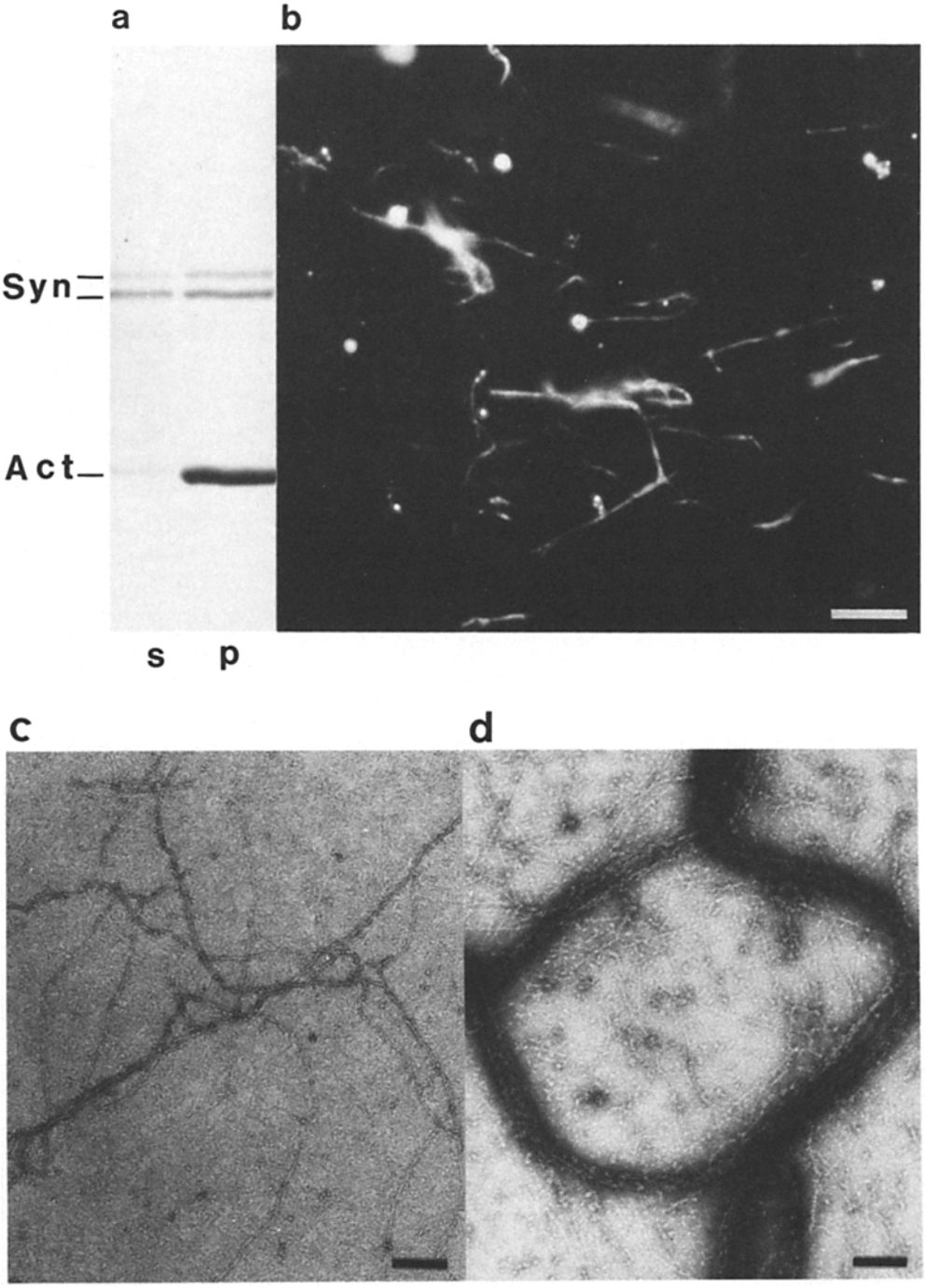

Figure 3. Actin bundle formation by synapsin I. (*a*) SDS-PAGE of a mixture of synapsin I and F-actin. Samples of pellet (*p*) and supernatant (*s*) after low speed sedimentation are shown. (*b*) Dark-field light micrograph of the mixture of F-actin and synapsin I before centrifugation. The bundled filaments are evident. Bar, 2.0 μm. (*c*) Negatively stained electron micrograph of the F-actin solution before the addition of synapsin. Bar, 160 nM. (*d*) Negatively stained electron micrograph of the same solution of actin as shown in *c* after the addition of synapsin. Bar, 160 nM.

since heat-denatured synapsin I (90°C, 5 min) did not sediment with F-actin.

As an alternative measure of actin binding, the effect of synapsin I on the high-shear viscosity of solutions of F-actin was determined. These results are presented in Table I. Very small amounts of synapsin I enhanced the viscosity slightly (105% of control), while increased amounts reduced the viscosity up to 61%.

Synapsin I Enhances the Sedimentation of F-actin Solutions Due to a Filament-bundling Action

The viscosity and high speed sedimentation studies suggested that synapsin I was interacting with actin filaments, and that it exhibited either a bundling or severing action. To distinguish these possibilities, the sedimentation studies were repeated under conditions in which F-actin alone would not sediment (10,000 *g* for 60 min). These results are shown in Fig. 2. In the absence of synapsin I, 26% of the actin sedimented. With the addition of increasing amounts of synapsin I, the amount of actin sedimenting rapidly approached 100%. This effect is similar to that observed with other actin-bundling proteins such as villin or fimbrin (e.g., see reviews by Pollard and Cooper, 1986; Mooseker, 1985).

The bundling activity of synapsin I was also evident by dark-field light microscopy or by electron microscopy. In the presence of synapsin I, under conditions that enhance the

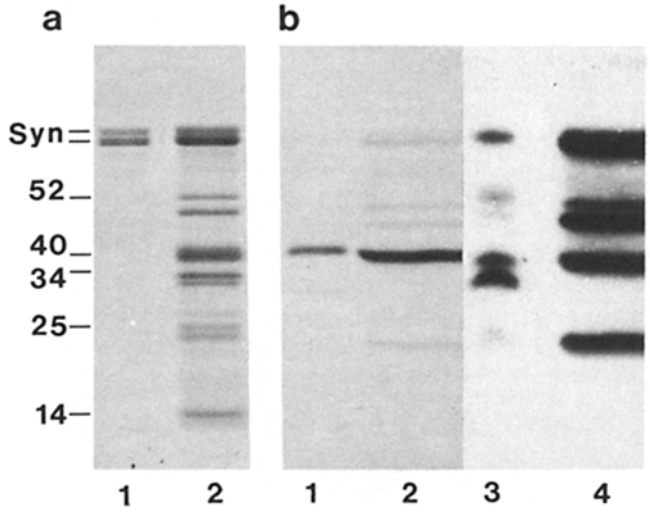

Figure 4. Specific NTCB peptide fragments bind F-actin. (*a*) Coomassie Blue–stained SDS gel of purified synapsin I (lane *1*) and of the peptides generated by NTCB cleavage of synapsin I (lane 2). The conditions of cleavage are given in the text. (*b*) Supernatant (lanes *1* and *3*) or pellet (lanes *2* and *4*) fractions of a mixture of F-actin with NTCB peptides generated from synapsin I. The conditions of this assay were 10 μM actin, 1.0 μM synapsin peptides, in G-actin buffer (see text), 0.1 M KCl, 2 mM magnesium chloride, pH 8.0. The samples were sedimented at 10,000 *g* for 1 h. The peptides that sediment with actin are difficult to discern by Coomassie Blue staining (lanes *1* and *2*), but are readily apparent in the autoradiogram after blotting with antibodies to synapsin and ^{125}I-labeled staphylococcus protein A. Note that while most bands contain fragments which will bind, no binding peptides of 34,000 M_r are evident.

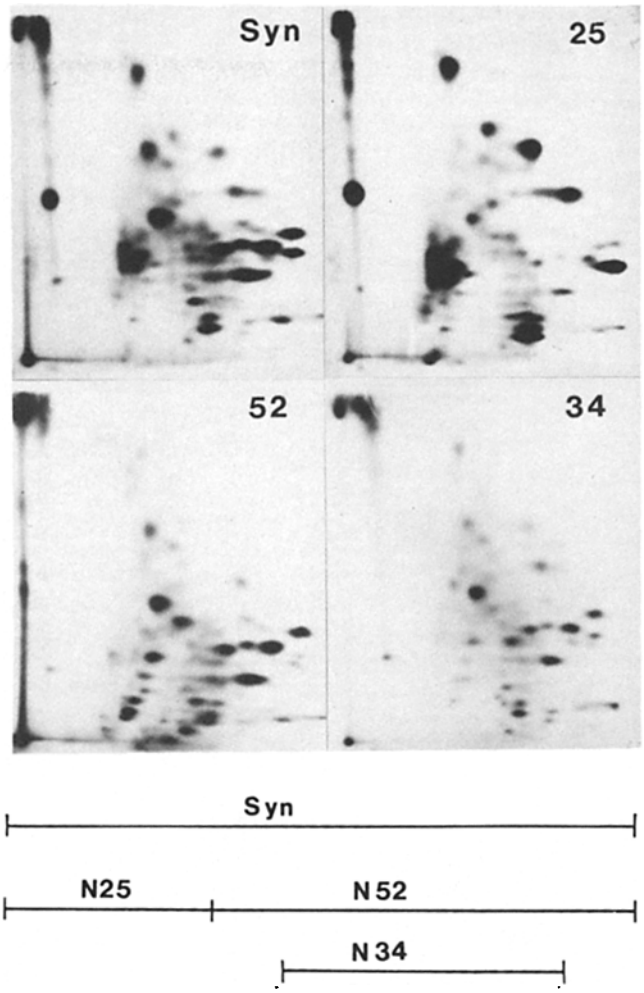

Figure 5. Two complementary NTCB peptides cosediment with actin. Prominent actin-binding and nonbinding fragments from the gel shown in Fig. 4 were mapped. The map of the parent molecule is labeled *Syn*. The maps of two binding fragments of 25,000 and 52,000 M_r are shown; they are almost completely complementary. However, the map of the 34,000 M_r nonbinding fragment is contained within the map of the 52,000 M_r fragment. Separate experiments (data not shown) indicate that the 34,000 band arises from the central portions of synapsin I. This alignment is shown in the line drawing.

sedimentation of actin (Fig. 3 *a*), large filamentous aggregates are observed by dark-field microscopy (Fig. 3 *b*). When these solutions are examined by electron microscopy after negative staining, the aggregates are found to consist of thick but relatively uniform bundles of actin filaments joined by densely packed molecules of synapsin I (Fig. 3 *d*). Identical solutions lacking synapsin I show only dispersed filaments of actin (Fig. 3 *c*).

The Actin-binding Activity of Synapsin I Is Confined to Specific Peptide Domains

To identify the site(s) in synapsin I responsible for its actin-binding and -bundling activity, the protein was cleaved at its cysteine residues by reaction with NTCB, after which fragments competent for actin-binding were identified by cosedimentation assay. The results of this experiment are shown in Figs. 4 and 5. Cleavage of synapsin I with NTCB after reduction with DTT yields many fragments, which on one-dimensional SDS-PAGE appear as approximately eight bands (Fig. 4 *a*, lane 2). The relationship of each of these fragments to the parent molecule has been determined (Petrucci, T. C., and J. S. Morrow, manuscript in preparation; also Petrucci and Morrow, 1986). When this mixture of peptides is sedimented with F-actin at 10,000 *g*, only some of the fragments precipitate. This is shown in Fig. 4 *b*. Lanes *1* and *2* are the supernatant and pellet fractions, respectively, from such an experiment, stained with Coomassie Blue. The prominent band near 40,000 M_r is actin; the bands arising from synapsin I are only faintly visible. However, when an identical gel from the same experiment was immunoblotted using polyclonal antisera to synapsin I, the distribution of the various NTCB fragments in the supernatant (Fig. 4 *b*, lane *3*) and pellet (lane *4*) is apparent. In addition to residual intact synapsin I, prominent peptides which bind actin are at 25,000, 40,000, and 52,000 M_r. Separate experiments indicate that the 14,000 M_r fragment will also bind, although this region of synapsin I is not recognized well by our antisera, and hence it does not appear in the blot shown in Fig. 4 *b*. Significantly, some peptides do not bind actin. The most prominent of these is represented by the band at 34,000 M_r (Fig. 4 *b*, lane *3*). There are no NTCB peptides of synapsin I of this molecular weight which are competent to bind actin.

The relationship of these peptides to the parent molecule was determined by two-dimensional cellulose peptide mapping of iodinated fragments cut from Coomassie Blue–stained gels. The relationship of the major binding and nonbinding

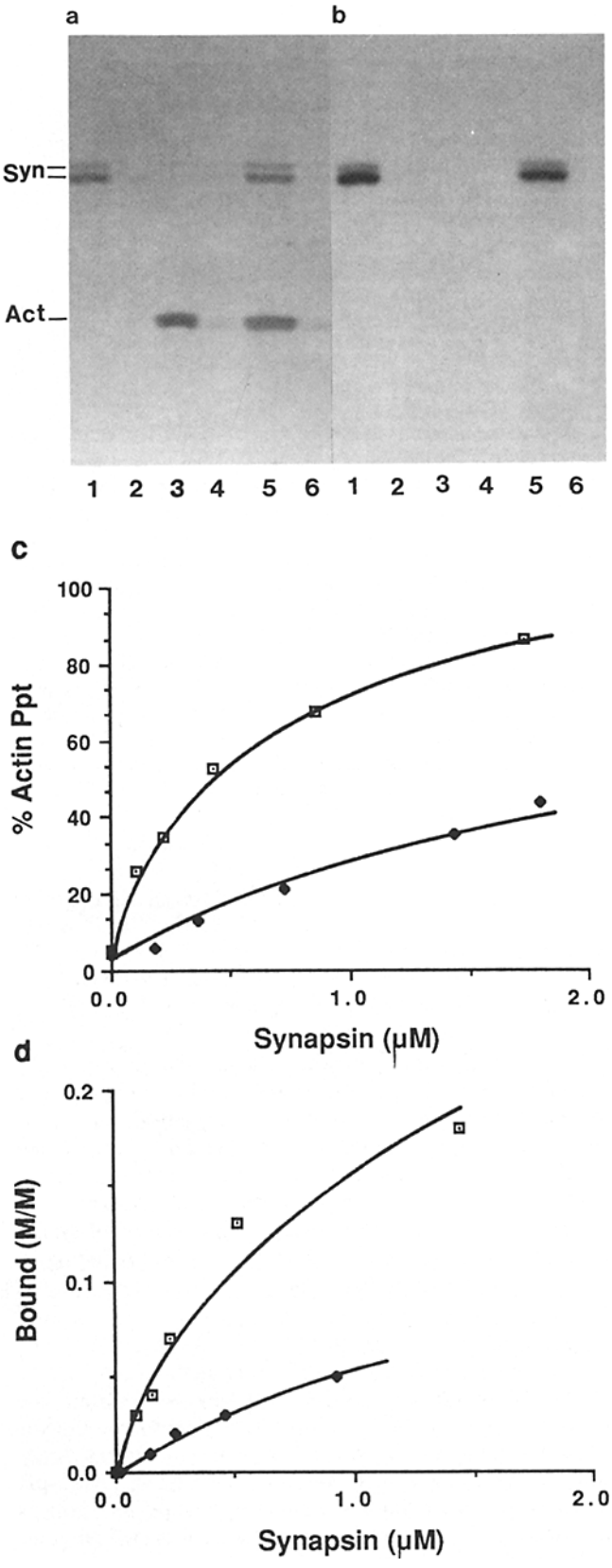

Figure 6. Phosphorylation of synapsin I by endogenous kinases inhibits its actin-bundling and -binding activity. Synapsin I was incubated with ^{32}P-γ-ATP and a crude brain homogenate extract, or with purified Ca^{++}/calmodulin-dependent kinase II, and after repurification, assayed for its ability to stimulate actin sedimentation at 10,000 *g* and to bind to F-actin. (*a*) Coomassie Blue–stained SDS gel. (*b*) Corresponding autoradiogram, showing the distribution ^{32}P-phosphorylated synapsin. Odd lanes are supernatants; even lanes are the pellets. (Lanes *1* and *2*) Synapsin alone; (lanes *3* and *4*) F-actin alone; (lanes *5* and *6*) phosphorylated synapsin and F-actin. Note that there is no stimulation of actin sedimentation by phosphorylated synapsin and no synapsin appears in the pellet (compare with Fig. 2). (*c*) Quantitation of the effect of phosphorylation of synapsin by Ca^{++}/calmodulin-dependent kinase on the ability of synapsin to bundle actin. The level of phosphate incorporation achieved in this experiment was 0.86 mol per mol of synapsin I. Closed symbols are the phosphorylated synapsin. Open symbols are for the same synapsin preparation before its phosphorylation. (*d*) Phosphorylation of synapsin by Ca^{++}/calmodulin kinase II also reduces its ability to bind to actin, as measured by its ability to cosediment with actin at 100,000 *g*. Symbols are as above.

fragments derived from these experiments is shown in Fig. 5. The complete molecule is represented by the 25,000 and 52,000 M_r peptides, both of which bind actin. The band at 40,000 M_r, which also binds actin, is a mixture of peptides which overlap either the 25,000 or the 52,000 M_r fragments (data not shown). The 34,000 M_r fragment, which demonstrates no actin-binding ability, is derived from the central portion of the 52,000 M_r peptide (Fig. 5) (Petrucci and Morrow, 1986). Separate studies in which nonequilibrium IEF was used (data not shown) indicate that the peptides comprising the 34,000 M_r band are the most basic in synapsin I. Therefore, these results indicate that the ability of certain peptides to bind actin or synapsin I–actin complexes does not simply reflect their degree of basicity. To cosediment with actin, a synapsin I peptide fragment must encompass either one or the other end of the intact molecule.

The Actin-bundling and -binding Activity of Synapsin I Is under Phosphorylation Control

Synapsin I contains three sites of potential phosphorylation (e.g., see Kennedy and Greengard, 1981, and references therein); one site is phosphorylated by a cAMP-dependent kinase, the others by Ca^{++}/calmodulin-dependent kinase(s). The synapsin I used for these studies, in which actin was actively bundled, contained less than 0.1 mol of inorganic phosphate per mole of protein, as determined by inorganic phosphate analysis. Phosphorylation of this synapsin I by incubation with a crude brain homogenate in the presence of ATP, Ca^{++}, and calmodulin (Kennedy and Greengard, 1981) rendered it incompetent for actin-bundling activity, as shown in Fig. 6. The Coomassie Blue–stained gel is shown in Fig. 6 *a*, and the corresponding autoradiogram depicting the behavior of the phosphorylated synapsin I is shown in Fig. 6 *b*. Note that the phosphorylated synapsin I has no effect on the sedimentation of actin at 10,000 *g*.

To determine if this effect of phosphorylation was specific for a certain kinase, the experiment was repeated using synapsin I phosphorylated by calcium- and calmodulin-dependent kinase II purified from bovine brain. These results are shown quantitatively in Fig. 6 *c*. In this preparation of synapsin I, 0.86 mol of ^{32}P was incorporated by the calmodulin-dependent kinase. This level of phosphorylation reduced the actin-bundling activity of synapsin I greater than fivefold, as judged by a comparison of the amount of phosphorylated synapsin I required to sediment a given amount of actin.

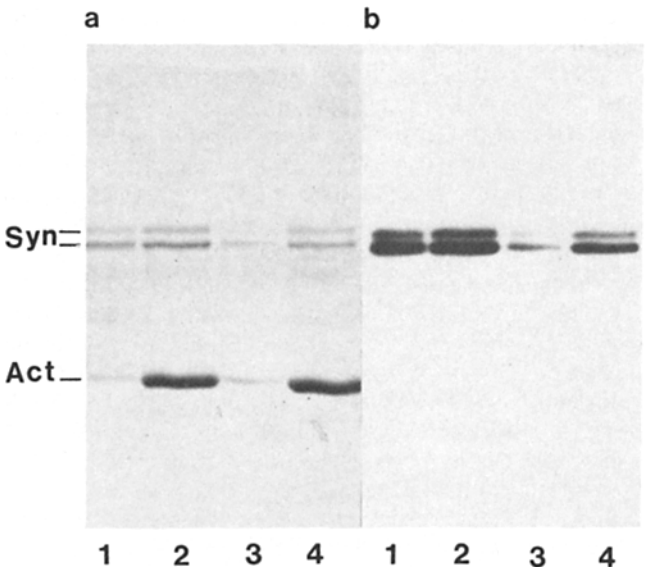

Figure 7. cAMP-dependent phosphorylation does not affect actin bundling. Synapsin I was phosphorylated by the catalytic subunit of cAMP-dependent kinase as described in the text. (*a*) Coomassie Blue–stained SDS gel. (*b*) Corresponding autoradiogram; even and odd lanes are as described in previous figure. (Lanes *1* and *2*) Synapsin/actin ratio of 1:3; (lanes *3* and *4*) synapsin/actin ratio of 1:7. Note that the phosphorylation of synapsin I by cAMP-dependent kinase does not diminish its ability to stimulate the low speed sedimentation F-actin.

Thus, phosphorylation of synapsin I by the purified calmodulin-dependent kinase II alone quantitatively inhibits its bundling activity.

Phosphorylation by the calcium- and calmodulin-dependent kinase II also reduced the ability of synapsin I to sediment with actin at 100,000 *g*, even at low concentrations of synapsin I (Fig. 6 *d*). The degree of reduction in its actin-binding activity paralleled its reduced actin-bundling activity (compare Fig. 6, *c* and *d*).

In contrast to the marked effect of phosphorylation by the calmodulin-dependent kinase, phosphorylation of synapsin I by the purified catalytic subunit of cAMP-dependent kinase was without effect on its actin-bundling activity. These results are shown in Fig. 7 for two different synapsin I concentrations. At either concentration, all of the actin is precipitated, together with a stoichiometric amount of phosphorylated synapsin I. Therefore, in contrast to the protein phosphorylated by the crude brain extract or by the calcium- and calmodulin-dependent kinase II, cAMP-dependent phosphorylation alone is without effect on synapsin I's ability to bundle actin.

Phosphorylation of Synapsin I Enhances Its Interaction with Microtubule Proteins

As an alternative measure of the activity of synapsin I phosphorylated by the brain homogenate, its ability to interact with bovine brain tubulin was tested. This was done by measuring the ability of synapsin I to copurify with tubulin after repeated cycles of warm polymerization and cold depolymerization. These results are shown in Fig. 8. An SDS gel analysis of the purified proteins is shown in Fig. 8 *a*. Both unlabeled and phosphorylated synapsin I cosediment with

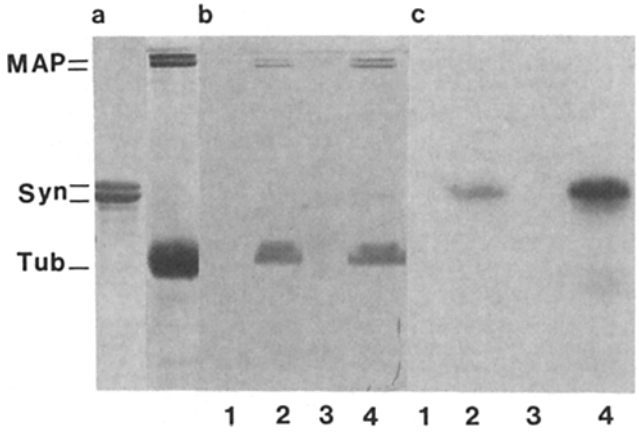

Figure 8. Phosphorylation of synapsin enhances its microtubule-binding ability. The ability of synapsin I phosphorylated by endogenous kinases (compare with Fig. 6) to co-purify with bovine brain tubulin was examined. (*a*) Coomassie Blue–stained gel showing the purified synapsin and tubulin (with MAPs) used for this experiment. (*b*) Coomassie Blue–stained gel showing supernatants (lanes *1* and *3*) and pellets (lanes *2* and *4*) from two experiments using either unlabeled (unphosphorylated) (lanes *1* and *2*) or phosphorylated (lanes *3* and *4*) synapsin I. While only a faint Coomassie Blue band for synapsin is visible in the microtubule pellets, it is easily discerned on the autoradiograms of the ^{125}I-labeled immunoblots shown in *c*. Note the greater intensity of the band for phosphorylated synapsin. This is not due to the presence of ^{32}P, since the blots were excised and quantitated by gamma counting. In these experiments, 4.7 times as much synapsin is present with the tubulin in lane *4* as in lane *3*.

tubulin (Fig. 8 *b*, lanes *2* and *4*, respectively), although the synapsin I is difficult to detect on these Coomassie Blue–stained gels. The presence of synapsin I in the pellet is clearly evident after immunoblotting with anti–synapsin I antibodies, as shown in Fig. 8 *c*. Quantitation of the amount of synapsin I recycling with the tubulin by gamma counting of the ^{125}I-labeled immunoblots indicated that phosphorylation of synapsin I by incubation with the brain homogenate enhanced the amount of material recycling with tubulin by four- to fivefold (e.g., compare lanes *2* and *4* in Fig. 8 *b* and *c*). Thus, while additional experiments will be required to quantitatively evaluate this phenomenon and determine its kinase specificity, it appears that phosphorylation of synapsin I may stimulate microtubule binding while reducing its ability to bundle actin filaments.

Discussion

The results presented here establish that synapsin I has actin-bundling activity which is subject to control by a Ca^{++}/calmodulin-dependent kinase. The evidence for this is threefold. (*a*) Synapsin I at physiologic ionic strength and pH produces bundled actin filaments morphologically, reduces the viscosity of F-actin solutions, and enhances the low speed sedimentation of F-actin solutions. (*b*) The actin-binding and/or -bundling properties of synapsin I are localized to specific peptide fragments generated after NTCB digestion, while other similarly sized and more basic fragments show no activity. (*c*) Finally, phosphorylation of synapsin I by Ca^{++}/calmodulin-dependent kinases but not the catalytic

Table I. Effects of Synapsin I on the Viscosity of F-actin Solutions

Actin	Synapsin	S:A	Specific Viscosity	Control
μM	*μM*		*cs*	*%*
7.0	0.00	0	0.182	100
7.0	0.06	1:120	0.191	105
7.0	0.29	1:24	0.146	80
7.0	2.91	1:2	0.107	61
10.0	0.00	0	0.310	100
10.0	0.59	1:17	0.276	89
10.0	3.13	1:3	0.251	81
10.0	5.00	1:2	0.260	83

All measurements were made by high-shear viscometry at 25°C. The values determined were independent of the time of incubation of synapsin with F-actin over periods between 1 and 60 min. Each entry is the mean of seven determinations. The error (SD) was <0.001 centistrokes (cs) for each value.

subunit of cAMP-dependent kinase inactivates its actin-binding/bundling properties.

Many macromolecules with basic isoelectric points will bind and gel actin solutions in vivo (Griffith and Pollard, 1982), an interaction often of dubious physiologic significance (Craig and Pollard, 1982). It is unlikely however that actin binding by synapsin I represents yet another example of this phenomenon. Not all peptides generated from synapsin I by NTCB cleavage bind actin, even though those that do not bind are more basic than the active fragments. Denatured synapsin I also does not bundle actin filaments, indicating that native conformation is required for the interaction. And finally, actin is a prominent component in the axon terminal (for review, see Fifkova, 1985), and is associated with synaptosomes in a Ca^{++}-dependent and reversible manner (Bernstein and Bamburg, 1985). Separate studies have implicated synapsin I as a protein link between synaptic vesicles and the neuronal cytoskeleton (Goldenring et al., 1986); while these studies focused on microtubule and neurofilament interactions, they do not exclude the participation of microfilaments.

The actin-bundling activity demonstrated here requires that synapsin I form cross-links between adjacent actin filaments. This requires either that synapsin I have multiple actin-binding sites, or that it attain such multivalency by self-association. We have not observed self-association of synapsin I in dilute solution (<0.6 μM) as judged by velocity sedimentation experiments (data not shown). This observation is consistent with earlier reports (Ueda and Greengard, 1977). However, it has been suggested that in more concentrated solutions (19 μM), tetramers of synapsin I may form (Baines and Bennett, 1986). The potential contribution of synapsin I dimers or tetramers to its actin-bundling activity is unknown, since the present experiments cannot differentiate between the presence of two actin-binding sites on a single synapsin I vs. one actin-binding site and one self-association site. However, it is clear that the active synapsin I complex is multivalent, as required for cross-linking. The weak positive cooperativity as suggested by the nonlinearity of the Scatchard curves of the binding data (not shown) and the Hill coefficient of 1.3 for this binding indicate that at least two binding interactions are involved, while the ability of two complementary NTCB fragments to sediment with actin suggests that the binding sites mediating actin–synapsin I and/or synapsin I–synapsin I interactions are topographically distinct.

Previous studies have noted that calcium- and calmodulin-dependent kinase II phosphorylates synapsin I in the "tail" region, a region involved in the binding of synapsin I to vesicles (DeCamilli and Greengard, 1986). Because of the strong inhibition of both actin-bundling and -binding activity observed in the present studies by such phosphorylation, it is tempting to speculate that the 52,000 M_r NTCB peptide, which encompasses this collagenase-sensitive region and which appears to be phosphorylated by the calmodulin-dependent kinase (Petrucci, T. C., and J. S. Morrow, unpublished observations), must also contain an actin-binding site. However, whether this fragment or the other active NTCB fragments each actually contain an actin-binding site is unknown, since fragments without actin-binding activity may remain noncovalently attached to an active fragment or to the residual intact synapsin I during the cosedimentation assays. Additional experiments with purified NTCB peptides will therefore be required to determine the exact mechanism by which synapsin I bundles actin filaments.

It is interesting that there are striking functional similarities between the activity of synapsin I and another neuronal phosphoprotein, the microtubule-associated protein 2 (MAP2) (e.g., see Selden and Pollard, 1986, and references therein). MAP2 is a 280,000 M_r protein preferentially associated with dendritic microtubules, and has also been implicated in the control of secretory granule interactions with the plasma membrane and with microtubules (Sherline et al., 1977; Suprenant and Dentler, 1979). Presumably, the membrane attachment of MAP2 to the granule is mediated by direct interactions between MAP2 and clathrin or a clathrin-like protein (Sattilaro et al., 1980). Unphosphorylated MAP2 has been shown to bind and bundle actin filaments (Sattilaro et al., 1981), while the phosphorylated protein does not (Nishida et al., 1981; Selden and Pollard, 1986; Sattilaro, 1986). Both forms of MAP2 bind microtubules. The ability of MAP2 to interact with multiple components of the cytoskeleton as well as indirectly with plasma or vesicle membranes (Dentler et al., 1980; Sherline et al., 1977; Suprenant and Dentler, 1979) may offer a mechanism whereby the organization of synaptic vesicles could be regulated. The properties of synapsin I appear to be remarkably similar. Its interaction with actin, as reported here, parallels that of MAP2. Previous work has established synapsin I as a microtubule-bundling (Baines and Bennett, 1986) and membrane-binding (e.g., see Huttner et al., 1983; or Schiebler et al., 1986, and references therein) protein.

If MAP2 and synapsin I are so functionally similar, why are they both needed on synaptic vesicles? The answer may be that they in fact characterize different classes of synaptic vesicles. Synapsin I is present only on small (40–60 nM) synaptic vesicles (Navone et al., 1984). MAP2 is found in association with secretory granules of many tissues (Sherline et al., 1977; Suprenant and Dentler, 1982). The large dense core granules present in axons most closely resemble secretory granules both morphologically and functionally (Navone et al., 1984; Palade, 1975). It is presumably with these vesicles that MAP2 associates. The small synaptic vesicles are a unique feature of nerve terminals since they are involved in the local processing and recyling of both membrane and neurotransmitter. Therefore, the association of synapsin

I with these vesicles offers one mechanism whereby their interactions with microfilaments and microtubules may be uniquely regulated, independent of dense core granule function.

Finally, it is perhaps important to emphasize that the actin-bundling activity of synapsin I described here does not require that synapsin I function as an actin-bundling protein in vivo. These results define synapsin I as a protein with the ability to interact in a multivalent fashion with F-actin, a function compatible with its presumed membrane–cytoskeleton linking role.

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