Actin and Tubulin Binding Domains of Synapsins Ia and Ib†

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ABSTRACT: Synapsins Ia and Ib are neuronal phosphoproteins involved with the regulated clustering of small synaptic vesicles at the presynaptic terminus. In vitro they bind and bundle filaments of both actin and tubulin. Previously, we identified an actin binding domain in the NH_2-terminal 25-kDa fragment (N25) generated by 2-nitro-5-thiocyanobenzoic acid (NTCB) cleavage of synapsin I and found that a complementary COOH-terminal 52-kDa portion of the molecule (N52) contained either a second actin binding site or a site of self-association [Petrucci, T. P., & Morrow, J. S. (1987) *J. Cell. Biol. 105*, 1355]. Using direct binding assays between actin, tubulin, and specific synapsin NTCB-derived peptides, we confirm the ability of purified N25 to bind but not bundle actin and demonstrate that the complementary N52 (or N50) fragments from synapsins Ia and Ib and a 14-kDa fragment derived from the middle of the molecule also associate directly with actin. An antibody specific for N25 inhibits the actin binding activity of N25 and the actin bundling but not the actin binding activity of intact synapsin I. Similar studies conducted with purified tubulin and tubulin immobilized on Sepharose demonstrate that both tubulin and actin bind at approximately the same sites in the NH_2-terminal half of synapsin I. Although the fragments derived from the COOH terminus of both synapsin Ia and synapsin Ib (N40b/N34) were devoid of measurable actin binding activity after NTCB cleavage, they were specifically labeled in the intact molecule by a photoactivated cross-linker bound to F-actin. Collectively, these results indicate that synapsins Ia and Ib possess two actin and tubulin binding domains located in the NH_2-terminal half of the molecule and suggest that a third actin binding domain is located in the COOH-terminal region. The NH_2-terminal sites are found in NTCB peptides N25 and N14, while the third site, apparently of lower affinity, resides in N40b/N34. It is hypothesized that, in the intact molecule, the two NH_2-terminal domains contribute to a single high-affinity actin and/or tubulin binding site in the "globular" head region of synapsin I, while the third actin binding domain constitutes the topographically distinct site required for the actin bundling activity of the native molecule. The 45-residue COOH extension that distinguishes synapsin Ia from synapsin Ib appears not to be involved with actin binding, since no differences were found in the ability of N40b and N34 to be photo-cross-linked to actin. These results provide a clear biochemical basis for the association of synaptic vesicles with microfilaments and microtubules.

The cytoskeleton plays a significant role in directing transmembrane vesicle traffic [for reviews, see Kelly (1988), Aunis and Bader (1988), Coleman et al. (1989), Morrow (1989), and Rodriquez-Boulan and Nelson (1989)]. Such trafficking is prominent at the presynaptic membrane, where the release of neurotransmitter involves the stimulated exocytosis and subsequent recycling of small synaptic vesicles [for reviews, see Kelly (1988) and Südhof et al. (1989)]. Correspondingly, several cytoskeletal elements have been identified in the presynaptic terminal, including tubulin, actin, and spectrin (fodrin), and synaptic vesicles have been found in association with these elements in vivo [e.g., see Hirokawa et al. (1989), Landis et al. (1988), and Goldenring et al. (1986)]. Presumably, pools of small synaptic vesicles are clustered at the presynaptic membrane by linkage to these cytoskeletal proteins and mobilized by means of posttranslational regulatory mechanisms at or about the time of Ca^{2+}-stimulated vesicle release [e.g., see Kelly (1988) for review of this hypothesis]. The molecular mechanisms by which this process may be mediated have only recently begun to be understood.

A central component in the linkage of small synaptic vesicles to the cytoskeleton appears to be synapsins Ia and Ib (collectively referred to as synapsin I) [for review, see DeCamilli and Greengard (1986)]. These two closely related phosphoproteins arise from a single gene by alternate mRNA processing and differ only in a short COOH-terminal region (Södhof et al., 1989). Synapsin I is multifunctional and can bind to sites on the membrane of synaptic vesicles (Schiebler et al., 1986; Benfenati et al., 1989), as well as to several cytoskeletal components including microtubules and spectrin (Baines & Bennett, 1986) and F-actin (Petrucci & Morrow, 1987, 1988; Bähler et al., 1987, 1989). Its interaction with actin has also been shown by in vitro assay to be strongly downregulated after phosphorylation by Ca^{2+} and calmodulin-dependent kinase II but only minimally altered after phosphorylation by cAMP-dependent kinase (Petrucci & Morrow, 1987). These activities correlate well with studies on the effects of synapsin I phosphorylation on synaptic vesicle release in the squid giant synapse (Llinas et al., 1985). The participation of synapsin I in the linkage of synaptic vesicles to the cytoskeleton has also been directly shown in vivo by deep-freeze etch electron microscopy, where proteins with the shape and dimensions of synapsin I link synaptic vesicles to microfilaments and microtubules in presynaptic zone membranes (Hirokawa et al., 1989; Landis et al., 1988).

Despite these advances, several aspects of the function of synapsin I remain unanswered. In vitro, synapsin I will bundle actin filaments, indicating that the functional synapsin I unit

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is at least bivalent for actin binding. The existence of at least two actin binding domains in synapsin I was also suggested by our initial NTCB[1] cleavage studies, in which NTCB-generated peptides of 25, 52, 40, and 14 kDa (N25, N52, N40, and N14, respectively) were found to cosediment with actin (Petrucci & Morrow, 1987; Petrucci et al., 1988). However, although an actin binding domain in the NH_2-terminal 25-kDa region of synapsin I was identified and a second one postulated in N14, the possibility could not be excluded that the NTCB peptides from the COOH portions of the molecule (N52, N40, N14) bound actin only by associating with intact synapsin or with N25. In addition, the site or sites of tubulin binding in synapsin I were completely unknown.

Recently, a partial answer to these questions has been provided by an independent study of the actin binding ability of the NTCB cleavage fragments of synapsin I (Bähler et al., 1989). These workers confirmed the identification of an actin binding domain in N25 and the inability of the N32/34 (35/36 in their nomenclature) fragments to bind and established that the purified COOH fragment (N52) was also competent for actin binding. They also postulated that the second actin binding domain was contained in N14 and on the basis of recent sequence data on synapsin I (Südhof et al., 1989) deduced that the N14 fragment must arise from the center of synapsin I.

In the present report, an alternative approach has been taken to establish the location and the number of actin binding sites in synapsin I and in NTCB digests of synapsin I, and the domains involved with tubulin binding have been identified. An antibody which specifically inhibits the binding of N25 to actin and the bundling activity of intact synapsin I, along with purified preparations of N25 or its complementary COOH-terminal NTCB fragments, has been used to establish the nature and location of the actin and tubulin binding domains. The location of these domains has been deduced by peptide mapping after two-dimensional NEPHGE–SDS–PAGE analysis of the NTCB peptides, and these alignments have been compared with recent sequence data on synapsin I. In addition, photo-cross-linking between F-actin and synapsin I has been used to study the sites of actin interaction with the intact molecule. We find that synapsin I has at least two actin and tubulin binding domains located in the NH_2-terminal half of the molecule and probably a third site for actin binding in the COOH-terminal domain. A portion of these results has been presented previously in abstract form (Petrucci & Morrow, 1987).

Materials and Methods

Purification of Proteins. Synapsin I was purified from frozen bovine brain by acidic extraction, followed by ion-exchange chromatography on CM-cellulose (Whatman) and hydroxyapatite (Bio-Rad). Rabbit skeletal muscle actin was prepared from an acetone powder extract and stored under conditions of continuous dialysis as G-actin in 2 mM Tris-HCl, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM DTT, and 0.2% NaN_3, pH 8.0. Immediately prior to use, actin was polymerized by the addition of KCl to 0.1 M and $MgCl_2$ to 2 mM in the above buffer. In general, the concentration of actin was 4–5 μM for all experiments. Tubulin was prepared from bovine brain homogenates by three cycles of temperature-dependent assembly and disassembly in 0.1 M Pipes, 1 mM EGTA, 0.1 mM $MgCl_2$, and 1 mM GTP, pH 6.6, followed by chromatography on Whatman DE52. Further details on the preparation of these proteins are described (Petrucci & Morrow, 1987).

Cysteine-Specific Cleavage of Synapsin I by S-Cyanylation and Purification of the Fragments. Purified synapsin I was cleaved as before with 2 mM NTCB (Sigma Chemical Co., St Louis, MO) in 7.5 M Gnd-HCl, 0.2 M Tris-HCl, and 0.1 mM EDTA, pH 8.0, for 1 h at room temperature, after which the pH was adjusted to 9.0 and the incubation continued overnight at 37 °C (Petrucci & Morrow, 1987). After cleavage, samples were dialyzed into 25 mM Tris-HCl, 1 mM EDTA, 0.1 mM 2-mercaptoethanol, 25 mM NaCl, and 4 M urea, pH 8.0, and purified by HPLC cation-exchange chromatography on a 7.5 × 75 mm column (Bio-Gel SP-5-PW, Bio-Rad Laboratories, Richmond, CA). Injection volumes were typically 2 mL. Effluent was monitored at 280 nm. A flow rate of 0.5 mL/min was used, with a series of step gradients to 0.5 M NaCl. The total time required to complete the separation was 70 min.

Two-Dimensional Cellulose Peptide Mapping. Two-dimensional chymotryptic cellulose peptide maps were prepared as before from the NTCB peptides, after they had been cut directly from SDS gels. The peptides were labeled with ^{125}I by use of the chloramine-T reagent (Sigma Chemical Co., St. Louis, MO) (Elder et al., 1977; Petrucci et al., 1987).

Antibody Preparation. Antibodies to the purified N25 fragment were raised in New Zealand rabbits as previously described (Petrucci et al., 1987). The IgG fraction was prepared by elution with 0.1 M glycine, pH 3.2, from immobilized protein A (Pierce Chemical Co., Rockford, IL).

Binding Assays. Actin binding assays were performed as before (Petrucci & Morrow, 1987) with 150-μL aliquots of 4 μM F-actin and various concentrations of synapsin I or the purified NTCB fragments from the HPLC column.

The interaction of the NTCB peptides with tubulin was measured by cosedimentation and by their ability to bind to tubulin immobilized on Sepharose affinity columns. Tubulin (5 μM final concentration) was stabilized with 10 μM taxol (generously provided by the Natural Products Branch, Division of Cancer Treatment of the National Cancer Institute) and was incubated with synapsin I or the synapsin I fragments for 30 min at room temperature. The total sample volume was 150 μL. Tubulin, with any bound peptides, was sedimented at 100000*g* (average) for 1 h at 4 °C in a 42.2 Ti rotor (Beckman Instruments, Inc., Palo Alto, CA) and then dissolved in a volume of SDS solubilization buffer equivalent to the original incubation volume. Equal aliquots of the supernatant and pellet from this sedimentation were analyzed by SDS–PAGE (Laemmli, 1970). Alternatively, 5 mg of purified tubulin was immobilized on 3 mL of CNBr-activated Sepharose CL-4B, equilibrated with PEMG buffer (0.1 M PIPES, 1 mM EGTA, 0.1 mM $MgCl_2$, 1 mM GTP, pH 6.9), and then 0.5 mL of this suspension was incubated overnight at 4 °C with synapsin I NTCB fragments. Unbound material was washed from the Sepharose with 15 volumes of PEMG buffer; bound material was eluted with 1% SDS in PEDG buffer; the fractions were analyzed by SDS–PAGE and stained with Coomassie blue.

Inhibition assays using the anti-N25 antibody were performed by incubating synapsin I or its NTCB fragments with aliquots of the purified anti-N25 IgG for 1 h at room temperature prior to the addition of actin or tubulin. Subsequent assays were as described above. Controls with antibody and synapsin I alone were carried out to be sure that the experi-

[1] Abbreviations: NTCB, 2-nitro-5-thiocyanobenzoic acid; NEPHGE, non equilibrium pH gel electrophoresis; HPLC, high-pressure liquid chromatography; SASD, sulfosuccinimidyl 2-(*p*-azidosalicylamido)ethyl 3,3′-dithiodipropionate; PBS, phosphate-buffered saline.

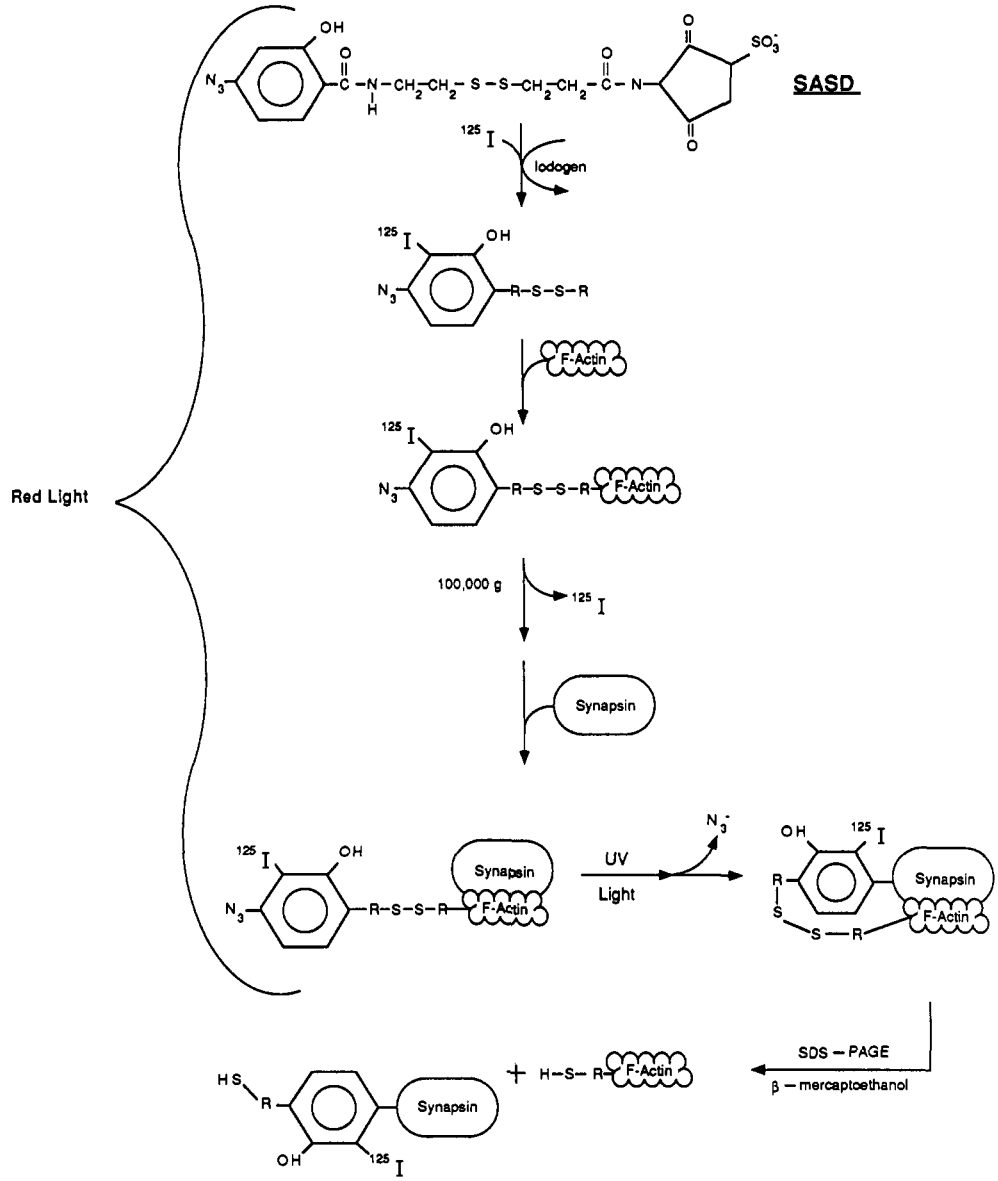

FIGURE 1: Outline of photolabel transfer experiment. In the dark, SASD is labeled on its aromatic ring with ^{125}I by use of iodogen, which is insoluble in aqueous solution. Subsequently, the ^{125}I-SASD reagent is transferred to a solution of F-actin (or BSA as a control), where it reacts spontaneously through its sulfosuccinimide group with amino groups in actin (or BSA). Proteins so labeled are purified from unreacted ^{125}I or SASD by either sedimentation or gel filtration, after which they are mixed with intact synapsin I and photolyzed briefly. Photolysis activates the aryl azide group, cross-linking it to any proteins bound within the effective radius of the SASD molecule (18.9 Å). Subsequent reductive cleavage releases the cross-link and transfers the ^{125}I label from F-actin (or BSA) to synapsin I. The site(s) of label transfer within synapsin I can then be analyzed by two-dimensional NEPHGE–SDS–PAGE after NTCB cleavage.

ments were conducted in the region of antibody excess and that the sedimentation of the fragments could not be attributed to the antibody alone.

Radiolabeling of Proteins with a Photoactive Label Transfer Agent. Prior to photolysis, all manipulations with the SASD were carried out either in the dark or under red illumination (15 W). An outline of the procedures followed is given in Figure 1. ^{125}I-SASD was prepared by dissolving 1 mg of SASD (Pierce Chemical Co.) in 40 μL of DMSO and then diluting this into 160 μL of 0.1 M phosphate buffer, pH 7.4 (Shephard et al., 1988). The dissolved SASD (≈200 μL) was added at room temperature to a dry tube containing 100 μg of iodogen (Pierce Chemical Co.), followed by 2 mCi of $Na^{125}I$ (Amersham). The iodination was terminated after 4 min, and the reaction of ^{125}I-SASD with actin or BSA was initiated by aliquoting the solution to second tubes containing either F-actin or BSA in PBS without iodogen. The molar ratio of ^{125}I-SASD to either BSA or actin was 1:5; this reaction was carried out for 30 min at room temperature. Free iodine and unreacted ^{125}I-SASD were separated from the F-actin by centrifugation at 4 °C through 5% sucrose in PBS and 2 mM Mg^{2+} for 1 h at 100000*g* in an SW50.1 (Beckman) rotor. The supernatant was discarded, and the pellet was redissolved in F-actin buffer. ^{125}I-SASD-labeled BSA was purified at room temperature in PBS by gel filtration on a Sephadex G-25 column (Pharmacia, Uppsala, Sweden). Fractions were collected and assayed for radioactivity by γ-counting (LKB 1282 Compugamma). Aliquots of the ^{125}I-SASD-labeled proteins were incubated with synapsin I (at 2.5 μM final concentration) for 30 min at room temperature in a total volume of 600 μL. Following this incubation, samples were irradiated with a long-wave (λ = 312 nm) UV lamp (International Biotechnologies, Inc.) held 12–15 cm above the open sample for 2 min, after which they were centrifuged at 100000*g* for 1 h at 4 °C in an SW50.1 rotor. Pellets were redissolved in 100 μL of 8 M urea, pH 8; equivalent aliquots

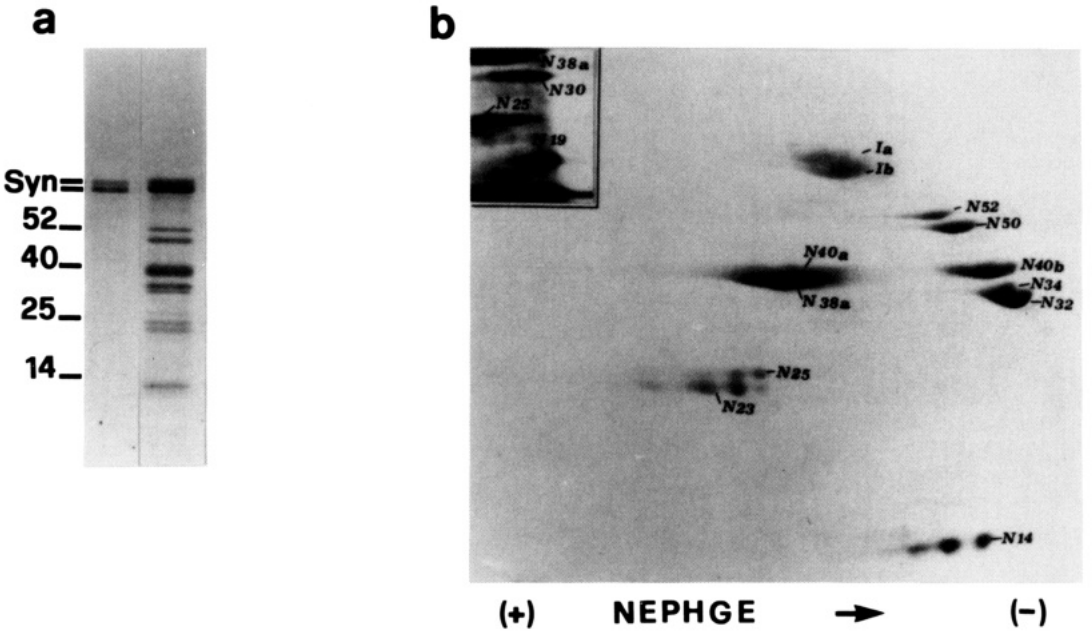

FIGURE 2: Digest of synapsin I with NTCB. (a) SDS–PAGE analysis of synapsin I and its NTCB digestion products. After NTCB digestion of purified synapsin I (left lane), approximately eight prominent fragments are apparent (right lane). (b) Analysis of the NTCB digestion mixture by two-dimensional NEPHGE–SDS–PAGE reveals that the digestion products cluster into acidic (+) or basis (−) fragments. In addition, it is clear that both acidic and basic fragments comprise the bands of about 38–40 kDa in the SDS–PAGE dimension. On heavily loaded gels (inset), additional acidic fragments of about 30, 19, and occasionally 14 kDa (N30 and N19 in the inset) are also evident. The gels shown here are Coomassie blue stained. Peptides are labeled by their apparent molecular mass (in kDa), preceded by an "N" to indicate that they arose from an NTCB digest.

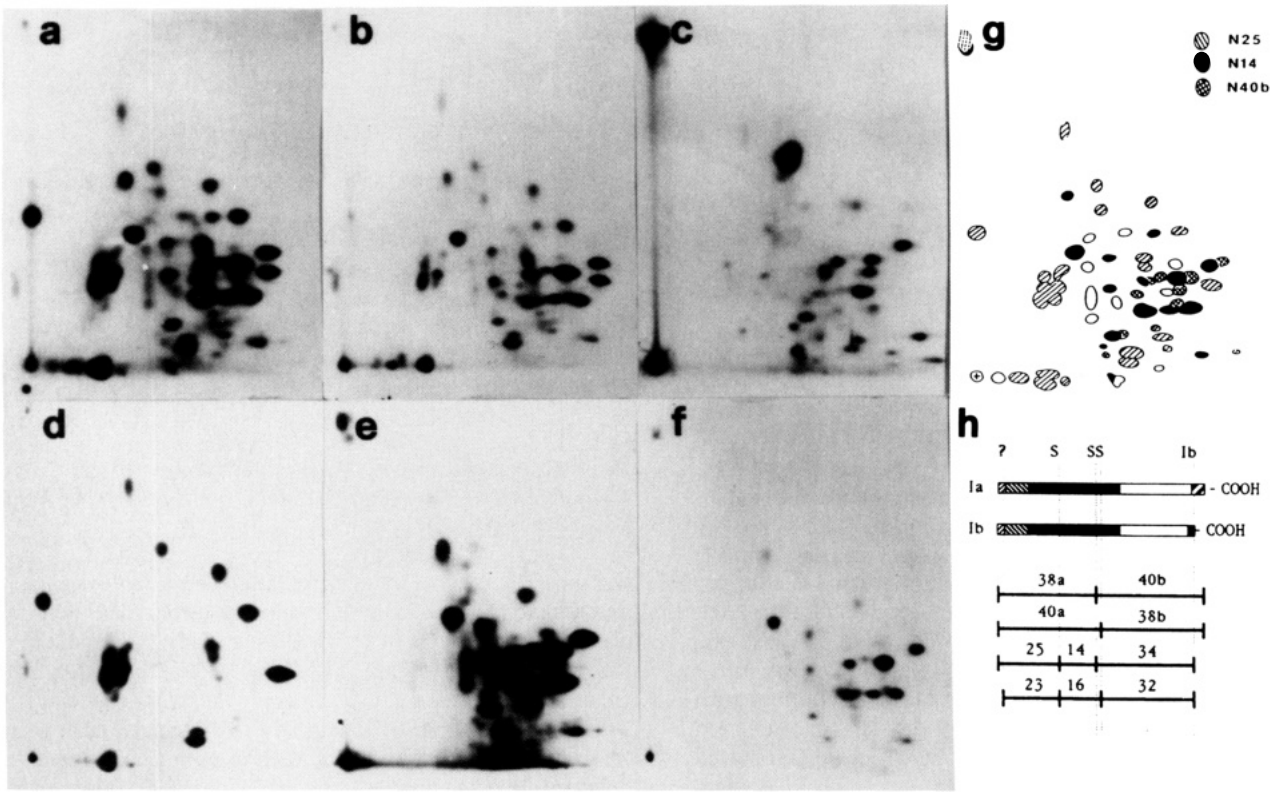

FIGURE 3: Peptide maps of the prominent complementary NTCB peptides and their alignment. The major fragments identified by two-dimensional NEPHGE–SDS–PAGE (Figure 2b) were each labeled with ^{125}I, digested with chymotrypsin, and mapped in two dimensions. The autoradiofluorograms of these maps are shown. They are (a) intact synapsin I, (b) N40a, (c) N40b, (d) N25, (e) N52, and (f) N14. In (g), the complementary nature of the N25, N14, and N40b maps is depicted. The derived alignment of these fragments with respect to the reported sequence of synapsin I is shown in (h). The shading of the bars in (h) and the position of the cysteine residues are taken from Südhof et al. (1989) and represent the domain structure of synapsins Ia and Ib as deduced from genomic DNA studies. It is apparent that the N23 peptide does not arise from cleavage at a terminal cysteine residue.

of both the supernatant and pellet fractions were analyzed by PAGE–SDS with and without 2-mercaptoethanol. Samples of the synapsin I–actin complex were cleaved with NTCB as above and analyzed by two-dimensional NEPHGE–SDS–PAGE. The labeled NTCB fragments were determined by autoradiography using Kodak XAR film with intensifying

screens and by γ-counting of the excised gel bands.

Other Procedures. SDS–PAGE and two-dimensional NEPHGE–SDS–PAGE were performed according to standard methods (Laemmli, 1970; O'Farrell, 1975). Protein determinations used the Lowry (1951) method. Western immunoblots were prepared after SDS–PAGE by electrophoretic transfer of the gels to nitrocellulose paper (Schleicher & Schuell, Inc., Keene, NH) (Towbin et al., 1979). Unless otherwise indicated, reactive bands were visualized with ^{125}I-labeled *Staphlococcus* protein A. Coomassie blue bands were quantized by the OD^{596} of the dye eluted with 25% pyridine (Fenner et al., 1975). Scanning densitometry was performed on a Biolmage Visage 2000 (Eastman Kodak, Inc.).

Results

Alignment of the NTCB-Derived Peptides within Synapsins Ia and Ib. Digestion of synapsin I with NTCB yielded approximately eight prominent fragments ranging in apparent size from 52 to about 14 kDa (Figure 2a). When this digest was analyzed by two-dimensional NEPHGE–SDS–PAGE (Figure 2b), it separated roughly into two clusters: acidic peptides composed of 40-, 38-, 25-, and 23-kDa fragments (labeled N40a, N38a, N25, and N23, respectively) and a more basic grouping composed of 52-, 50-, 40-, 38-, 34-, 32-, 16-, and 14-kDa fragments (labeled N52, N50, N40b, N38b, N34, N32, N16, and N14, respectively). Additional complexity in the digest could often be appreciated on heavily loaded gels (Figure 2, inset). Specifically, both the N25 and N23 fragments were resolved into at least four species in the NEPHGE dimension, and minor fragments associated with the basic peptides were apparent at about 30 and 19 kDa (and sometimes 14 kDa) (labeled N30 and N19, respectively).

The arrangement of these peptides within their parent proteins was determined by a comparison of their two-dimensional ^{125}I-labeled peptide maps (Figure 3) [see Speicher et al. (1982) for a description of this procedure]. The maps of N25 and N23 were identical; the maps of N52 and N50 were identical; the maps of N40a and N38a were identical; the maps of N16 and N14 were identical; and the maps of N40b, N38b, N34, and N32 were nearly identical. The various N25 and N23 species separated in the NEPHGE dimension also shared a common map. The alignment of the complementary peptides which compose the complete synapsin I molecule is shown in Figure 3. The map of the N25/23 peptide was complementary to the map of the N52/50 fragments, and together they accounted for the map of intact synapsin I. Similarly, the maps of N40A/38a and N40b/38b/34/32 were complementary and accounted for the intact molecule. The map of N16/14 was contained in N52/50, and both N25/23 and N16/14 were contained in N40a/38a. Thus, given that the acidic N25 peptide occurs at the NH_2-terminus of synapsin I (Petrucci & Morrow, 1987), the alignment of the major NTCB peptides in synapsin I must be as shown in Figure 3. This alignment is in complete agreement with the anticipated fragments on the basis of the position of the cysteine residues as most recently reported (Südhof et al., 1989), except for the existence of the N23 fragment, which is consistently observed and which cannot be explained by a cysteine-specific cleavage. From this analysis, it is clear that N40a and N38a arise from alternate cleavages at two closely spaced cysteine residues near the center of the molecule. The N34/32 fragments derive from synapsin Ib, while the N40b/38b peptides are the analogous fragments from synapsin Ia (Figure 3). The alignment presented here is similar to the tentative layout we had previously proposed on the basis of maps prepared from one-dimensional SDS gels of the digest

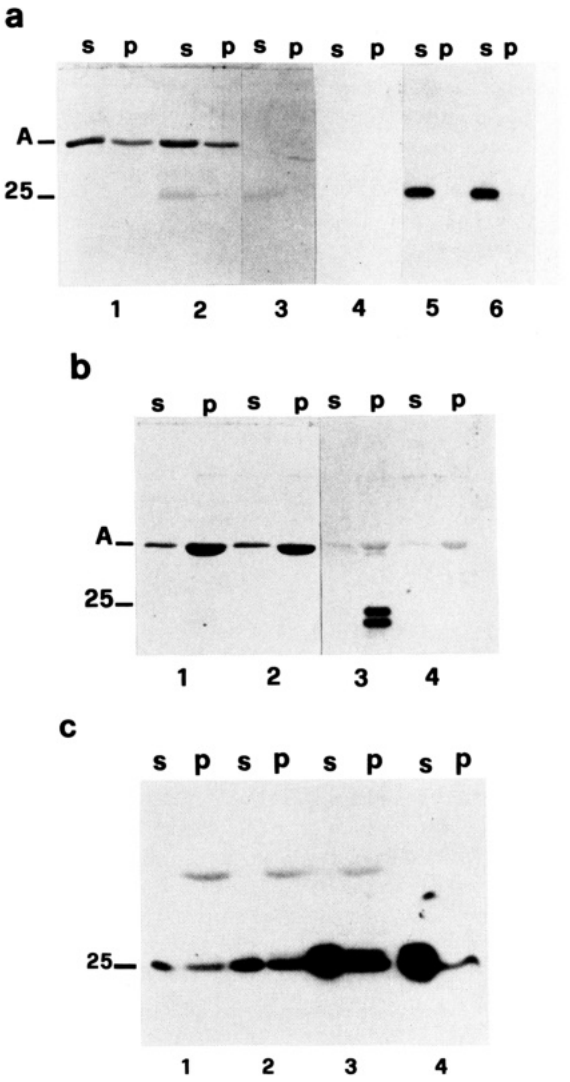

FIGURE 4: The NH_2-terminal N25/23 fragment binds but does not bundle actin. (a) Both supernatant (s) and pellet (p) fractions are shown. The conditions of each experiment were as follows: (lanes 1 and 4) actin alone; (lanes 2 and 5) N25/23 + 4 μM actin; (lanes 3 and 6) N25/23 alone. In these experiments the samples were sedimented at 10000*g*; lanes 1–3 are Coomassie blue stained; lanes 4–6 are autoradiofluorgraphs of Western blots with anti-N25 antibody and ^{125}I-labeled staph protein A. (b) Experiment similar to that in (a), except that the samples were sedimented at 100000*g*. (Lanes 1 and 3) N25/23 with 4 μM F-actin; (lanes 2 and 4) F-actin alone. The left two lanes are Coomassie blue stained; the right two are autoradiofluorograms. Note the quantitative sedimentation of N25/23 with F-actin. (c) Autoradiofluorgram of a Western blot demonstrated the effect of increasing amounts (lanes 1–3) of N25/23 on its ability to bind actin. The Western blot was prepared as in (b). Sedimentation was at 100000*g*. The peptide alone was sedimented in lane 4. The trace labeling observed at 42 kDa arises from nonspecific binding between the ^{125}I-labeled staph protein A and actin. Quantization of the sedimentation experiment shown in (c) demonstrated an approach to saturation and that the sedimentation of the N25/23 required actin.

(Petrucci et al., 1988), with one exception. Previously, it seemed that there might be multiple NTCB fragments of about the size of N14, a notion supported by the appearance of anomalous cleavages by NTCB in the digests (see below) and by the limited sequence data then available (McCaffery & DeGennaro, 1986). Because of this, there was ambiguity in the earlier study as to the location of the N14 peptide that had actin binding activity [see below, also Petrucci et al. (1988)]. The present alignments taken from two-dimensional gels, together with more complete sequence data, indicate that there is probably only a single 14-kDa peptide produced in high

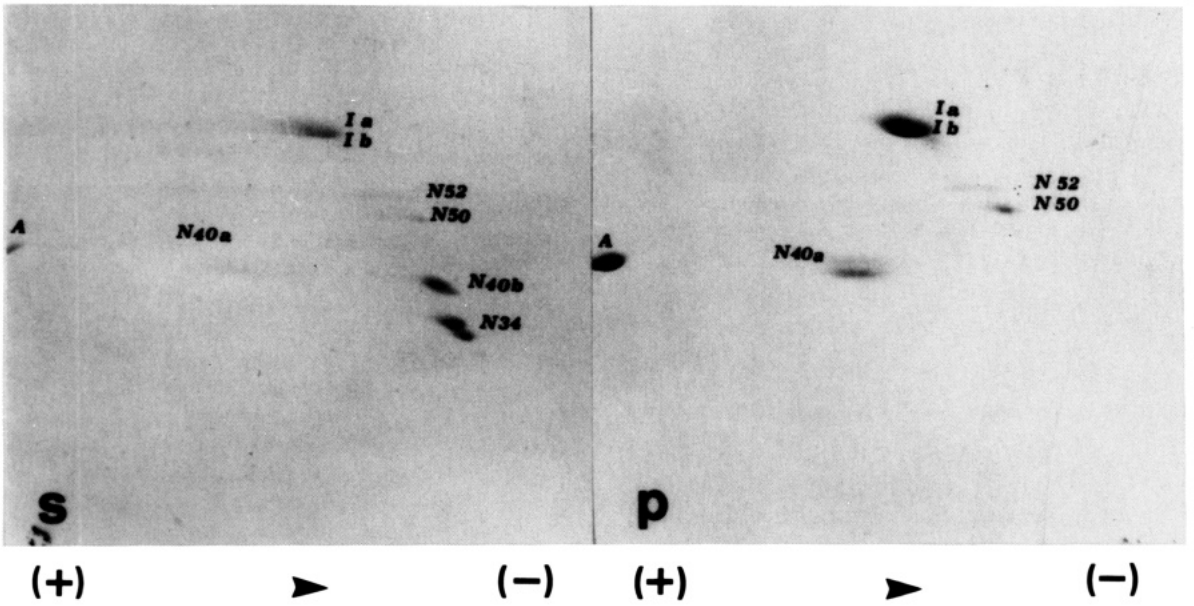

FIGURE 5: Binding of the basic NTCB fragments to actin. The ability of semipurified pool of basic NTCB fragments containing N52/50, N40b/38b, and N34/32 to bind to 4 μM actin was examined by two-dimensional NEPHGE–SDS–PAGE. Coomassie blue stained supernatant (s) and pellet (p) fractions are shown, after a 100000g sedimentation. Note the absence of N40b/38b and N34/32 in the pellet fraction.

yield (N14) and that this peptide lies adjacent to N25 in the NH_2-half of the molecule.

In addition to the major NTCB-cleavage products characterized above, three minor products were encountered which could not be explained by cysteine-specific cleavage if the most recently proposed sequence of synapsin I is correct. These peptides were N30, N19, and a minor N14 (Figure 2b, inset). The maps and alignments of the two most abundant of these are presented as Figure S-1 in the supplementary material (see paragraph at end of paper regarding supplementary material). These data have been evaluated by the reviewers of this paper, and all supplementary material is also available by writing directly to the authors. It is likely that these minor fragments arise from anomalous NTCB cleavages, although this has not been clearly demonstrated.

The Purified 25-kDa Amino-Terminal Fragment of Synapsin I Contains One Actin Binding Site. Although our previous studies indicated that the N25/23 terminal peptide was competent for actin binding, it was important that this peptide be obtained in a pure state so that its actin bundling ability could be evaluated. It was purified by HPLC ion-exchange chromatography in 4 M urea on a carboxymethyl HPLC column (Figure S-2, supplementary material). It was found that, under these mildly denaturing conditions, the major acidic NTCB peptides (N40a/38a, N25/23) could be separated completely from the basic NTCB digestion products. In these experiments, the amount of the N40a/38a peptide produced in the digestion mixture was highly variable and, as expected, was inversely related to the amount of N25/23 and N14 generated (cf. Figure 3h). Factors that controlled the distribution of product between the N40a/38a and the N25/23 and N16/14 peptides were not explored.

The ability of the purified N25/23 peptide to bind and bundle actin was investigated by cosedimentation assay. These results are shown in Figure 4. The purified N25/23 peptide was incubated either alone or with actin and then sedimented at either 100000g or 10000g for 1 h. Under the less stringent sedimentation conditions, only agents that bundle actin will be sedimented, while at 100000g agents that simply bind actin will be sedimented. In the absence of actin, only trace amounts of the N25/23 sediment, even at 100000g (Figure 4a,b).

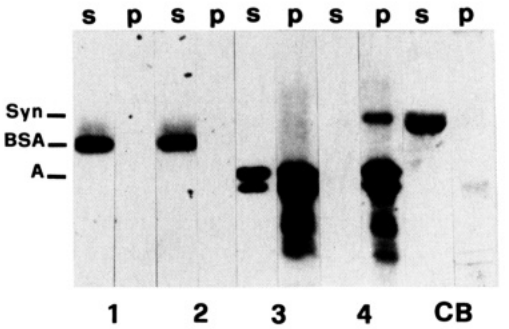

FIGURE 6: Photolabel is only transferred to synapsin I bound to F-actin. Synapsin I was incubated with either ^{125}I-SASD-labeled F-actin or ^{125}I-SASD-labeled BSA, photolyzed, and then sedimented at 100000g. The resulting supernatants (s) and pellets (p) were analyzed by SDS–PAGE and autoradiofluorography after reduction with 2-mercaptoethanol. (a) Autoradiofluorogram of (lane 1) BSA alone, (lane 2) BSA + synapsin I, (lane 3) F-actin alone, and (lane 4) F-actin + synapsin I. The pair marked CB is the Coomassie blue stained gel of synapsin + F-actin. Note that while most of the synapsin remains in the supernatant in these experiments (due to the small amount of F-actin present), most of the labeled synapsin is found with the F-actin pellet.

When sedimented at 100000g, the N25/23 peptide binds to actin in a specific and saturable fashion (Figure 4c). However, the N25/23 peptide does not enhance the sedimentation of actin at 10000g, indicating that it does not bundle actin (Figure 4a,b). Therefore, this region of synapsin I appears to be monovalent for actin binding.

An Antibody against N25 Inhibits Synapsin I Actin Bundling Activity but Not Its Ability To Bind Actin. An antibody was raised to the purified N25/23 peptide. The specificity of this antibody for peptides in the NTCB digest mixture of synapsin I is shown in Figure S-3 of the supplementary material. As expected from the peptide mapping results, this antibody recognized epitopes only in intact synapsin I and in the N40a/38a and N25/23 fragments. When this antibody was incubated with mixtures of synapsin I and actin, it inhibited the ability of synapsin I to enhance the low-speed (10000g) sedimentation of actin, a measure of its actin bundling activity (Figure S-4a,b, in supplementary material). However, this antibody did not reduce the amount of intact

synapsin I that bound to actin after a 100000g sedimentation (Figure S-4c, supplementary material). Since the anti-N25 antibody can inhibit the binding of purified N25 peptide to actin (data not shown), these results indicate that synapsin I must have at least two distinct actin binding sites, one of which is not inhibited by anti-N25.

The N52/50 Fragment Binds Actin Independently of the Activity Resident in N25. The ability of the purified N25/23 peptides and the semipurified pool of basic NTCB fragments containing predominantly N52/50, N40b/38b, and N34/32 to bind to actin is shown in Figure S-5 (supplementary material) and in Figure 5. Since the anti-N25 antibody completely inhibited the actin binding ability of N25/23, this antibody was used in the assays of the basic NTCB peptides to assure that any binding observed was not due to noncovalent association with contaminants that included the N25 actin binding domain (such as residual intact synapsin I). Two components from the basic pool of NTCB peptides were found to bind actin regardless of the presence of the anti-N25 antibody: residual intact synapsin I and the N52/50 peptides. Although there was a suggestion in these experiments that a small amount of the N40b might also sediment with actin (Figure S-5), separate experiments on the complete digestion mixture, using two-dimensional IEF–SDS–PAGE analysis of both the supernatant and pellet fractions, failed to demonstrate binding of either the N40b or the N34/32 fragment to actin (Figure 5). The ability of the isolated N16/14 fragment to bind actin could not be examined since it was present in low abundance in the fractionated pool of basic NTCB peptides and was poorly reactive with our anti-synapsin antibody. However, the two-dimensional analysis shown in Figure 5 as well as previous studies employing ^{125}I-labeled protein (Petrucci et al., 1988) indicates that this fragment does sediment with actin (albeit in the presence of other NTCB peptides).

Label Transfers from F-Actin to both the NH_2- and COOH-Terminal Domains of Intact Synapsin I. In order to investigate the sites of F-actin binding in intact synapsin I, the ability of ^{125}I-SASD-labeled F-actin to phototransfer label to specific domains in synapsin I was investigated. The ability of ^{125}I-SASD-labeled BSA to also transfer label to synapsin I was used as a measure of the extent of random collisional labeling. After cross-linking, samples of synapsin I with either the ^{125}I-SASD-labeled BSA or the ^{125}I-SASD-labeled F-actin were sedimented, and the amount of label in synapsin I was analyzed by SDS–PAGE (Figure 6). No detectable label was transferred to synapsin I from BSA, and only trace labeling of synapsin I could be detected in the F-actin supernatant. Conversely, the synapsin I that bound to F-actin (as evidenced by its ability to cosediment with F-actin) was heavily labeled after photo-cross-linking and reduction. Quantization of these results indicated that ^{125}I transferred to the bound synapsin I from F-actin $\approx$7 times more efficiently than it did to the unbound synapsin I (Figure S-6, supplementary material).

Two-dimensional NEPHGE–SDS–PAGE analysis of an NTCB digest of the bound synapsin I (containing the transferred label) indicated that all major NTCB domains became labeled significantly above the levels attributable to background or random collisional processes (Figure 7). The most strongly labeled domains were N25 and N14, consistent with the ability of the NTCB peptides encompassing these regions to bind F-actin avidly. Also significantly labeled (3 times background + collisional labeling) was the COOH-terminal region of intact synapsin I, as encompassed in the N40b/38b and N34/32 peptides. A pairwise comparison of each of these COOH-region peptides failed to find significant differences between the labeling of the N40b/38b and the N34/32 peptides, representing synapsins Ia and Ib, respectively.

The Actin Binding Domains of Synapsin I Also Bind Tubulin. Synapsin I can bind and cross-link tubulin. The domains responsible for this activity were sought by identifying which NTCB fragments would sediment with taxol-stabilized microtubules and/or would be retained by purified tubulin immobilized on a Sepharose affinity column. These results, which parallel those obtained for actin, are shown in Figure 8. Specifically, the only fragments of synapsin I which do not sediment with tubulin and which are not retained by immobilized tubulin are N40b/38b and N34/32. Using the purified fractions, it is clear that one tubulin binding site must reside in the N25/23 peptide. A second site lies in the N52/50 fragment. Although the binding of N14 to microtubules cannot be discerned due to the poor reactivity of our antibodies to this region of synapsin, a fraction of this peptide is retained on the tubulin–Sepharose column. As with actin, no binding of the N40b/38b and N34/32 peptides could be demonstrated after NTCB cleavage. It is unknown whether the COOH-terminal domain will react with tubulin in intact synapsin I.

Discussion

The results presented here contribute to our understanding of the way that synapsin I can link synaptic vesicles to the cytoskeleton, establish unequivocally that this protein can be polyvalent with respect to both actin and tubulin binding, define two putative sites of tubulin binding within synapsin I, and identify a heretofore unrecognized ability of the COOH-terminal regions of synapsin I to interact with F-actin. These results are summarized in Figure 9, where a schematic model representing our current state of knowledge of the structure and function of synapsins Ia and Ib is presented.

Our earlier conclusion (Petrucci & Morrow, 1987) that one actin binding domain resides in the 25-kDa NH_2-terminal region of synapsin I has been confirmed. In previous studies (Petrucci et al., 1988), we had also identified other fragments complementary to N25, including a 14-kDa NTCB fragment, that would sediment with actin and concluded that these fragments either bound actin directly or associated with portions of the molecule that did. It is now clear that the region encompassed by the N14 peptide also binds actin directly, since this region is directly photolabeled by ^{125}I-SASD-labeled F-actin and since the binding of isolated peptides that contain this region is unaffected by the absence of N25. In addition, on the basis of limited mapping data from one-dimensional SDS–PAGE analyses of the NTCB fragments and the sequence of synapsin I as originally reported (McCaffery & DeGennaro, 1986), we had previously thought that there were at least three NTCB fragments of about 14 kDa and that a second actin binding domain or a site of self-association probably lay in a 14-kDa peptide that was derived from the COOH terminus of synapsin I (Petrucci et al., 1988). In the present report, with the aid of more extensive mapping data from two-dimensional NEPHGE–SDS–PAGE gels and more complete sequence data (Südhof et al., 1989), it is now clear that while minor 14-kDa fragments may arise from anomalous cleavages, there is only a single major NTCB product of 14–16 kDa and that this peptide is derived from the center of synapsin I. A similar conclusion has also been recently reported in an independent analysis (Bähler et al., 1989).

Although the region encompassed by the N14 peptide does have actin binding properties, we do not believe that this creates a second actin binding domain in intact synapsin, since

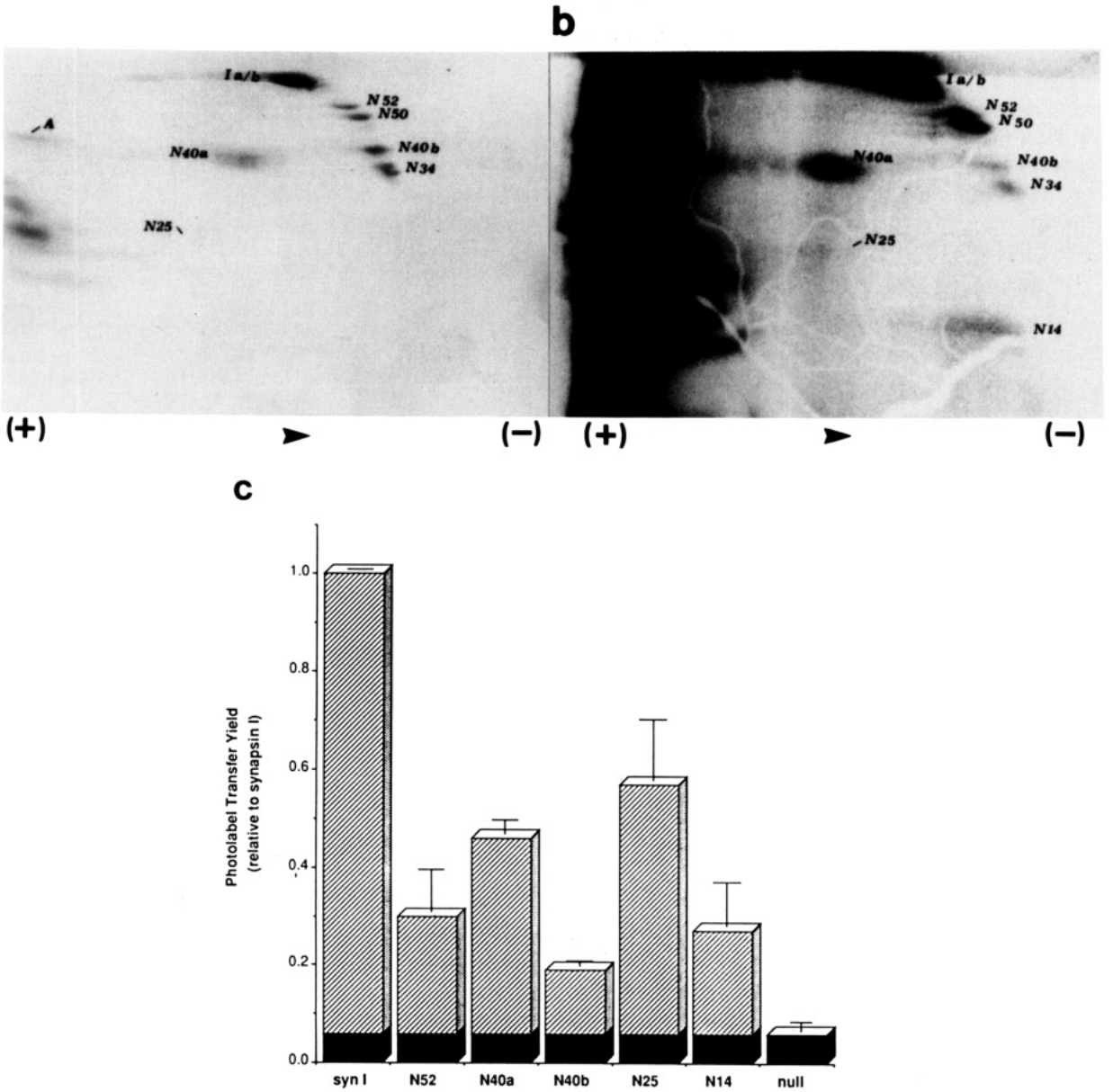

FIGURE 7: Photolabel is transferred from F-actin to three domains in synapsin I. Synapsin I that pelleted with the ^{125}I-SASD-labeled F-actin was photolyzed, reduced, and then subjected to NTCB cleavage. The resulting peptide mixture was analyzed by two-dimensional NEPHGE–SDS–PAGE, followed by Coomassie blue staining (a) and autoradiofluorography (b). Note the significant transfer of ^{125}I label into all of the major NTCB peptides. The strong labeling at the left (acidic) end of the gel is actin and its NTCB breakdown products. (c) Relative amount of label transferred from F-actin to bound synapsin I. The filled bar (null) represents the amount of label transfer that can be accounted for by background in these gels and actually exceeds the amount of label transferred to synapsin in the supernatant of the ^{125}I-SASD-labeled F-actin solution or in either fraction of the ^{125}-SASD-labeled BSA solution (cf. Figure 10). The identity of each bar is as indicated: syn I, intact synapsin I; N52, the sum of the N52/50 fragments; N40a, the sum of the N40a and N38a fragments; N40b, the sum of the N40b, N38b, N34, and N32 fragments; N25, the sum of the N25 and N23 fragments; N14, the sum of the N14 and N16 fragments. Note that while the strongest labeling occurs in the peptides containing N25 and N14, significant label (several times background) is also transferred into those domains encompassing the COOH terminus of synapsin I (N40b, N38b, N34, N32). Error = 1 SD (n = 3).

antibodies directed to N25 inhibit the actin bundling (but not binding) activity of intact synapsin I. The regions of actin binding in N25 and N14 must therefore by closely approximated in the intact molecule; we propose that collectively these two fragments form a single high-affinity actin binding site in the "globular head region" of synapsin Ia and Ib.

Evidence has also been presented for a third actin binding site in the COOH-terminal half of synapsin I. Although isolated peptides derived by NTCB digestion from this region do not bind actin in vitro, it appears that this region is competent for such binding in the intact molecule. Presumably, the denaturing conditions required for cleavage and/or cysteine cleavage near the center of the molecule destroy the actin binding ability of the COOH terminal derived peptides. While it is formally possible that the photolabel transferred to the COOH region represents random labeling (or "spill-over" labeling from a nearby actin binding site), we do not favor this interpretation. The observed level of label incorporated into the COOH region, although about half that transferred to the N25 or N14 domains, is still many times the level of collisional labeling observed either with BSA or with unbound synapsin in the presence of ^{125}I-SASD-labeled F-actin. Second, the reported interaction distance (18.9 Å, Pierce Chemical Catalog, 1989) of the SASD probe is not large, restricting its ability to interact with sites on synapsin I distant from a site of F-actin binding. Finally, the identification of an actin binding site in the COOH region of synapsin I is consistent with the actin bundling properties of the intact molecule

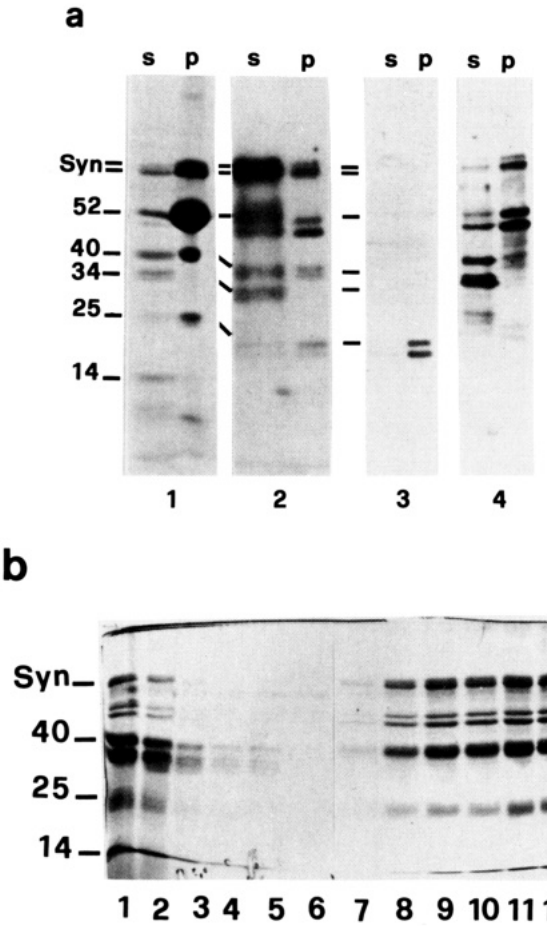

FIGURE 8: Tubulin binding NTCB fragments of synapsin I. The ability of intact synapsin, the NTCB digestion mixture of synapsin I, or the purified N25/23 and the pool of basic NTCB peptides to bind to tubulin was measured by cosedimentation with taxol-stabilized microtubules or by their ability to be retained on immobilized tubulin affinity columns. (a) Cosedimention of synapsin I NTCB fragments with taxol-stabilized tubulin. Supernatant (s) and pellet (p) fractions are shown. (Lane pair 1) Coomassie blue stain of digestion mixture; (lane pair 2) autoradiofluorogram of the digestion mixture after Western blotting with anti-synapsin I antibodies; (lane pair 3) cosedimentation of the purified N25/23 peptide; (lane pair 4) cosedimentation of the semipurified basic NTCB mixture. The gels shown in lane pairs 3 and 4 were visualized by Western blot with anti-synapsin I antibodies, alkaline phosphatase stain. (b) SDS–PAGE analysis of NTCB synapsin I peptides eluted from a Sepharose–tubulin affinity column, Coomassie blue stained. Fractions 1–6, flow-through fragments; fractions 7–12, fragments eluted by PEMG with 1% SDS.

(Petrucci & Morrow, 1987) and the isolated N52/50 fragment (Bähler et al., 1989), as well as with reported ultrastructural investigations of the presynaptic membrane that indicate that monomers of synapsin I cross-link small synaptic vesicles to actin filaments and microtubules (Hirokawa et al., 1989; Landis et al., 1988). In separate in vitro experiments, we have also been unable to demonstrate significant self-association of intact synapsin I at concentrations up to 29 μM by sedimentation velocity (unpublished observations), lending further evidence that each isolated synapsin I molecule must be polyvalent for actin binding.

Although not directly investigated in the present study, it will be of interest in the future to see if the phosphorylation of the CAM II kinase site in the COOH-terminal region of synapsin I (Figure 9) downregulates the ability of the COOH-terminal domain to bind actin and whether tubulin and actin compete for shared or closely approximated binding sites in the intact molecule.

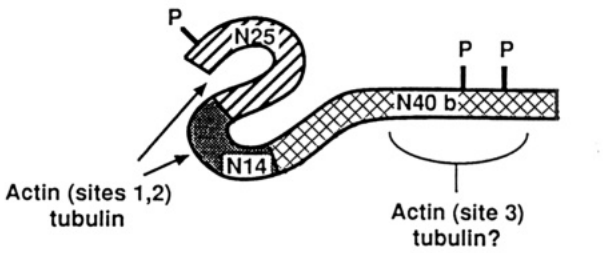

FIGURE 9: Functional domain structure of synapsin I. Both synapsin Ia and synapsin Ib contain an actin and tubulin binding domain in the region encompassed by the NH_2-terminal N25 peptide. Both also contain a second actin and tubulin binding site in the region encompassed by the N14 peptide. Since antibodies to the N25 alone appear to block the activity of both the N25 and the N14 site in intact synapsin, these two domains probably are closely apposed in the intact molecule and may participate in the formation of only a single actin (and tubulin) binding site near the NH_2 terminus. It also appears that an additional actin binding site is present in the COOH-terminal portion of intact synapsin I and that this activity is destroyed or of low affinity in the isolated NTCB cleavage fragments. Since both synapsin Ia and synapsin Ib have this actin binding activity, it probably does not involve the unique COOH-terminal extensions at the end of the collagenase-resistant sequence that distinguish synapsin Ia from synapsin Ib. The regions of phosphorylation in synapsin I are also depicted [see Benfenati et al. (1989) and references cited therein]. On the basis of the measurements of Hirokawa et al. (1989), the globular head region is estimated to be ≈14 nm in diameter, and the "tail" region is estimated to be ≈30 nm long.

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Supplementary Material Available

Six figures depicting peptide maps of the minor N30 and N19 NTCB fragments and their approximate alignment in synapsin I (Figure S-1), purification of the NH_2-terminal N25 fragment (Figure S-2), reactivity of antibodies to synapsin and N25 (Figure S-3), antibodies to N25 inhibiting actin bundling by synapsin I but not its ability to bind actin (Figure S-4), binding of the N25 and the basic NTCB fragments to actin (Figure S-5), and quantitation of photolabel transfer to synapsin I bound to F-actin (Figure S-6) (8 pages). Ordering information is given on any current masthead page.

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Kinetics of the Interaction of 2′(3′)-*O*-(*N*-Methylanthraniloyl)-ATP with Myosin Subfragment 1 and Actomyosin Subfragment 1: Characterization of Two Acto·S1·ADP Complexes†

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ABSTRACT: We have used a fluorescent analogue of ATP, mantATP [2′(3′)-*O*-(*N*-methylanthraniloyl)-adenosine 5′-triphosphate; Hiratsuka, T. (1983) *Biochim. Biophys. Acta 742*, 496-508], and made a detailed kinetic study of the interaction of mantATP and mantADP with S1 and acto·S1. We have shown that these analogues behave like ATP and ADP, respectively. In addition, we have demonstrated that this analogue can distinguish between two acto·S1 complexes, the A-M·N (attached) and A·M·N (rigor-like) states [Geeves, M. A., Goody, R. S., & Gutfreund, H. (1984) *J. Muscle Res. Cell Motil. 5*, 351-361]. Previously, these two states were observed with a pyrene label on Cys 374 of actin. This isomerization can now be monitored at two spatially distinct sites on the ternary complex, indicative of a major conformational change in the ternary complex. Also, we have measured the rate of ADP dissociation from both A-M·N and A·M·N directly and shown these to differ by a factor of 1000. Thus the results presented here support the model of Geeves et al. and are consistent with the A-M·N to A·M·N transition being coupled to the force-generating event of the crossbridge cycle.

The mechanism of the ATPase reaction of myosin and its proteolytic subfragments (subfragment 1 and heavy meromyosin, S1[1] and HMM, respectively) has been studied by a wide variety of transient kinetic and isotope exchange techniques [for reviews, see Trentham et al. (1976), Taylor (1979), and Sleep and Smith (1981)]. A generally accepted mechanism of the first part of the ATPase reaction is shown in Scheme I.

Scheme I

$$M + T \underset{}{\overset{k_1}{\rightleftharpoons}} M\cdot T \overset{k_2}{\rightleftharpoons} M^*\cdot T \overset{k_3}{\rightleftharpoons} M^{**}\cdot D\cdot P$$

Step 1 is the formation of the binary collision complex (K_1 = 500 M^{-1}) followed by a rapid almost irreversible isomerization to the M*·ATP complex ($k_{+2} > 1000\ s^{-1}$, $K_2 > 10^7$) which is then reversibly hydrolyzed ($k_{+3} = 100\ s^{-1}$, $K_3 = 10$). (These kinetic and equilibrium constants are for pH 7.5, 20 °C, I = 0.15 M.) In subsequent steps (not shown) phosphate is released in the rate-limiting reaction, and then ADP is released in a relatively slow two-step reaction which is similar to a reversal of the two-step association of ATP.

Stopped-flow studies of the association of actin with S1 and S1-nucleotide complexes are compatible with a single-step binding reaction (Finlayson et al., 1969; White & Taylor, 1976; Marston, 1982; Konrad & Goody, 1982; Criddle et al., 1985). However, relaxation methods have shown this to be a three-step reaction: collision complex formation followed by two isomerization events (Scheme II).

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§ University of Bristol.

[1] Abbreviations: S1, myosin subfragment 1; mantATP, 2′(3′)-*O*-(*N*-methylanthraniloyl)adenosine 5′-triphosphate; mantdATP, 2′-deoxy-3′-*O*-(*N*-methylanthraniloyl)adenosine 5′-triphosphate; εATP, 1,N^6-etheno-ATP; DEDA-ATP, 2′(3′)-*O*-[*N*-[2-[[[5-(dimethylamino)-naphthyl]sulfonyl]amino]ethyl]carbamoyl]-ATP. The 5′-diphosphates are similarly abbreviated.

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