amino acids that may be critical for voltage gating.

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Limited Proteolysis of Synapsin I. Identification of the Region of the Molecule Responsible for Its Association with Microtubules

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ABSTRACT: Synapsin I is a highly asymmetric neuronal structural phosphoprotein implicated in the regulation of neurotransmitter release probably by the multiple interactions it can contract with membranous and cytoskeletal elements of the neuronal cell. In order to locate the region(s) of synapsin I responsible for its association with microtubules, we have first studied synapsin I limited digestion by trypsin. The resulting polypeptides were localized in the synapsin I molecule by using three different criteria: their kinetics of appearance, their collagenase sensitivity, and the presence of the synapsin phosphorylation site 1 (cyclic AMP dependent). Synapsin I digestion kinetics are not affected by phosphorylation at this site. Analysis of the ability of various synapsin I tryptic fragments in mixture to cosediment with microtubules shows that a 44-kDa fragment corresponding to the NH_2-terminal hydrophobic head of the molecule contains a binding site for polymerized tubulin. This fragment competes with native synapsin I for binding on microtubules. None of the polypeptides belonging to the tail region of synapsin I (COOH-terminal half of the molecule) were found to cosediment with microtubules.

Synapsin I is a neuronal phosphoprotein implicated in the regulation of neurotransmitter release [for a review, see DeCamilli and Greengard (1986)]. It is concentrated in the presynaptic terminals where it is associated with the cytoplasmic surface of small synaptic vesicles (DeCamilli et al., 1983; Huttner et al., 1983; Navone et al., 1984). Synapsin I is a major substrate for cyclic AMP dependent and for (calcium, calmodulin)-dependent protein kinases which

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phosphorylate it on different sites (Huttner et al., 1981; Kennedy & Greengard, 1981; Nairn & Greengard, 1987). Phosphorylation of synapsin I at sites 2 and 3 by the (calcium, calmodulin)-dependent protein kinase II reduces its ability to bind to synaptic vesicles (Huttner et al., 1983; Schiebler et al., 1986). Furthermore, synapsin I has been detected by immunoelectron microscopy in association with neurofilaments, axonal membranes, and microtubules (Goldenring et al., 1986). A probable function of synapsin I as an interlinker between synaptic vesicles and membranes or between vesicles and the neuronal cytoskeleton was suggested by the protein's ability to interact in vitro with various cytoskeletal elements: spectrin (Baines & Bennett, 1985), microtubules (Baines & Bennett, 1986), F-actin (Bähler & Greengard, 1987, Petrucci & Morrow, 1987), and the 68-kDa neurofilament subunit (Steiner et al., 1987). According to this idea, synapsin I could have a primary role in synaptic vesicle transport and availability for neurotransmitter release (Baines & Bennett, 1985; Goldenring et al., 1986; Steiner et al., 1987).

Synapsin I is a highly basic and asymmetric protein composed of two closely related polypeptides, synapsin Ia and synapsin Ib, with apparent molecular mass of 86 000 and 80 000 daltons, respectively (Ueda & Greengard, 1977). These two forms would differ in only about 40 additional amino acids at the C-terminal end of the molecule (Huttner et al., 1981; McCaffery & DeGennaro, 1986). Synapsin I has a globular NH_2-terminal hydrophobic head region which comprises the cyclic AMP dependent phosphorylation site (site 1) and an elongated collagenase-sensitive hydrophilic tail phosphorylated at two sites (sites 2 and 3) by (calcium, calmodulin)-dependent protein kinase II (Ueda & Greengard, 1977; McCaffery & DeGennaro, 1986; Czernik et al., 1987). The ability of synapsin I to bind to different proteins could involve distinct binding sites for each potentially interacting protein, or, alternatively, synapsin I could have a common binding domain that recognizes consensus sequences or conformations present in different proteins. In vitro recent studies concerning synapsin I interaction with synaptic vesicles reveal that the head of the protein seems to be involved in phospholipid binding whereas the tail domain appears to bind to a vesicle protein component (Benfenati et al., 1989). On the other hand, Bähler et al. (1989) have localized two F-actin binding sites in synapsin I fragments originating from the head portion of the molecule and have shown that the tail is necessary for synapsin F-actin bundling activity. Their study was performed using purified 2-nitro-5-thiocyanobenzoic acid (NTCB)-generated fragments of synapsin I. In the present report, we describe obtainment and characterization of polypeptides produced in the course of native synapsin I limited tryptic proteolysis. It is demonstrated that a 44-kDa tryptic fragment corresponding to the head portion of synapsin I can cosediment with microtubules. Intact synapsin I inhibits this interaction. In contrast, synapsin I tryptic polypeptides which comprises the COOH-terminal tail region that is hydrophilic and strongly basic fail to cosediment with microtubules.

Experimental Procedures

Materials. *Clostridium histolyticum* collagenase (form VII), trypsin (type XI), soybean trypsin inhibitor (type I.S), and catalytic subunit of cyclic AMP dependent protein kinase were from Sigma. Taxol was kindly supplied by the Drug Synthesis & Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD.

Preparation of Proteins. Bovine brain synapsin I was purified by the method of Bennett et al. (1986). Microtubule proteins were obtained from bovine brain by two temperature-dependent assembly–disassembly cycles (Shelanski, 1973) using the protocol described by Berkowitz et al. (1977) and stored at −70 °C in 0.1 M Na-PIPES, pH 6.9, 2 mM EGTA, and 1 mM $MgSO_4$ (polymerization buffer) containing 4 M glycerol and 0.5 mM GTP. Immediately before use, a third cycle of assembly–disassembly was performed, yielding 3 times cycled microtubule proteins (3×MT). Tubulin depleted of microtubule-associated proteins (MAPs) was purified by phosphocellulose chromatography according to the method of Weingarten et al. (1975) as described by Williams and Detrich (1979) and stored at −70 °C.

Precycling of Tubulin. Purified tubulin (3 mg/mL in polymerization buffer containing 2 mM DTT and 0.5 mM GTP) was first concentrated about 3 times using Centricon 10 (Amicon) at 4 °C in conditions recommended by the manufacturer. This concentrated tubulin was then submitted to one cycle of assembly–disassembly and immediately used.

Preparation of Taxol–Tubulin. Purified precycled tubulin (43 μM) was polymerized 20 min at 37 °C in buffer containing 0.5 mM GTP, 2 mM DTT, and 32 μM taxol. The resulting microtubules were resistant to cold depolymerization and dilution.

Tryptic Digestion of Synapsin I. Synapsin I (1 mg/mL) in polymerization buffer (0 °C) was incubated with or without trypsin at an enzyme to substrate ratio of 1:400 (w/w). At time zero, trypsin was added, and the samples were transferred into a 30 °C bath for various times. Aliquots were withdrawn, and the digestion was stopped with soybean trypsin inhibitor (trypsin to inhibitor ratio of 1:1, w/w).

Synapsin I Phosphorylation at Site 1. The incubation medium was as follows: 25 mM Na-PIPES, pH 7.2, 5 mM $MgCl_2$, 1 mM EGTA, 0.1 M NaCl, 27 μM [γ-^{32}P]ATP [(1–5) × 10^6 cpm/nmol], synapsin I (0.2 mg/mL), and catalytic subunit of cyclic AMP dependent protein kinase (300 units/mL). After incubation for 10 min at 30 °C, the reaction was terminated by the centrifuge column procedure according to Penefsky (1977) using 1-mL columns filled with G50-Sephadex previously equilibrated with cold polymerization buffer. In these conditions, we checked that synapsin I phosphorylation occurred only at site 1 by the procedure described by Huttner and Greengard (1979). This verification relies on the appearance of a single labeled polypeptide of about 12 kDa after V8 limited proteolysis of phosphorylated synapsin I according to Cleveland et al. (1977).

Collagenase Treatment. Synapsin I or synapsin I tryptic fragments (0.27 mg/mL) were incubated at 32 °C in the presence or absence of collagenase (30 units/mL) in 27 mM Na-PIPES buffer (pH 6.9) containing 0.5 mM EGTA, 0.3 mM $MgSO_4$, and 6 mM $CaCl_2$. After 1 h, the samples were denatured and run on SDS–polyacrylamide gel electrophoresis.

Other Procedures. An anti-synapsin I immune serum from rabbit was prepared by a standard procedure. SDS–PAGE were performed by the discontinuous buffer system according to Laemmli (1970) using linear 8–13% polyacrylamide gradient gels. After migration, proteins were electrophoretically transferred onto nitrocellulose sheets according to Szewczyk and Kozloff (1985). Immunodecoration of synapsin I was accomplished by using a horseradish peroxidase labeled second antibody whose activity was revealed with α-naphthol as substrate (Archinard et al., 1986). Tubulin or microtubule protein concentration was determined by the method of Bradford (1976) using the ready to use Coomassie Blue G-250 based reagent from Pierce. Synapsin I concentration was measured by the method of Peterson (1977) with bovine serum albumin as standard.

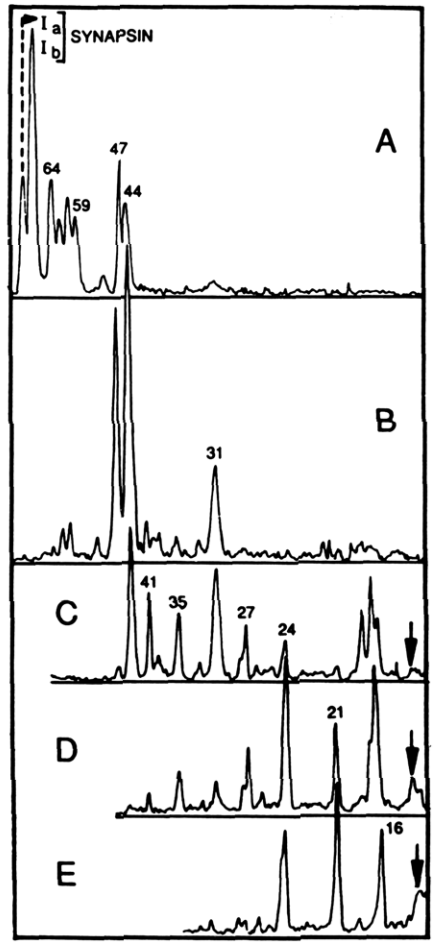

FIGURE 1: Time evolution of tryptic digestion of synapsin I estimated by densitometry of the Coomassie Blue stained gel. Synapsin I was digested by trypsin as described under Experimental Procedures for 30 s (A), 2 min (B), 5 min (C), 15 min (D), or 30 min (E). Samples were boiled in SDS-containing buffer for 2 min; the fragments in the mixture were separated by SDS–PAGE and stained with Coomassie Blue. Arrows correspond to the migration front.

RESULTS

Kinetics of Tryptic Digestion of Synapsin I. Time-dependent limited proteolysis of bovine synapsin I by trypsin is illustrated in a typical experiment (Figure 1). Intact synapsin Ia and Ib doublet (apparent molecular mass 80 kDa and 76 kDa, respectively) were absent from the gel after digestion for 2 min. For very short times (between 0 and 2 min), there was a transient formation and decay of 64–59-kDa polypeptides. Two more stable polypeptides of 47 and 44 kDa accumulated and were the prominent species present after 2 min of incubation together with a 31-kDa fragment (Figure 1B). Their digestion occurred with the generation of smaller polypeptides of 41, 35, 31, 27, and 24 kDa (Figure 1C). For longer times (30 min, Figure 1E), three major fragments of 24, 21, and 16 kDa were present in significant amounts.

Localization of the Fragments in the Synapsin I Molecule. We used two different characteristics of synapsin I to localize the tryptic fragments: the collagenase sensitivity of the C-terminal moiety (Ueda & Greengard, 1977) and the existence of a single specific serine residue phosphorylated by cyclic AMP dependent protein kinase at the N-terminal end (Huttner & Greengard, 1979). This serine (site 1) represents amino acid 9 in the rat brain synapsin I sequence renumbered according to the corrections published by Czernick et al. (1987).

Synapsin I phosphorylated at this site in the presence of [γ-^{32}P]ATP was mixed with a 100 molar excess of non-

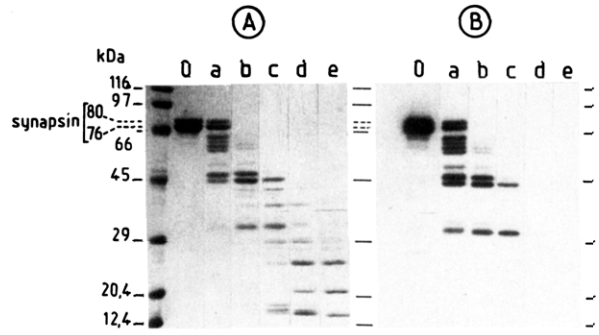

FIGURE 2: Limited proteolysis of a mixture of phosphorylated and nonphosphorylated synapsin I. Synapsin I phosphorylated in the presence of [γ-^{32}P]ATP as described under Experimental Procedures was mixed with a 100 times excess of nonphosphorylated synapsin I to give a concentration of 1 mg/mL. This mixture was incubated without (0) or with trypsin for 30 s (a), 2 min (b), 5 min (c), 15 min (d), or 30 min (e) and subjected to electrophoresis. The fragments were identified by Coomassie Blue staining (A) and the phosphorylated species by autoradiography of the same gel (B). Markers of electrophoretic mobility were β-galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20.4 kDa), and cytochrome *c* (12.4 kDa).

phosphorylated synapsin I (used as an internal control in order to have identical experimental conditions) and incubated with trypsin during various times. The proteolytic fragments of phosphorylated and nonphosphorylated synapsin I were identified by autoradiography and staining with Coomassie Blue, respectively. Figure 2 shows the proteolytic fragment pattern identified by Coomassie Blue staining (A) and the phosphate-labeled peptides revealed after autoradiography (B). It must be specified that if synapsin I was digested prior to phosphorylation, the same results were obtained (not shown). As a consequence, all fragments present for a 30-s digestion period of the synapsin I doublet contain their N-terminal end (lanes a). In contrast, for a 5-min digestion time (lanes c), the only labeled polypeptides are the 44- and 31-kDa fragments. Their further digestion (lanes d and e) parallels a disappearance of any detectable phosphorylated fragment. Limited proteolysis of a protein is dependent not only on the presence of specific amino acids but also on the conformation of the protein. Here, no differences in the tryptic cleavage patterns of phosphorylated and nonphosphorylated synapsin I were seen. This result suggests that cyclic AMP dependent phosphorylation of synapsin I does not lead to a dramatic conformational change of this protein.

To further locate the fragments on the synapsin I molecule, nonphosphorylated synapsin I was digested by trypsin for various periods and then incubated in the presence or in the absence of collagenase. When bovine synapsin I (0.27 mg/mL) was digested for 1 h at 30 °C by collagenase (10 units/mL), it was degraded to what appeared to be a single, stable 48-kDa peptide through a series of intermediate peptides with apparent molecular mass of 65, 59, and 50 kDa (not shown). This behavior, first reported by Ueda and Greengard (1977), reflects the presence of 11 potential sites for collagenase digestion (cleavage at the Y–Gly bond in the sequence Y–Gly–Pro) in the C-terminal third of the predicted rat synapsin I sequence. For the experiment described in Figure 3, we used a higher collagenase concentration (30 units/mL) to obtain the disappearance of intermediate peptides. Under these conditions, synapsin I digestion by collagenase produced mainly two polypeptides of 47 and 44 kDa. Only a faint additional 59-kDa band remained (Figure 3, lane a). Synapsin I digested for 30 s by trypsin produced the same fragmentation

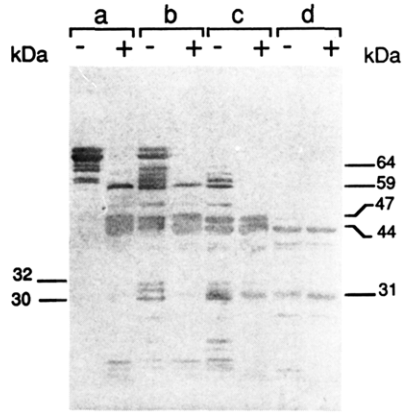

FIGURE 3: Western immunoblot of synapsin I tryptic fragments treated by collagenase. Synapsin I was first digested by trypsin for 0 s (lane a), 30 s (lane b), 2 min (lane c), or 5 min (lane d) and then incubated in the presence (+) or the absence (−) of collagenase. Collagenase treatment, gel electrophoresis, electrotransfer, and immunostaining were carried out as described under Expermental Procedures.

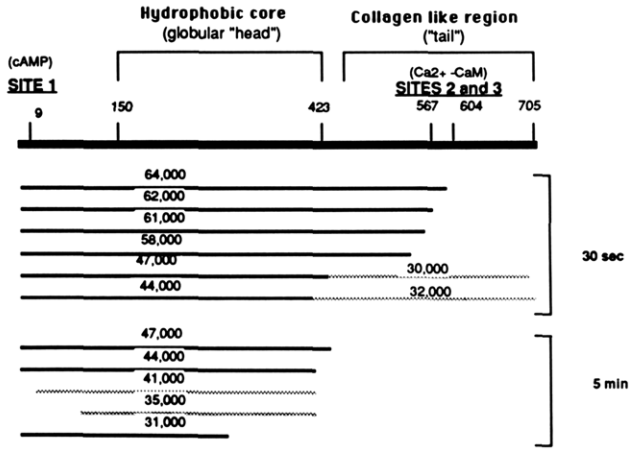

FIGURE 4: Schematic representation of the synapsin I molecule and location of the major tryptic fragments. Polypeptides corresponding to 30-s or 5-min tryptic digestion are aligned according to results presented in the text. Fragments in dotted lines cannot be unequivocally located at one or both of their extremities.

pattern as in Figures 1 or 2 in a parallel gel stained with Coomassie Blue (not shown), namely, synapsin Ia and Ib and polypeptides of 64–59, 47, and 44 kDa. However, on the immunoblot, additional fragments of 32–30 kDa are revealed (Figure 3, lane b). These fragments belong to the "tail" (C-terminal end) of synapsin I since they disappear following collagenase treatment. They can be visualized only by immunodecoration owing to the strong antigenicity of the collagenase-sensitive region (see Discussion). For a 5-min incubation time in the presence of trypsin, all the fragments present were collagenase-insensitive. Among these fragments, the 44- and 31-kDa fragments are visible in Figure 3 (lane d), and the 41-, 35-, and 27-kDa fragments are only faintly revealed because of the poor recognition of fragments belonging to the head region of synapsin I by our antiserum. Nevertheless, the parallel gel stained with Coomassie Blue demonstrated their collagenase resistance (not shown).

Cosedimentation of Synapsin Tryptic Fragments with Microtubules. We focused our attention on synapsin I fragments corresponding to 30-s and 5-min tryptic digestions. Figure 4 summarizes the polypeptides present for these two times and their location in the synapsin I molecule. Untreated or 30-s trypsinated synapsin I was incubated with 3 times cycled microtubule proteins in the polymerization buffer at 37 °C in the presence of 0.5 mM GTP. Sedimentation of

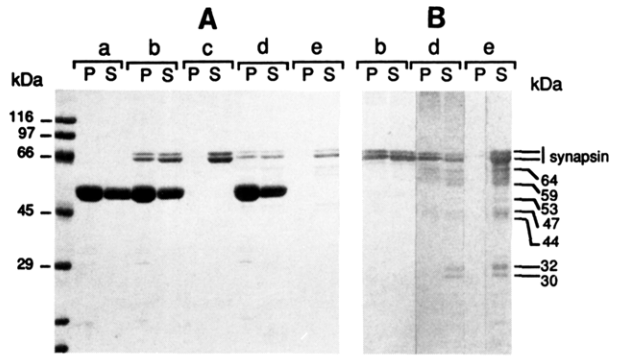

FIGURE 5: Cosedimentation of native or 30-s trypsinated synapsin I with microtubule proteins. Synapsin I (1 mg/mL) in the polymerization buffer was treated for 30 s with (lanes d–e) or without (lanes b–c) trypsin (1:400, w/w), and the proteolytic attack was stopped by the addition of soybean trypsin inhibitor (1:1, w/w relative to trypsin). Aliquots were withdrawn and further incubated (0.5 mg/mL final concentration) in the presence (lanes b and d) or absence (lanes c and e) of 3 times cycled microtubule proteins (2.2 mg/mL) for 30 min at 37 °C in polymerization buffer containing 0.5 mM GTP. In lane a, no synapsin I was present in the incubation mixture. The microtubules that formed during the incubation were collected by centrifugation at 100000*g* for 30 min at 32 °C. Pellets (P) and supernatants (S) were treated in the same final volume with SDS-containing buffer, and their content was analyzed by Coomassie blue staining (A) or immunodecoration (B).

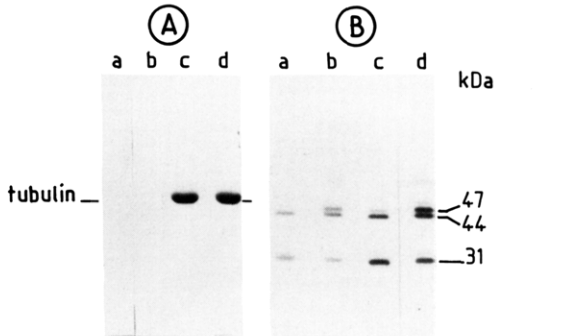

FIGURE 6: Cosedimentation of ^{32}P-labeled synapsin I tryptic fragments with purified tubulin. (A) Coomassie Blue stained gel. (B) Autoradiogram. Synapsin I phosphorylated at site 1 was mixed with nonphosphorylted synapsin I (in a 1:70 ratio), trypsin-treated 2 min (lanes b and d) or 5 min (lanes a and c), and incubated (0.35 mg/mL final concentration) with (c and d) or without (a and b) purified tubulin (3.2 mg/mL) in the polymerization buffer containing glycerol 20% (v/v), 0.5 mM GTP, and 1.6 mM DTT. The mixture was brought to 37 °C for 30 min, and polymers were collected by centrifugation at 100000*g* for 30 min at 32 °C. Supernatants were aspirated, and pellets superficially rinsed with the polymerization buffer (37 °C) were resuspended in SDS-containing buffer and subjected to electrophoresis.

assembled polymers and associated polypeptides resulted in pellet and supernatant fractions which were analyzed by Coomassie Blue staining of the SDS gel (Figure 5A) or Western immunoblot (Figure 5B). Synapsin I or synapsin fragments alone were entirely recovered in the supernatant fractions (lanes c and e). In the presence of microtubule proteins, synapsin I was significantly present associated with the microtubule pellet (lanes b). All the fragments of synapsin I revealed by immunodecoration also cosedimented with microtubules except 32–30-kDa polypeptides (and other faster migrating ones faintly visible, lanes d). From these experiments, it can be concluded that fragments originating from the collagenase-sensitive region of synapsin I are unable to cosediment with polymerized microtubule proteins.

When the association of fragments corresponding to 5-min tryptic digestion of synapsin I was studied by using the same

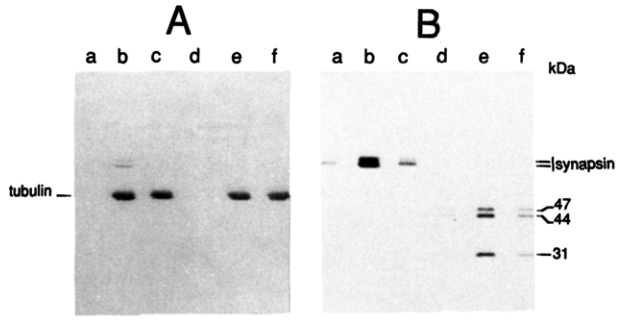

FIGURE 7: Effect of 0.35 M NaCl on cosedimentation of synapsin I or synapsin tryptic fragments with taxol-stabilized microtubules preformed from pure tubulin. (A) Coomassie Blue stained gel. (B) Autoradiogram. Synapsin I phosphorylated at site 1 in the presence of [γ-^{32}P]ATP was mixed with nonphosphorylated synapsin I (in a 1:75 ratio) in polymerization buffer (pH 6.4) and treated for 7 min with (lanes a–c) or without (lanes d–f) trypsin. The digestion was stopped, and 20-μg aliquots were incubated 30 min at 37 °C in a final volume of 100 μL with (lanes b, c, e, and f) or without (lanes a and d) taxol–tubulin (3 μM relative to dimer) in the polymerization buffer (pH 6.4) containing 0.5 mM GTP/4 μM taxol (lanes a, b, d, and e) or in the same medium in the presence of 0.35 M NaCl (lanes c and f). Samples of 80 μL were layered over a 100-μL cushion of 20% sucrose/5 μM taxol in the polymerization buffer (pH 6.4) containing 0.5 mM GTP and 7 μg/mL soybean trypsin inhibitor in Beckman Airfuge tubes. The same cushion containing 0.35 M NaCl was used for samples corresponding to lanes c and f. Taxol-stabilized microtubules with bound synapsin I fragments were pelleted during centrifugation at 100000g for 10 min. Pellets were superficially rinsed with the polymerization buffer, resuspended in SDS-containing buffer, and subjected to electrophoresis.

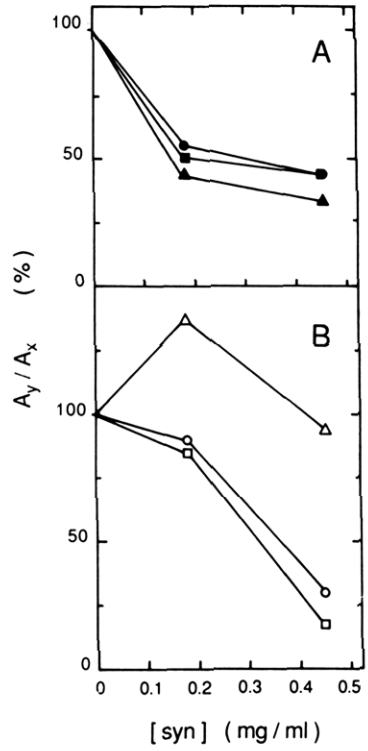

FIGURE 8: Competition between phosphorylated tryptic fragments of synapsin I and nonphosphorylated synapsin I for binding on taxol–tubulin. Synapsin I phosphorylated at site 1 was trypsinated for 30 s (A) or 5 min (B) as in Figure 6. Twenty-microgram aliquots were incubated 30 min at 37 °C in a final volume of 100 μL with or without taxol–tubulin (1.3 μM relative to dimer) and various amounts of nonphosphorylated synapsin I (0–50 μg) in the polymerization buffer containing 2 mM DTT, 0.5 mM GTP, and 20 μM taxol. Samples of 80 μL were layered over 100 μL of 20% sucrose/20 μM taxol in the polymerization buffer containing 0.5 mM GTP and 2.1 μg/mL soybean trypsin inhibitor in Beckman Airfuge tubes. Taxol-stabilized microtubules with bound synapsin I fragments were centrifuged and further treated as in the legend of Figure 7. After electrophoresis, the Coomassie Blue stained gel was autoradiographied and the amount of phosphorylated species estimated by densitometry of the autoradiogram. Results of the experiments in the presence of tubulin are corrected by the values obtained in the absence of tubulin (A_y) and are expressed in percent with respect to the values measured in the absence of undigested synapsin I (A_x). Intact synapsin I (●), tetraplet 64–59-kDa (■), 47–44-kDa (▲), 47-kDa (○), 44-kDa (□), and 31-kDa (△) fragments.

experimental conditions as those described for Figure 5, no information could be obtained because controls without microtubules revealed the strong tendency of these polypeptides to precipitate (not shown, but see Figure 6 and Discussion). We thus phosphorylated synapsin I at site 1 in the presence of [γ-^{32}P]ATP prior to trypsinization to measure the ability of resulting labeled polypeptides to cosediment with microtubules. In fact, the increased detection sensitivity afforded by autoradiography when compared to Coomassie Blue staining allowed us to use lower concentrations of these fragments. This approach has the further advantage of overcoming any risk of misinterpretation due to mixed polypeptides migrating as a simple electrophoretic band since radiolabeled peptides can unambiguously be identified as N-terminal fragments. Figure 6 illustrates the results of a typical experiment and shows that the 5-min tryptic digestion products of synapsin I of 47, 44, and 31 kDa cosediment with microtubules obtained by polymerization of purified tubulin. This cosedimentation of synapsin I fragments with microtubules did not result from pellet trapping since their interaction with taxol-stabilized microtubules prepared from PC–tubulin can almost be abolished in the presence of 0.35 M NaCl (Figure 7).

In another set of experiments, we tested the competition between intact synapsin I and these polypeptides for cosedimentation ability using taxol–tubulin. The results presented in Figure 8 show that 47- and 44-kDa fragments can cosediment with taxol–tubulin and that they were displaced by intact nonphosphorylated synapsin I. This behavior was still true for a 5-min tryptic digestion of synapsin I. In consequence, the association of 47- and 44-kDa fragments with microtubules is not reliable to a possible self-association of intact synapsin I with itself or with its degradation products (see Discussion). Concerning the 31-kDa phosphorylated fragment, it was present in significantly larger amounts in the pellet fractions when tubulin or taxol–tubulin was present than in control samples without tubulin (see Figures 6 and 7), but this "association" could not be diminished when the intact synapsin I concentration was raised (Figure 8B).

The effect of synapsin I fragment concentration on their binding to taxol-stabilized tubulin was also studied. Binding data of intact synapsin I phosphorylated at site 1 gave a classical saturation curve with half-saturation obtained with synapsin I concentrations of about 2–3 μM (not shown). This value is of the same order of magnitude as the K_d value published by Baines and Bennett (1986). On the other hand, cosedimentation of tryptic fragments of synapsin I phosphorylated on site 1 with polymerized taxol–tubulin revealed a more complex situation and suggests apparent cooperativity for 47-, 44-, and 31-kDa polypeptide binding (not shown).

Discussion

The advantage of enzymatic partial digestion of a protein under nondenaturing and well-controlled conditions lies in the fact that endoproteases, especially if they have a narrow specificity, cleave their proteic substrate at a restricted number of sites. For example, regions of a native protein which are relatively unfolded are more sensitive to proteases than com-

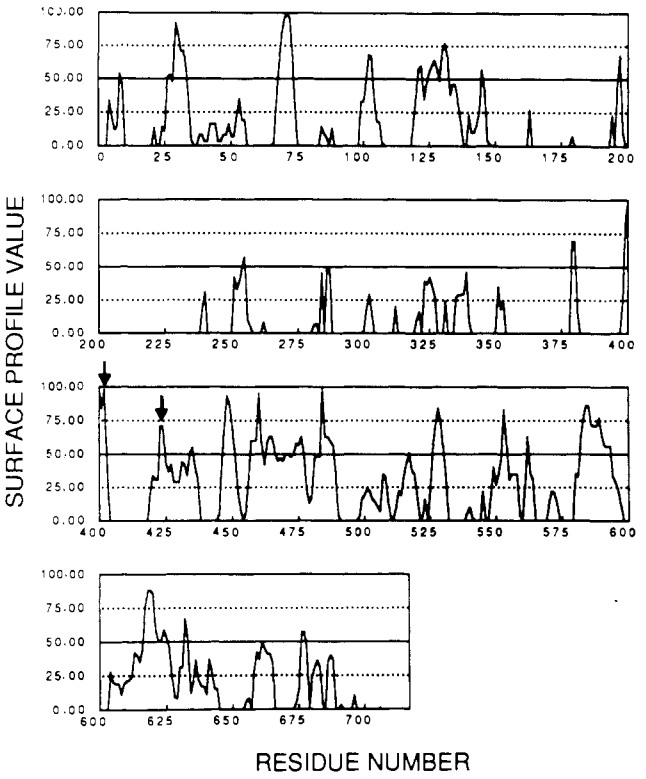

FIGURE 9: Predictive antigenicity plot for rat brain synapsin I according to the method of Parker et al. (1986). The residue position is plotted on the horizontal axis using the rat brain synapsin I sequence deduced from the cDNA clone pSyn5 (McCaffery & DeGennaro, 1986) and renumbered according to the corrections published by Czernick et al. (1987).

pact domains. Tryptic digestion of synapsin I proceeds very rapidly in the tail region (at time zero, the incubation mixture temperature is 0 °C and the trypsin to synapsin I weight ratio is 1:400), resulting in complete disappearance of intact synapsin I and the concomitant accumulation of a series of shorter polypeptides belonging to the N-terminal portion of the protein (see Figures 1 and 4). The major fragments present after a 2-min incubation period are the 47- and 44-kDa polypeptides. They contain the cyclic AMP dependent phosphorylation site (site 1) and are at the edge of the hydrophobic core. In fact, initial proteolytic attack concerns exclusively the collagen-like region of synapsin I. A predictive composite surface profile for synapsin I obtained as described by Parker et al. (1986) by combination of HPLC hydrophobicity (Parker et al., 1986), accessibility (Janin, 1979), and flexibility (Karplus & Schulz, 1985) is shown in Figure 9. This plot is in agreement with the fact, already outlined under Results, that the central portion of synapsin I is poorly recognized by our antiserum whereas fragments arising from the tail of the protein are strongly immunoreactive. Furthermore, it is tempting to speculate, using this predictive antigenicity plot, that 47- and 44-kDa polypeptides arise from tryptic digestion at the level of Arg-422 and Lys-403, respectively (arrows in Figure 9), in the rat synapsin I sequence or the equivalent basic amino acids in the bovine protein. Further digestion of these fragments produces a "family" of polypeptides lacking the native synapsin I NH_2-terminal end, the longest of which is the 41-kDa fragment. In parallel, a 31-kDa fragment which has conserved serine-9 and whose COOH end lies probably around amino acid 281 is present. Synapsin I phosphorylated at site 1 shows the same kinetics of tryptic digestion as dephosphosynapsin in our experimental conditions (Figure 2). This fact is not especially amazing since we follow the appearance or the disappearance of relatively long fragments which have the synapsin I NH_2-terminal end. However, Bähler et al. (1989) reported a conformatinal change in the head domain induced by (Ca^{2+}, calmodulin)-dependent tail phosphorylation. Our results suggest that cyclic AMP dependent phosphorylation of synapsin I, whose physiological significance is not clearly understood, does not lead to a noticeable conformational change in the tail domain.

To identify the structural domain of synapsin I involved in binding to microtubules, mixtures of proteolytic fragments corresponding to 30-s or 5-min tryptic digestion were incubated with microtubule proteins (Figure 5) or polymerized pure tubulin (Figure 6). Their cosedimentation ability with assembled polymers was tested. In these experiments, we obtained evidence that the microtubule binding activity is located in the head region of synapsin I. The 44-kDa fragment, which comprises the NH_2-terminal extremity and the hydrophobic head domain of synapsin I, cosediments with microtubules and competes with intact synapsin I for binding. The interaction of native synapsin I and 47-, 44-, and 31-kDa polypeptides with taxol-stabilized microtubules prepared from PC–tubulin is mainly electrostatic in nature since their cosedimentation with assembled polymers is greatly reduced when the ionic strength of the medium increases (Figure 7). On the other hand, fragments of the tail region, particularly the 30–32-kDa fragments visualized in immunoblots (see Figure 5), are entirely present in the supernatant fraction after cosedimentation experiments with microtubules. It can first be concluded that these polypeptides do not interact either with intact synapsin I or with other synapsin I fragments present in the microtubule pellet. Furthermore, they do not bind to microtubules in spite of their high basicity. An unexpected result lies in the increased cosedimentation of the 31-kDa NH_2-terminal proteolysis product of synapsin I with microtubules when undigested synapsin I is present (see Figure 8B). A constant problem encountered during this study was the loss of solubility of fragments originated from the head of synapsin I. In fact, this 31-kDa NH_2-terminal tryptic polypeptide is very similar to the NTCB (2-nitro-5-thiocyanobenzoic acid)-generated 29-kDa fragment purified under denaturing conditions and tested for F-actin binding activity by Bähler et al. (1989). These authors localized one of the two F-actin binding sites in this fragment and emphasized its insolubility in aqueous media and tendency to self-associate. The binding of this NH_2-terminal 29-kDa fragment to F-actin was complex and occurred with a strong apparent positive cooperativity. As a consequence, the lack of competition between native synapsin I and the 31-kDa tryptic fragment for binding to microtubules cannot be unequivocally interpreted as nonspecific adsorption or coprecipitation. Furthermore, both the apparent cooperativity in binding of 47-, 44-, and 31-kDa synapsin I tryptic polypeptides to taxol-stabilized microtubules (not shown) and their strong tendency to precipitate at high concentrations make saturation curves difficult to obtain. These experimental data suggest that although the tail fragments obtained by tryptic digestion of synapsin I do not bind to microtubule proteins (Figure 5B, lanes d), removal of the tail domain of synapsin I could lead to conformational modifications of the head portion of the molecule.

Synapsin I is a cross-linking protein. It has been shown to bundle microtubules (Baines & Bennett, 1986) and actin filaments (Petrucci & Morrow, 1987; Bähler & Greengard, 1987) and to cross-link synaptic vesicles to brain membranes (Steiner et al., 1987) and vesicles to themselves (Hirokawa et al., 1989). On the other hand, the strong tendency of

synapsin I to self-associate has been evoked by several groups (Baines & Bennett, 1986; Bähler & Greengard, 1987), and we have obtained experimental evidence (Font & Aubert-Foucher, 1989) that native synapsin I in solution interacts with itself in a head to head association. During this oligomerization, cysteines belonging to the hydrophobic core from two different synapsin I molecules are in close apposition and can form a Cu^{2+}–phenanthroline-catalyzed intermolecular disulfide bond. A simple dimerization process could then explain the cross-linking abilities of synapsin I. However, recent studies have pointed out the fact that the numerous interactions that synapsin I can contract with cytoskeletal elements or membranes are complex in nature and could involved both synapsin I self-association and multisite existence. As a matter of fact, in vitro reconstitution experiments followed by quick-freeze deep-etch electron microscopic visualization (Hirokawa et al., 1989) suggest that a single synapsin I molecule can cross-link actin filaments. An extensive study of NTCB (2-nitro-5-thiocyanobenzoic acid)-generated synapsin I fragment interaction with F-actin (Bähler et al., 1989) has shown that F-actin binding activity is located within two fragments of synapsin I, a 29-kDa NH_2-terminal fragment and a 15–16-kDa middle fragment. Furthermore, the tail fragment of synapsin I is involved in F-actin bundling, although this fragment is not able to bundle F-actin by itself. Concerning in vitro synapsin I interaction with microtubules, the work of Hirokawa et al. (1989) using quick-freeze deep-etch electron microscopy reveals that the spherical head of synapsin I is often localized on one microtubule while on the adjacent microtubule a tail is found. In addition, when adjacent microtubules are futher apart, these authors describe globular masses from which fine strands radiate and cross-link microtubules. From this, they assumed that a simple synapsin I molecule has the ability to cross-link two microtubules and that synapsin I molecules can alternatively connect with each other at their heads and cross-link microtubules by their tail. As a consequence, synapsin I should have a tubulin binding site in its tail domain in addition to the site located in the head of the molecule revealed in the present work. We cannot completely rule out by our experiments the existence of a microtubule binding domain in the tail of native synapsin I. As a matter of fact, a conformational binding site in the tail could be inforced by the head portion in the intact synapsin I molecule. This site could disappear following the proteolysis-induced separation of synapsin I in its two constitutive functional domains. Future work, involving synapsin I digestion after its chemical cross-linking to polymerized tubulin, should give interesting information concerning this point.

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