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Synapsin IIa: Expression in Insect Cells, Purification, and Characterization†

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ABSTRACT: Synapsin IIa belongs to a family of neuron-specific phosphoproteins called synapsins, which are associated with synaptic vesicles in presynaptic nerve terminals. In order to examine the biochemical properties of synapsin IIa, and ultimately its physiological function, purified protein is required. Since attempts to purify significant quantities of synapsin IIa, an isoform of the synapsins, from mammalian brain have proven difficult, we undertook the production of recombinant synapsin IIa by utilizing the baculovirus expression system. Rat synapsin IIa cDNA was introduced into the baculovirus genome via homologous recombination, and the recombinant baculovirus was purified. *Spodoptera frugiperda* (Sf9) cells infected with this virus expressed synapsin IIa as 5% of the total cellular protein. The recombinant protein was extracted from the particulate fraction of the infected Sf9 cells with salt and a nonionic detergent and purified by immunoaffinity chromatography. The purified synapsin IIa was phosphorylated by the catalytic subunit of cAMP-dependent protein kinase to a stoichiometry of 0.8 mol of phosphate/mol of protein. Metabolic labeling with [^{32}P]P_i demonstrated synapsin IIa phosphorylation in infected Sf9 cells. Using a homogenate of uninfected Sf9 cells, a cAMP-dependent protein kinase activity which can phosphorylate synapsin IIa was detected. Limited proteolysis of recombinant synapsin IIa phosphorylated in vitro and in vivo resulted in identical phosphopeptide maps. Further, synapsin IIa, like synapsin I, binds with high affinity in a saturable manner to synaptic vesicles purified from rat cortex.

The synapsins are a family of neuronal phosphoproteins that are localized on the cytoplasmic surface of small synaptic vesicles [for a review, see De Camilli et al. (1990)]. This family is comprised of four proteins, synapsins Ia and Ib (collectively termed synapsin I) and synapsins IIa and IIb (collectively termed synapsin II). The mRNAs of synapsins Ia and Ib and of synapsins IIa and IIb are generated by alternative splicing of transcripts from two different genes (Südhof et al., 1989). A comparison of the nucleotide and deduced amino acid sequences of the synapsins reveals a high degree of identity in more than half of each protein. The NH_2-terminal domains, A, B, and C, of synapsin I and synapsin II show an overall sequence identity of 70% covering 420 residues (Südhof et al., 1989). The synapsins differ extensively, however, in their COOH-termini, except for synapsin Ia and synapsin IIa which contain 76% identical amino acids in the final 50 residues.

The functional behavior of the synapsins is affected by their phosphorylation states, which are regulated by a variety of physiological and pharmacological stimuli that alter synaptic efficacy (Nestler & Greengard, 1983). All four synapsins share an NH_2-terminal phosphorylation site (site 1) for cAMP-dependent protein kinase and Ca^{2+}/calmodulin-dependent protein kinase I; synapsins Ia and Ib have two ad-

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ditional phosphorylation sites (sites 2 and 3) for Ca^{2+}/calmodulin-dependent protein kinase II (Huttner & Greengard, 1979; Huttner et al., 1981; Nairn & Greengard, 1987) and another site for proline-directed protein kinase (Hall et al., 1990) near the COOH-terminal. It has been shown that phosphorylation by Ca^{2+}/calmodulin-dependent protein kinase II decreases the in vitro binding of synapsin I to synaptic vesicles and to F-actin and virtually abolishes the synapsin I-induced bundling of actin filaments (Schiebler et al., 1986; Bähler & Greengard, 1987; Petrucci & Morrow, 1987; Benfenati et al., 1989a). In addition, synapsin I has been reported to bind to tubulin, spectrin, and calmodulin in vitro (Baines & Bennett, 1986; Okabe & Sobue, 1987; Sikorski et al., 1991). These data, along with data from microinjection experiments (Llinás et al., 1985, 1991; Hackett et al., 1990; Lin et al., 1990), have led to the hypothesis that synapsin I may regulate the number of synaptic vesicles available for release by cross-linking them to the cytoskeleton and thereby preventing their access to fusion sites at the presynaptic membrane (Benfenati et al., 1991).

The biochemical properties and functional role of the synapsin II isoforms have not yet been studied as extensively as those for synapsin I. Although the high degree of homology in the NH_2-terminal regions of synapsins I and II suggests that the proteins share some of their functions, the significant divergence in the COOH-terminal regions suggests as yet unidentified functional distinctions (Südhof et al., 1989). Furthermore, the role of synapsin I in regulating neurotransmitter release is dependent on the state of phosphorylation of sites 2 and 3, which are located near the COOH-terminal. The likelihood of distinct functions for each of the four synapsins is supported by the demonstration of their heterogeneous distribution in brain (Südhof et al., 1989) and among conventional synapses in the vertebrate retina (Mandell et al., 1990, 1992). In order to define the specific functions of the homologous and divergent domains of the synapsins and the overall functions of synapsin II, biochemical and physiological studies of synapsin II are necessary.

Attempts to purify significant amounts of synapsin II from mammalian brain have proven difficult, in part because synapsin II is more hydrophobic than synapsin I and because it is more easily proteolyzed (Y. L. Siow and P. Greengard, unpublished observations). Additionally, the isolation of individual isoforms of the synapsins is technically difficult using conventional purification techniques, because of the extent of homology between primary sequences. We were recently able to express various NH_2-terminal domains of synapsin II in bacteria (Thiel et al., 1990), which led to the discovery of a new site for binding of synapsin II to synaptic vesicles. The expression of the entire synapsin II molecule in bacteria, however, resulted in a completely insoluble recombinant protein which could not be used for biochemical studies (G. Thiel, T. Südhof, and P. Greengard, unpublished observations). We therefore employed the baculovirus expression system in an effort to produce soluble synapsin II. We report here the purification and characterization of synapsin IIa, one isoform of the synapsin family, produced by *Spodoptera frugiperda* (Sf9)[1] cells infected with recombinant baculovirus. The recombinant synapsin IIa so produced is immunologically indistinguishable from rat brain synapsin IIa, is an excellent substrate for cAMP-dependent protein kinase, and binds with high affinity to purified synaptic vesicles from rat cortex.

Experimental Procedures

Materials. The baculovirus transfer vector, pVL1393, wild-type *Autographa californica* nuclear polyhedrosis virus (AcNPV) (high-titer stock), and AcNPV viral DNA were purchased from Invitrogen. SeaPlaque agarose for plaque assays was obtained from FMC BioProducts. The insect cell line Sf9 (CRL 1711) was from American Type Culture Collection. Grace's supplemented insect medium, Sf-900 serum-free insect medium, methionine-free insect medium, fetal bovine serum, gentamicin, fungizone, and Lipofectin were from GIBCO-BRL. Ex-cell 401 phosphate-free insect medium was from JRH Biosciences. [γ-^{32}P]ATP and EXPRE^{35}S^{35}S (L-[^{35}S]methionine and L-[^{35}S]cysteine) were from New England Nuclear. ^{125}I-Labeled protein A was from Amersham Corp. The catalytic subunit of cAMP-dependent protein kinase was a gift from A. Horiuchi and A. C. Nairn of our laboratory. COOH-terminal amidated peptide corresponding to residues 5–24 of the peptide inhibitor of cAMP-dependent protein kinase (Walsh inhibitor) was synthesized by the Yale University Protein and Nucleic Acid Chemistry Facility.

Construction of Recombinant Transfer Vector and Transfection. The rat synapsin IIa cDNA was excised from the plasmid pSKA by *Bam*HI and *Eco*RI and cloned in the *Bam*HI/*Eco*RI sites of the baculovirus transfer vector pVL1393. The resultant transfer vector, pVLSyIIa, contained one copy of the synapsin IIa cDNA, including a 130 bp 5′-untranslated region, in the correct orientation downstream of the viral polyhedrin promoter. The fusion gene contained the entire 5′-untranslated region of the baculovirus polyhedrin gene. Because of a point mutation of the polyhedrin ATG, the fusion gene product utilized the initiating ATG codon of synapsin IIa. Transfer of the cDNA of synapsin IIa into the AcNPV genome was accomplished by homologous recombination after cotransfection of CsCl-purified pVLSyIIa plasmid and wild-type AcNPV DNA into Sf9 cells. Briefly, 3.5×10^6 Sf9 cells were seeded in a 25 cm^2 T-flask. After 1 h, the cells were washed twice with serum-free Grace's medium and then transfected by lipofection (Felgner & Holms, 1989) using 2 μg of AcNPV DNA, 4 μg of pVLSyIIa plasmid, and 30 μg of Lipofectin. The lipofection was stopped after 20 h by the addition of 1 volume of Grace's medium containing 20% fetal bovine serum. The insect culture medium containing the recombinant viruses was harvested 5-days posttransfection.

Isolation of Recombinant Baculovirus. Recombinant virus was isolated by limiting dilution of the virus and slot-blot hybridization followed by immunoblot analysis and plaque purification. A 96-well plate was seeded with 2×10^4 Sf9 cells in 80 μL of insect cell culture medium per well. After cells were allowed to attach for 1 h at 27 °C, 50 μL of a 10^{-5}–10^{-7} dilution of the transfection supernatant was added to each well. The 96-well plate was then sealed tightly with parafilm and was incubated for 1 week. On the eighth day, the medium from each well was transferred to the corresponding well of a new 96-well plate and stored at 4 °C. Two hundred microliters of 0.5 M NaOH was added to each well of the plate containing the cells. After incubation at room temperature for 15 min, 20 μL of ammonium acetate was added to each well to neutralize the NaOH, followed by the addition of 3 μL of proteinase K (20 mg/mL). The plate was then incubated at 37°C for 0.5–2 h. A BioTrace HP hybridization membrane (Gelman Sciences) was premoistened with 10× SSC (1× SSC: 15 mM sodium citrate buffer, pH 7.0, con-

[1] Abbreviations: AcNPV, *Autographa californica* nuclear polyhedrosis virus; bp, base pair(s); BCIP, 5-bromo-4-chloro-3-indolyl phosphate; BSA, bovine serum albumin; MOI, multiplicity of infection; NBT, *p*-nitroblue tetrazolium chloride; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; Sf9, *Spodoptera frugiperda*.

taining 150 mM NaCl) for 15 min and was assembled in a slot-blot apparatus (Bio-Rad). Two hundred microliters of 1 M ammonium acetate/0.02 M NaOH was added to each well followed by addition of the lysed cell samples. Each well was then washed with 200 μL of 2X SSC. The membrane was hybridized with ^{32}P-labeled oligonucleotides corresponding to the synapsin II coding region. The supernatants of those samples which hybridized with the ^{32}P-labeled oligonucleotides were transferred to a 24-well plate previously seeded with 6 × 10^5 cells per well. This plate was incubated for 5 days at 27 °C. After the supernatant was saved, the cells were analyzed for synapsin IIa expression by immunoblotting. The recombinant viruses which expressed the highest level of synapsin IIa were then plaque-purified according to Summers and Smith (1987). Two rounds of plaque-purification were performed to obtain the pure recombinant virus. Immunoblot analysis was performed after each round of plaque purification. Virus stocks were prepared and stored at 4 °C.

Expression of Synapsin IIa and Immunoblotting. Sf9 cells were grown and maintained either as monolayers or in suspension in spinner flasks according to Summers and Smith (1987) and Piwnica-Worms (1988). Cells were infected as described by Summers and Smith (1987) at a multiplicity of infection (MOI) of 10 and were harvested either periodically (for determining the time course of maximum expression) or at 68–72-h postinfection. Cells were washed with ice-cold PBS containing 0.2 mM PMSF and lysed by sonication on ice for two 10-s bursts at setting 11 (Microson ultrasonic cell disruptor, Heat Systems–Ultrasonic, Inc., Farmingdale, NY) in 50 mM Tris-HCl buffer (pH 8.0) containing 1% SDS and 50 mM DTT. Samples were subjected to electrophoresis on 6.5% SDS–polyacrylamide gels. For immunoblotting, proteins were transferred to nitrocellulose membranes (pore size, 0.2 μm) as described by Towbin et al. (1980). Blots were developed using either alkaline phosphatase-conjugated secondary antibody or ^{125}I-protein A.

Subcellular Fractionation and Purification. All procedures were performed at 4 °C unless otherwise stated. Buffer A was 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, and 5 mM 2-mercaptoethanol; buffer B consisted of 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 0.1 mM EGTA, and 5 mM 2-mercaptoethanol; buffer C was composed of 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 0.1 mM EGTA, and 5 mM 2-mercaptoethanol; buffer D was 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% Tween 20; buffer E contained 10 mM Tris-HCl (pH 8.0) and 200 mM NaCl. Where indicated, protease inhibitors were added to the buffers at the following final concentrations: 0.2 mM phenylmethanesulfonyl fluoride (PMSF), 20 μg/mL each of leupeptin and antipain, and 2 μg/mL each of chymostatin and pepstatin.

Sf9 cells infected with synapsin IIa recombinant virus were harvested and washed as described in the previous section. The cell pellet was lysed by sonication in buffer A plus protease inhibitors and aprotinin (25 KIU/mL final concentration). The lysate was centrifuged at 27000*g* for 30 min, and the resulting supernatant was centrifuged at 200000*g* for 20 min. The 27000*g* pellet was resuspended in buffer B (same volume as the supernatant) plus protease inhibitors, sonicated, and stirred for 45 min prior to recentrifugation at 27000*g* for 30 min. Samples from each step were analyzed by electrophoresis on 7.5% SDS–polyacrylamide gels and by immunoblotting.

Ten to fourteen 150 cm^2 T-flasks each containing 2 × 10^7 Sf9 cells were infected for 1 h at room temperature with 4 mL of recombinant synapsin IIa virus at an MOI of 10. The viral supernatant was removed and replaced by 20 mL of Sf-900 serum-free insect cell culture medium. After incubation at 27 °C for 70–72 h, the infected Sf9 cells were harvested and washed with 100 mL of ice-cold PBS containing 0.2 mM PMSF. Cells were centrifuged at 300*g* for 10 min, and the resulting pellet was sonicated in 160–230 mL of buffer A plus protease inhibitors and aprotinin (25 KIU/mL final concentration). This homogenate was centrifuged at 27000*g* for 30 min. The pellet was resuspended and homogenized in 160–230 mL of buffer B plus protease inhibitors. The mixture was stirred for 45 min prior to centrifugation at 27000*g* for 30 min. The resulting supernatant was diluted 5-fold with buffer C containing protease inhibitors and applied, at a flow rate of 1–2 mL/min, onto a synapsin II immunoaffinity column. The affinity column had been prepared by coupling a synapsin II-specific monoclonal antibody (c19.21) to CNBr-activated Sepharose 4B. Before each sample application, the column was equilibrated with buffer D. After sample application, the column was washed with 2 column volumes of buffer D followed by 4 column volumes of buffer E. Recombinant synapsin IIa was eluted from the affinity column with 0.1 M glycine (pH 2.7). The eluate was adjusted immediately to pH 8.0 with 1 M Tris (pH 11) and dialyzed for 16–18 h against buffer C containing protease inhibitors and 1 mM 2-mercaptoethanol.

Phosphorylation Assays. In vitro phosphorylation by purified catalytic subunit of cAMP-dependent protein kinase was carried out according to the method described by Huttner et al. (1981). cAMP-dependent protein kinase activity in Sf9 cells was determined as described by Walaas et al. (1989) except that exogenous synapsin IIa was used as substrate and 1 mM Na_3VO_4 (final concentration) was included in the reaction mixture.

Metabolic Labeling. Sf9 cells infected with the recombinant baculovirus were labeled at 69-h postinfection for 2 h with 0.5 mCi/mL [^{32}P]P_i (specific activity 8500–9120 Ci/mmol) in 2 mL of phosphate-free medium to detect synapsin IIa phosphorylation in vivo. Parallel experiments using 300 μCi/mL EXPRE^{35}S^{35}S (specific activity of L-[^{35}S]methionine, 1186 Ci/mmol) in 2 mL of methionine-free medium were performed to label total protein. After being labeled, the cells were washed twice with ice-cold PBS/0.2 mM PMSF, and synapsin IIa was immunoprecipitated according to the procedure described by Grebb et al. (1989) except for the addition of 1 mM Na_3VO_4 (final concentration) and the use of antibody G-38 (rabbit polyclonal antibody made against rat synapsin II). Immunoprecipitates were analyzed by SDS–PAGE. Gels containing ^{35}S-labeled proteins were fluorographed in ENTENSIFY (Dupont-NEN).

Assay of Synapsin IIa Binding to Synaptic Vesicles. Small synaptic vesicles were purified from rat neocortex (male Sprague-Dawley, 150–200-g body weight) as described by Huttner et al. (1983). Endogenous synapsins were stripped from the purified vesicles as described (Thiel et al., 1990). The binding of synapsin IIa to synaptic vesicles (5 μg of total protein per sample) was performed as described by Benfenati et al. (1989a,b). The amounts of synapsin IIa bound to synaptic vesicles were quantitated either by dot immunobinding as described by Jahn et al. (1984) or by immunoblotting using a synapsin IIa-specific antibody. The individual values of synapsin IIa bound to synaptic vesicles were corrected for vesicle recovery determined by dot immunobinding using an anti-synaptophysin antibody.

Miscellaneous Techniques. SDS–PAGE was performed according to Laemmli (1970). One-dimensional phospho-

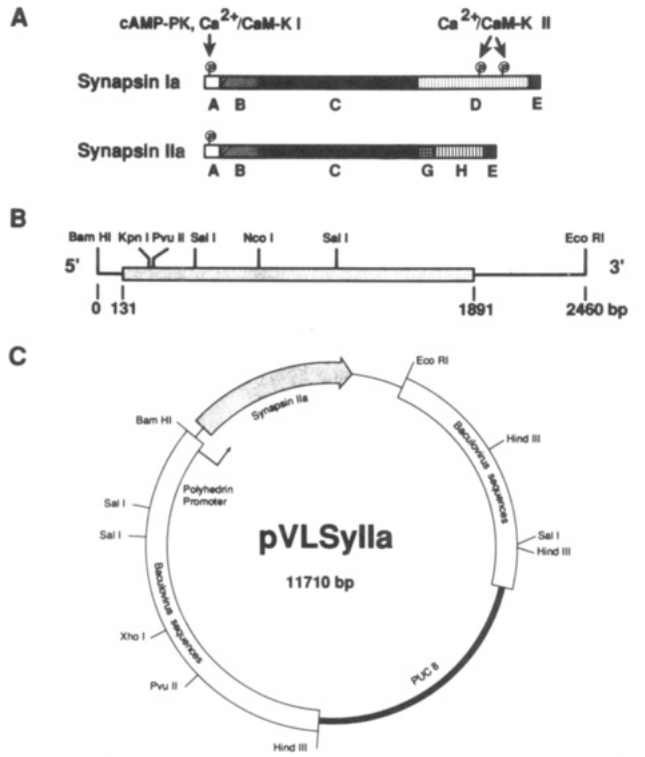

FIGURE 1: Domain structure of synapsins Ia and IIa and construction of the baculovirus transfer vector. (A) Domain structure of synapsin Ia and synapsin IIa (Südhof et al., 1989). The phosphorylation site for cAMP-dependent protein kinase and Ca^{2+}/calmodulin-dependent protein kinase I and the two additional phosphorylation sites for Ca^{2+}/calmodulin-dependent protein kinase II are indicated. (B) The cDNA of synapsin IIa was inserted into the baculovirus vector pVL1393, leading to the baculovirus transfer vector for synapsin IIa, pVLSyIIa [shown in (C)].

peptide mapping was performed as described by Cleveland et al. (1977). Saturation curves were analyzed using the computer program RECEPT (Benfenati & Guardabasso, 1984). Protein was determined according to the method of Bradford (1976) or Peterson (1977) using BSA as a standard.

RESULTS

Construction and Purification of the Recombinant Baculovirus. Synapsin IIa represents one isoform of the synapsin family, and shows a high degree of homology to synapsin Ia in domains A, B, C, and E (Figure 1A). Synapsin IIa diverges from the Ia isoform by replacing the proline-rich domain D with domains G and H, which are of unknown function. To express synapsin IIa in insect cells using recombinant baculovirus, the rat synapsin IIa cDNA (Figure 1B) was cloned initially into a baculovirus vector, generating the transfer vector pVLSyIIa (Figure 1C). This plasmid was cotransfected with AcNPV wild-type DNA into Sf9 cells, and recombinant baculovirus was isolated using a combination of screening methods. This procedure placed synapsin IIa expression under the control of the strong viral polyhedrin promoter of AcNPV. Throughout the screening, expression of synapsin IIa was monitored by immunoblot analysis.

Expression of Recombinant Protein. The expression of the recombinant synapsin IIa in Sf9 cells infected with purified recombinant synapsin IIa baculovirus is shown as a function of postinfection time in Figure 2. The identity of the expressed protein was confirmed by immunoblotting using a synapsin II-specific monoclonal antibody (Figure 2A). The expression of another protein which migrated at an apparent molecular mass of 55–60 kDa was detected beginning at 24–36-h postinfection. This protein was not recognized by any of the

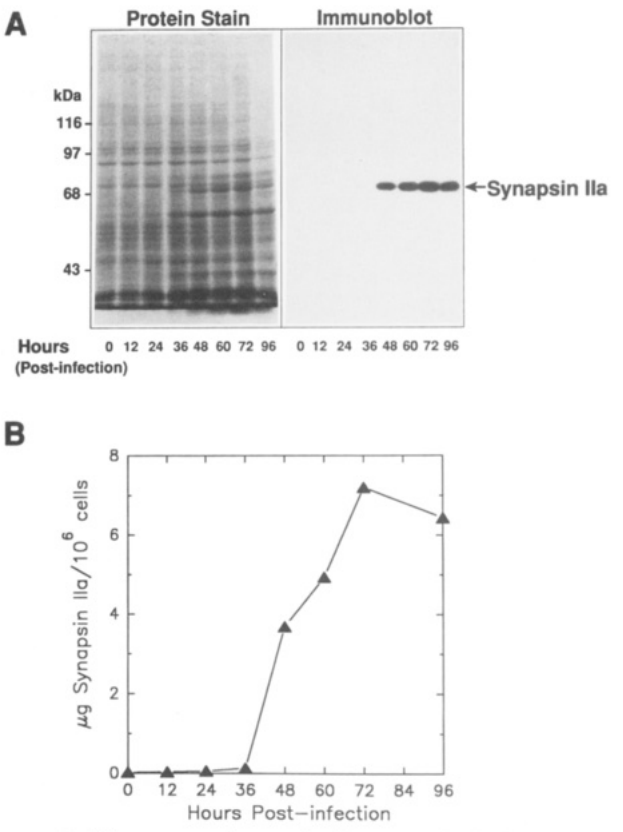

FIGURE 2: Time course of synapsin IIa expression in baculovirus-infected Sf9 cells. (A) Left panel: cell lysates (25 μg of protein/lane) from synapsin IIa baculovirus-infected Sf9 cells at 0–96-h postinfection were analyzed on a 6.5% SDS–polyacrylamide gel, and the Coomassie-stained gel is shown. Right panel: the same samples (5 μg of protein/lane) were subjected to SDS–PAGE and transferred to a nitrocellulose membrane which was then developed with c19.21 monoclonal antibody and ^{125}I-protein A. An autoradiogram of the immunoblot is shown. (B) Synapsin IIa bands on the immunoblot shown in the right panel of (A) were excised and counted, and the amount of synapsin IIa was quantitated.

synapsin II-specific antibodies, suggesting that it was a baculovirus-encoded late gene product. Uninfected Sf9 cells and cells infected with wild-type baculovirus did not show any immunoreactivity (data not shown). Synapsin IIa was detected in the Sf9 cells beginning at 36 h and could still be detected 96-h postinfection. The maximal level of expression occurred at 72-h postinfection (Figure 2B). Therefore, a 72-h postinfection time was chosen for harvesting the infected Sf9 cells. A densitometric scan of the Coomassie-stained protein gel (Figure 2A) indicated that recombinant synapsin IIa represented approximately 5% of the total cellular protein.

Subcellular Fractionation and Purification. Prior to attempting to purify the recombinant synapsin IIa from infected Sf9 cells, it was necessary to determine whether synapsin IIa was in the soluble or particulate fraction of Sf9 cells. The fractionation was based on the purification procedure[2] for synapsin II from mammalian brain. Figure 3 shows the Coomassie-stained SDS–polyacrylamide gel (left panel) and the corresponding immunoblot (middle panel) of the cell fractionation analysis. The recombinant synapsin IIa was expressed as a major protein in infected cells (Figure 3, left panel, homogenate lane, and Figure 2A). Most of the synapsin IIa in the Sf9 cells was pelleted at 27000*g* while the majority of other proteins remained in the supernatant (Figure 3, left

[2] Y. L. Siow, A. J. Czernik, M. D. Browning, and P. Greengard, manuscript in preparation.

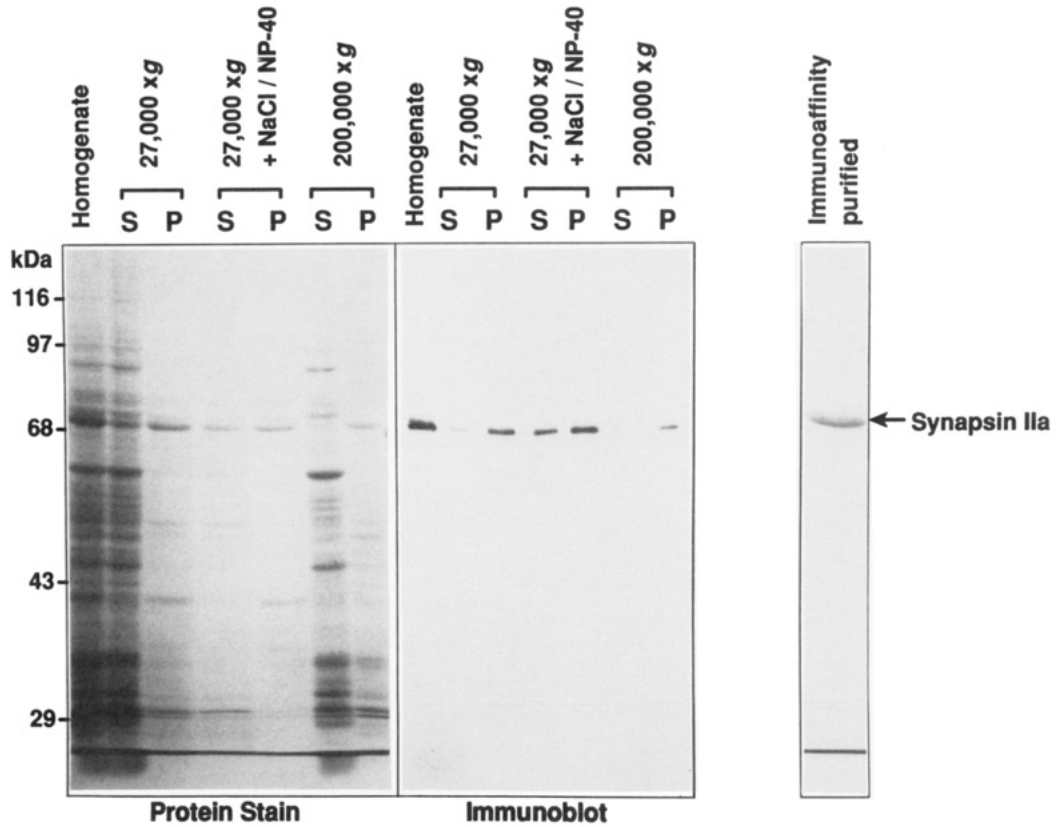

FIGURE 3: Subcellular fractionation and purification of recombinant synapsin IIa. (Left panel) Cell fractionation was performed as described under Experimental Procedures. The proteins were analyzed on a 7.5% SDS–polyacrylamide gel, and the Coomassie-stained gel is shown. (Middle panel) The same samples were subjected to SDS–PAGE and transferred to a nitrocellulose membrane which was then developed with c19.21 monoclonal antibody and alkaline phosphatase-conjugated secondary antibody using NBT and BCIP as substrates. (Right panel) Immunoaffinity-purified synapsin IIa (7 μg) was analyzed by SDS–PAGE, and the Coomassie-stained gel is shown. S, supernatant; P, pellet.

panel). The pellet was extracted with detergent/salt, and approximately 40% of the recombinant protein was recovered in the supernatant obtained after recentrifugation. When the initial 27000*g* supernatant which contained a minute fraction of the recombinant protein (Figure 3, middle panel) was centrifuged at 200000*g*, all of the remaining synapsin IIa was found in the pellet.

Since the detergent/salt extract of the 27000*g* pellet was relatively enriched in the recombinant protein (Figure 3, left panel, middle lane), the same detergent/salt extraction procedure was used in the scaled-up purification process. The solubilized synapsin IIa was free of most of the cellular proteins and was therefore applied directly to an immunoaffinity column. As shown in the Coomassie-stained SDS–polyacrylamide gel (Figure 3, right panel), synapsin IIa was purified to homogeneity. A recovery of approximately 12% was obtained from the original cell lysate. On average, 200–300 μg of synapsin IIa protein was recovered from a total of 2×10^8 infected insect cells (in 100 mL of insect media).

By all parameters measured, the recombinant synapsin IIa behaved identically to that from rat brain. Both had the same migration pattern on SDS–polyacrylamide gels (data not shown). Antibodies which recognized rat brain synapsin IIa also recognized recombinant synapsin IIa (data not shown). Attempts to sequence the NH_2-terminal of the recombinant synapsin IIa by Edman degradation (performed by the Rockefeller University Protein Sequencing Facility) were unsuccessful, suggesting that the protein possessed a blocked NH_2-terminus, as has been observed for rat brain synapsin II (A. J. Czernik and P. Greengard, unpublished observations).

Phosphorylation of Recombinant Protein. Each of the four synapsins from mammalian brain can be phosphorylated by cAMP-dependent protein kinase on a seryl residue located in the NH_2-terminal domain (Figure 1A). The recombinant synapsin IIa was tested for its ability to serve as a substrate for this kinase. Recombinant synapsin IIa was phosphorylated by the catalytic subunit of cAMP-dependent protein kinase to a stoichiometry of 0.8 mol of phosphate/mol of protein. An identical stoichiometry was obtained when purified rat brain synapsin I was used as a substrate in a parallel experiment (data not shown).

In order to determine whether the recombinant synapsin IIa was phosphorylated in insect cells, infected Sf9 cells were labeled with [^{32}P]P_i for 2 h at 69-h postinfection. Synapsin IIa was immunoprecipitated and analyzed by SDS–PAGE and autoradiography. A band at the position corresponding to that of synapsin IIa was detected after overexposure of the autoradiogram (Figure 4A, lane 2). This indicates that synapsin IIa was phosphorylated only to a minor extent in infected Sf9 cells under basal conditions. Parallel metabolic labeling with [^{35}S]methionine and [^{35}S]cysteine demonstrated that synapsin IIa was synthesized during the labeling period (Figure 4A, lane 1). Limited proteolytic digestion with *Staphylococcus aureus* V8 protease (Cleveland et al., 1977) of recombinant synapsin IIa phosphorylated in vivo or in vitro yielded an identical 18-kDa phosphopeptide fragment (Figure 4B, lanes 1 and 2, respectively). This one-dimensional phosphopeptide map was indistinguishable from that obtained with mammalian brain synapsin II phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (Huang et al., 1982; Grebb et al., 1989).

We further characterized the protein kinase responsible for the in vivo phosphorylation of synapsin IIa. A homogenate of uninfected Sf9 cells was able to catalyze the phosphorylation

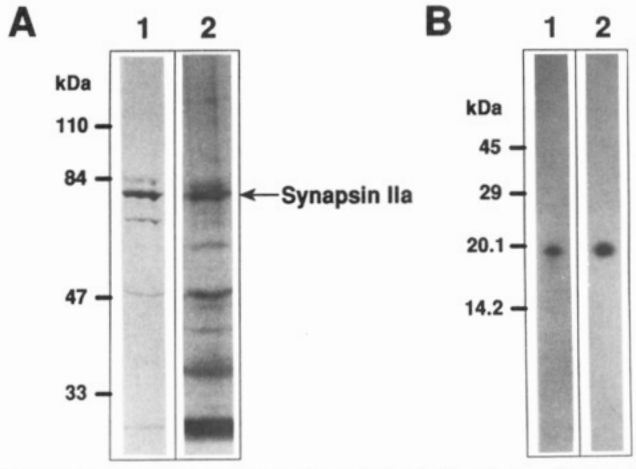

FIGURE 4: Metabolic labeling of synapsin IIa baculovirus-infected Sf9 cells. Sf9 cells infected with synapsin IIa baculovirus were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine or with [^{32}P]P_i at 69-h postinfection. (A) Synapsin IIa was immunoprecipitated with G-38 antibody and analyzed on 7.5% SDS–polyacrylamide gels. The gels were dried, and the ^{35}S-labeled and ^{32}P-labeled immunoprecipitates were visualized by fluorography (lane 1) or autoradiography (lane 2), respectively. The very dark dye front (lane 2) indicates that overexposure of the autoradiogram was necessary to visualize phosphosynapsin IIa. The amounts of immunoprecipitated proteins were from equal numbers of cells (2.5×10^6 cells sample^{-1} lane^{-1}). (B) Autoradiogram depicting the one-dimensional phosphopeptide map of immunoprecipitated recombinant synapsin IIa metabolically-labeled with [^{32}P]P_i (lane 1) and of purified recombinant synapsin IIa phosphorylated in vitro by the catalytic subunit of cAMP-dependent protein kinase (lane 2).

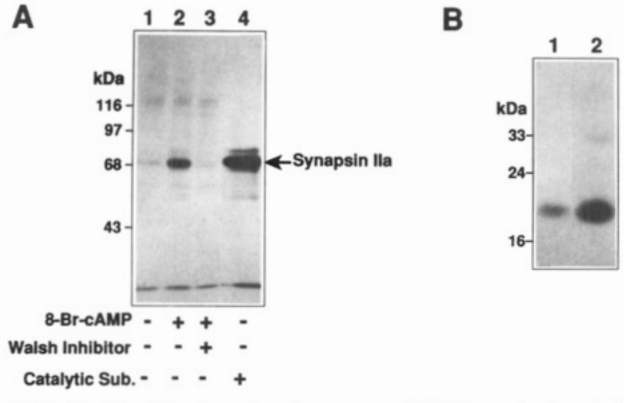

FIGURE 5: Identification of endogenous cAMP-dependent protein kinase activity in Sf9 cells. (A) Autoradiogram showing the phosphorylation of exogenously added recombinant synapsin IIa by homogenate preparations from uninfected Sf9 cells. Homogenate samples were incubated for 5 min with 100 μM [γ-^{32}P]ATP in the absence or presence of 8-Br-cAMP (10 μM) and the protein inhibitor of cAMP-dependent protein kinase (Walsh inhibitor, 5 μM), as indicated. Synapsin IIa was also phosphorylated with the purified catalytic subunit of cAMP-dependent protein kinase (lane 4). Proteins were subjected to SDS–PAGE, and phosphosynapsin IIa was visualized by autoradiography. (B) Autoradiogram depicting the one-dimensional phosphopeptide map of recombinant synapsin IIa phosphorylated by endogenous cAMP-dependent protein kinase of Sf9 cells (lane 1) and of purified recombinant synapsin IIa phosphorylated in vitro by the catalytic subunit of cAMP-dependent protein kinase (lane 2).

of synapsin IIa in vitro (Figure 5A, lane 1). Addition of 10 μM 8-Br-cAMP further increased the ^{32}P incorporation into the protein about 3-fold above basal levels (Figure 5A, lane 2). An inhibitor of the catalytic subunit of cAMP-dependent protein kinase (Walsh inhibitor) prevented the basal phosphorylation (data not shown) of and the 8-Br-cAMP-stimulated increase in ^{32}P incorporation into synapsin IIa (Figure 5A, lane 3). Proteolytic digestion of the ^{32}P-labeled synapsin IIa with *S. aureus* V8 protease resulted in a one-dimensional

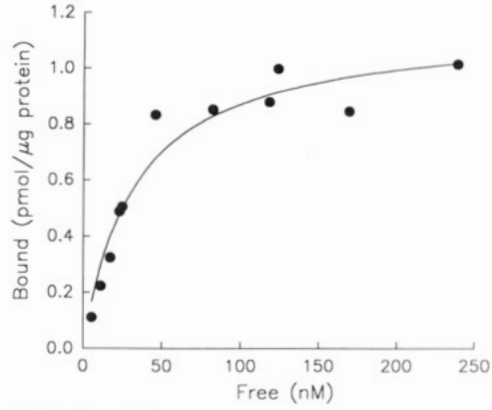

FIGURE 6: Binding of dephosphosynapsin IIa to synaptic vesicles as a function of synapsin IIa concentration. (A) Synaptic vesicles (5 μg of vesicle protein/sample) depleted of endogenous synapsins were incubated with various concentrations of synapsin IIa under standard conditions (40 mM NaCl equivalent) for 1 h at 4 °C. Synapsin IIa bound to vesicles was separated from free synapsin IIa by pelleting the vesicles through a 5% sucrose cushion. The amount of bound synapsin IIa was quantitated by dot immunobinding and then corrected for vesicle recovery. Free synapsin IIa was calculated by subtracting the corrected bound synapsin IIa value from the total synapsin IIa added to each sample. A nonlinear least-squares regression analysis was used to fit the data. The values obtained were as follows: K_d, 32 nM; B_{max}, 1.153 pmol/μg of vesicle protein.

phosphopeptide map identical to that of synapsin IIa phosphorylated in vitro by the catalytic subunit of cAMP-dependent protein kinase (Figure 5B, cf. Figure 4B; Huang et al., 1982; Grebb et al., 1989).

Binding of Synapsin IIa to Synaptic Vesicles. Synapsin IIa is localized on the cytoplasmic surface of small synaptic vesicles. Thus far, only the NH_2-terminal region of synapsin II has been shown to interact with synaptic vesicles (Thiel et al., 1990). To investigate the interaction of the entire synapsin IIa molecule with synaptic vesicles and to compare the binding properties of synapsin II and synapsin I, synaptic vesicles were purified from rat brain and salt-extracted to remove the endogenous synapsins. In vitro binding assays were carried out with recombinant synapsin IIa. As depicted in Figure 6, synapsin IIa bound with high affinity in a saturable manner to synaptic vesicles. Nonlinear regression analysis of the saturation curves gave a K_d of 32 nM and a B_{max} of 1.153 pmol/μg of vesicle protein. Similar binding isotherms had been described for synapsin I (Schiebler et al., 1986; Benfenati et al., 1989a).

Discussion

As part of our effort to elucidate the neurophysiological processes mediated by synapsins in the brain, we report here the purification and characterization of synapsin IIa, one of the neuron-specific phosphoproteins which coat the cytoplasmic surface of synaptic vesicles. The synapsin IIa described in this paper is a recombinant protein expressed in baculovirus-infected Sf9 cells. Immunological analyses established that the recombinant synapsin IIa is identical to the native form from rat brain.

The purified recombinant synapsin IIa is also an excellent substrate for cAMP-dependent protein kinase. It was phosphorylated to a stoichiometry of 0.8 mol of phosphate/mol of protein. The high stoichiometry of phosphorylation in vitro suggests that the purified recombinant synapsin IIa was essentially unphosphorylated. Consistent with this suggestion, in vivo metabolic labeling of Sf9 cells infected with the recombinant baculovirus revealed that only a minor portion of the synapsin IIa pool was phosphorylated in intact cells under

basal conditions. It has been shown by others that some proteins, such as the recombinant $p34^{cdc2}$-kinase (Furukawa et al., 1990), are also expressed in insect cells in unphosphorylated forms. There are, however, other phosphoproteins, e.g., the human retinoblastoma gene product (Wang et al., 1990), the cytoplasmic domain of the human insulin receptor (Herrera et al., 1988), chicken $p60^{c\text{-}src}$ (Morgan et al., 1989), and the rat glucocorticoid receptor (Alnemri et al., 1991), which are expressed in phosphorylated form.

Two pieces of data suggest that the small extent of synapsin IIa phosphorylation observed in the metabolic labeling studies is due to a cAMP-dependent protein kinase. First, the metabolically-labeled synapsin IIa was phosphorylated in the same region as that phosphorylated in vitro by cAMP-dependent protein kinase. Second, cAMP-dependent protein kinase activity was demonstrated in a homogenate of Sf9 cells using synapsin IIa as a substrate. The kinase activity was stimulated by 8-Br-cAMP and blocked by the highly specific inhibitory peptide Walsh inhibitor. To our knowledge, this is the first demonstration of cAMP-dependent protein kinase activity in Sf9 cells, although the kinase appears to be ubiquitously expressed in the animal kingdom (Kuo & Greengard, 1969), including *Drosophila* (Kalderon & Rubin, 1988).

From a functional standpoint, the synapsins are among the best characterized synaptic vesicle proteins. As has been shown for other synaptic vesicle proteins (Elferink et al., 1989; Knaus et al., 1990; Trimble et al., 1990; Geppert et al., 1991), the synapsins are present in different isoforms in the brain. Considerably more information is available for synapsin I than for synapsin II. Domains for binding of synapsin I to synaptic vesicles have already been identified (Benfenati et al., 1989a,b). The COOH-terminal region of synapsin I was shown to interact with the protein portion of the vesicles while the NH_2-terminal region was shown to interact with the lipid bilayer. In this study, synapsin IIa has been demonstrated to bind with high affinity and in a saturable manner to synaptic vesicles (Figure 6). The K_d and B_{max} for synapsin IIa binding to vesicles are almost identical to those for synapsin I (Schiebler et al., 1986). This suggests that synapsin IIa and synapsin I interact with synaptic vesicles in a similar manner. Since domains A, B, and C of synapsins IIa and I are highly homologous, it is likely that synapsin IIa also interacts with the lipid bilayer. Thiel et al. (1990) have demonstrated recently, using truncated synapsin II fusion proteins, that there may also be protein binding regions within the NH_2-terminal domain of synapsin II. The affinity of synapsin II for synaptic vesicle proteins relative to that for the lipid bilayer of the vesicles is currently being investigated. Whether the COOH-terminal region of synapsin II contains additional protein binding sites, analogous to synapsin I, remains to be studied.

Synapsin I can be classified as an amphitropic protein (Burn, 1988); i.e., it interacts with lipid bilayers but is a nonintegral membrane protein. Synapsin IIa is probably also an amphitropic protein as suggested by two pieces of evidence. First, in this study, subcellular fractionation showed that synapsin IIa was present mainly in the particulate fraction, and after purification it remained soluble in the absence of detergent. Second, the NH_2-terminal region of synapsin IIa is highly homologous with the corresponding region of synapsin I, which contains a lipid interaction domain.

Studies are now in progress, using recombinant synapsin IIa, to analyze its actin binding and actin bundling activities and its role in neurotransmitter release. The establishment of the baculovirus expression system should also enable a study of mutant forms of the synapsins. Experiments in which domains are interchanged between synapsin I and synapsin II should provide further information about the homologous and divergent domains of the synapsins and ultimately about the common and distinct functions of the four synapsin isoforms.

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Luminescent/Paramagnetic Probes for Detecting Order in Biological Assemblies: Transformation of Luminescent Probes into π-Radicals by Photochemical Reduction†

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ABSTRACT: The spectroscopic methods of fluorescence polarization and electron paramagnetic resonance (EPR) are used to study order and orientation of extrinsically labeled protein elements of ordered biological systems. These methods generate complementary information about the order of the system, but a consistent quantitative interpretation of the related data is complicated because the signals arise from different donors. We introduce a new method that allows us to detect both signals from the same donor. Unsubstituted xanthene dyes (eosin, erythrosin, and fluorescein) were irradiated by laser light at their absorption maximum in the presence of different reducing agents. Due to photochemical reduction, the quinoidal structure of the xanthene ring is transformed into a semiquinone, and a π-radical is formed having a characteristic EPR signal of an unpaired electron spin with proton hyperfine interactions. A strong EPR signal is observed from the dye in solution or when specifically attached to a protein following irradiation in the presence of dithiothreitol or cysteine. We applied this technique to the study of skeletal muscle fibers. The fluorescent dye (iodoacetamido)fluorescein was covalently attached to the reactive thiol of the myosin molecule in muscle fibers. Fluorescence polarization and EPR spectroscopy were performed on the labeled fibers in rigor. Both signals indicate a highly ordered system characteristic of cross-bridges bound to actin. Our use of the same signal donor for fluorescence and EPR studies of probe order is a promising new technique for the study of order in protein elements of biological assemblies.

The techniques of luminescence polarization and electron paramagnetic resonance (EPR) spectroscopy employing extrinsic probes are useful in quantitating orientation and order in biological assemblies because of their high sensitivity to differences or changes in probe orientation (Griffith & Jost, 1976; Arata & Shimizu, 1981; Morales et al., 1982; Gergely & Seidel, 1983; Vanderkooi & Berger, 1989). In the highly ordered skeletal muscle fiber system, two spatially separated points on the surface of the myosin cross-bridge, the reactive thiols SH1 and SH2, are routinely specifically modified by covalent probes of this type (Morales et al., 1982; Gergely & Seidel, 1983; Ajtai & Burghardt, 1989; Burghardt & Ajtai,

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