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Amino acid sequences surrounding the cAMP-dependent and calcium/calmodulin-dependent phosphorylation sites in rat and bovine synapsin I

(protein kinases/phosphoprotein/nervous system)

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ABSTRACT **The amino acid sequences surrounding three major phosphorylation sites in rat and bovine synapsin I have been determined by employing automated gas-phase sequencing and manual Edman degradation of purified phosphopeptide fragments. Site 1 is a serine residue phosphorylated by cAMP-dependent protein kinase and by calcium/calmodulin-dependent protein kinase I. The sequence around site 1 was derived from tryptic/chymotryptic phosphopeptides and overlapping cyanogen bromide cleavage fragments. This sequence, identical in rat and bovine synapsin I, is Asn-Tyr-Leu-Arg-Arg-Arg-Leu-Ser(*P*)-Asp-Ser-Asn-Phe-Met. Site 1 is located at the NH_2 terminus of the protein, within the collagenase-resistant head region. Sites 2 and 3 are serine residues phosphorylated by calcium/calmodulin-dependent protein kinase II. The sequences surrounding bovine site 2 and site 3 were derived from tryptic phosphopeptides and overlapping fragments generated by cleavage with chymotrypsin, collagenase, and endoproteinase Lys-C. The sequence around bovine site 2 is Thr-Arg-Gln-Thr-Ser(*P*)-Val-Ser-Gly-Gln-Ala-Pro-Pro-Lys, and the sequence around bovine site 3 is Thr-Arg-Gln-Ala-Ser(*P*)-Gln-Ala-Gly-Pro-Met-Pro-Arg. Sites 2 and 3 are located within the COOH-terminal, collagenase-sensitive tail region of the molecule, separated by 36 amino acids. The sequences surrounding rat site 2 and site 3 were derived from tryptic phosphopeptides. The sequence around rat site 2 is Gln-Ala-Ser(*P*)-Ile-Ser-Gly-Pro-Ala-Pro-Pro-Lys, and the sequence around rat site 3 is Gln-Ala-Ser(*P*)-Gln-Ala-Gly-Pro-Gly-Pro-Arg. Thus, the sequences surrounding the four sites that are phosphorylated by calcium/calmodulin-dependent protein kinase II, namely sites 2 and 3 in rat and bovine synapsin I, exhibit a high degree of homology.**

Synapsin I, a neuron-specific, synaptic-vesicle-associated phosphoprotein, is a major substrate for cAMP-dependent and calcium/calmodulin-dependent protein kinases (1–5). Phosphopeptide mapping of synaptosomal preparations and of the purified protein demonstrated that rat synapsin I was phosphorylated on serine residues at three major sites (6). Site 1, phosphorylated by cAMP-dependent protein kinase (cAMP kinase), is located in the collagenase-resistant head region of synapsin I. Site 1 is also phosphorylated by calcium/calmodulin-dependent protein kinase I [Ca^{2+}/CaM kinase I, ref. 7]. Sites 2 and 3, located in the collagenase-sensitive tail region of synapsin I, are phosphorylated by calcium/calmodulin-dependent protein kinase II [Ca^{2+}/CaM kinase II, refs. 6 and 8].

Synapsin I is believed to play an important role in synaptic vesicle function and neurotransmitter release (9, 10). Phosphorylation-dependent regulation of several effects of synapsin I has been reported (11–15). As part of our efforts to characterize the effects of phosphorylation on synapsin I function at the molecular level, in the present study we have determined the amino acid sequences surrounding the three major phosphorylation sites in rat and bovine synapsin I.

MATERIALS AND METHODS

Materials. Reagents were obtained from the following sources: [γ-^{32}P]ATP (New England Nuclear); chymotrypsin and L-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Cooper Biomedical, Malvern, PA); cellulose TLC plates (Merck); collagenase (Advanced Biofactures, Lynbrook, NY); cyanogen bromide (CNBr) and phenylisothiocyanate (Pierce); endoproteinases Arg-C and Lys-C and pyroglutamyl aminopeptidase (Boehringer Mannheim); *Staphylococcus aureus* V8 protease (Miles); Sephadex G-75 and Sephacryl S-200 (Pharmacia). Columns were purchased from the following sources: C_4 and C_8 reverse-phase (0.21 × 3 and 0.21 × 10 cm, Brownlee); C_{18} reverse-phase (0.45 × 25 cm, Vydac); Mono-S (5/10, Pharmacia); TSK-CM cellulose (0.75 × 15 cm, LKB); TSK-3000 SW (0.75 × 60 cm, Altex, Berkeley, CA). Purified samples of Ca^{2+}/CaM kinase I and the catalytic subunit of cAMP kinase were gifts of Angus Nairn and Atsuko Horiuchi (our laboratory). Purified calmodulin and Ca^{2+}/CaM kinase II were gifts of Yvonne Lai and Teresa McGuinness (our laboratory).

Purification of Synapsin I. Calf forebrain was homogenized as described (14) with a Polytron apparatus. Synapsin I was extracted using a modification of the acid-extraction procedure (16). After centrifugation at 13,700 × *g* for 30 min, the pellet was resuspended in 10 mM H_3PO_4, adjusted to pH 3.0 with 1 M HCl, and recentrifuged under the same conditions. The supernatant was adjusted to pH 6.0 with 1 M NaOH and recentrifuged, and the supernatant was adjusted to pH 8.0. Subsequent chromatography using CM-cellulose, hydroxylapatite, and Sephacryl S-200 was performed as described (14).

Rat brain synapsin I was prepared by homogenization [brain/buffer, 1:10 (wt/vol), final dilution] of 100 brains as described above, except for the use of a glass homogenizer and a motor-driven Teflon pestle. Subsequent steps were identical to those used in the preparation of bovine synapsin I, with an appropriate reduction in scale.

Phosphorylation of Synapsin I. Phosphorylation of bovine and rat synapsin I employed the incubation conditions described for the catalytic subunit of cAMP kinase (6), Ca^{2+}/CaM kinase I (7), and Ca^{2+}/CaM kinase II (8). The final concentrations of synapsin I and [γ-^{32}P]ATP were 5–10 μM and 80–100 μM, respectively. The determination of stoichiometry and two-dimensional phosphopeptide mapping were performed as described (6, 8).

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Abbreviation: Ca^{2+}/CaM, calcium/calmodulin-dependent.

Enzymatic and Chemical Cleavage of Synapsin I. The ^{32}P-labeled synapsin I was precipitated from the phosphorylation reaction mixture by addition of trichloroacetic acid prior to reduction and carboxymethylation with iodoacetic acid (17). Primary enzymatic digestions were performed with *S. aureus* V8 protease, chymotrypsin, or endoproteinase Arg-C in 100 mM NH_4HCO_3 at 37°C with an enzyme/substrate weight ratio of 1:200. Chymotryptic digests were incubated for 30 min; other digestions proceeded for 12–16 hr. Secondary cleavage was performed with endoproteinase Lys-C, trypsin, or a combination of trypsin and chymotrypsin, using the conditions described above for 5–12 hr. Secondary cleavage with collagenase was performed in 50 mM Tris·HCl, pH 7.3/10 mM $CaCl_2$ for 5 hr at 37°C. Chemical fragmentation of synapsin I was accomplished by incubation in the presence of a 1000-fold molar excess of CNBr in 70% (vol/vol) trifluoroacetic acid.

Purification of ^{32}P-Labeled Phosphopeptides. Cation-exchange chromatography was performed using Mono-S or TSK-CM columns with linear gradient elution. Gel filtration employed either Sephadex G-75 in 100 mM NH_4HCO_3 or TSK-3000 SW in 20 mM Tris·HCl, pH 7.4/1 mM EDTA/250 mM NaCl. Reverse-phase HPLC was performed with C_4, C_8, or C_{18} columns using linear gradient elution. Two HPLC solvent systems were used: system A was 0–100% acetonitrile (CH_3CN) in 0.1% trifluoroacetic acid; system B was 0–80% CH_3CN in 20 mM triethylamine acetate, pH 6. [Hereafter for CH_3CN, % (vol/vol) values will be simply percentages.] Peptide elution was monitored at 210 and 280 nm. Radioactivity of column fractions was monitored by Cerenkov counting.

Amino Acid Analysis. Amino acid analysis of the peptides was determined by HPLC of phenylthiocarbamyl amino acid derivatives after hydrolysis and derivatization with phenylisothiocyanate using the Waters Pico-Tag system (18).

Sequence Determination. Peptide sequences were determined using an Applied Biosystems (Foster City, CA) AB-470A gas-phase sequencer as described (19). Identification of phenylthiohydantoin amino acid derivatives was accomplished with C_{18} HPLC and was semiquantitative. Phosphorylated serine residues were identified by subtractive manual Edman degradation (20). Individual aliquots of the ^{32}P-labeled phosphopeptides were subjected to an increasing number of Edman cycles and analyzed for the release of $^{32}P_i$ by either C_{18} HPLC or by electrophoresis at pH 1.9 (21). The position of the phosphorylated serine residue is indicated for each peptide that was subjected to the manual sequencing analysis.

RESULTS AND DISCUSSION

Two-Dimensional Phosphopeptide Maps of Bovine and Rat Synapsin I. Phosphopeptide maps of bovine and rat synapsin I are displayed in Fig. 1 *A*, *C*, and *E*. Peptide 1 (6) was derived from bovine synapsin I that had been phosphorylated with cAMP kinase (Fig. 1*A*). Peptide 1 was also obtained when rat synapsin I was phosphorylated with cAMP kinase and when either rat or bovine synapsin I was phosphorylated with Ca^{2+}/CaM kinase I (refs. 6 and 7 and data not shown). A minor phosphopeptide, peptide 1′, was also observed in these maps (Fig. 1*A*). Peptides 2–5 (6) were derived from rat synapsin I phosphorylated by Ca^{2+}/CaM kinase II (Fig. 1*C*). It has been suggested that peptides 2 and 4 represent one phosphorylation site and that peptides 3 and 5 represent a second, distinct site (6). Phosphopeptides derived from bovine synapsin I phosphorylated by Ca^{2+}/CaM kinase II are shown in Fig. 1*E*. In Fig. 1 *B*, *D*, and *F*, maps of the purified phosphopeptide fragments used in the sequence analysis are shown.

Determination of the Amino Acid Sequence Around Site 1 in Bovine and Rat Synapsin I. A 5-mg aliquot of bovine synapsin I, phosphorylated to a stoichiometry of 0.9 mol of phosphate per mol of protein with the catalytic subunit of cAMP kinase, was digested with endoproteinase Arg-C. After Sephadex G-75 chromatography, the major peak (78% of the recovered radioactivity) was digested with trypsin and chymotrypsin. The phosphopeptides were separated by C_{18} HPLC in system A (10–25% CH_3CN gradient in 30 min at a flow rate of 1 ml/min). The major peak (73% of the recovered radioactivity), which eluted at 19% CH_3CN, was purified by C_{18} HPLC using isocratic elution at 12.5% CH_3CN (system A). This peptide, BTC-1, yielded the sequence Arg-Arg-Leu-Ser(*P*)-Asp-Ser-Asn-Phe (Fig. 2). Amino acid analysis of BTC-1 was consistent with the sequence data (Table 1). The migration of BTC-1 in two-dimensional peptide maps was identical to that of peptide 1′ (Fig. 1*B*).

Another 2-mg aliquot of ^{32}P-labeled bovine synapsin I was cleaved with CNBr, and the fragments were isolated using C_8 HPLC in system B (0–30% CH_3CN in 40 min at 0.25 ml/min). The major peak of radioactivity (65%), which eluted at 16.2% CH_3CN, was rechromatographed using C_4 HPLC in system A (5–30% CH_3CN in 50 min at 0.25 ml/min). A phosphopeptide, BCB-1, which eluted at 14.5% CH_3CN, yielded the sequence Asn-Tyr-Leu-Arg-Arg-Arg-Leu-Ser-Asp-Ser-Asn-Phe-Met (Table 1 and Fig. 2).

To determine the sequence around site 1 in the rat protein, 0.65 mg of ^{32}P-labeled rat synapsin I, phosphorylated with cAMP kinase to a stoichiometry of 0.9 mol of phosphate per

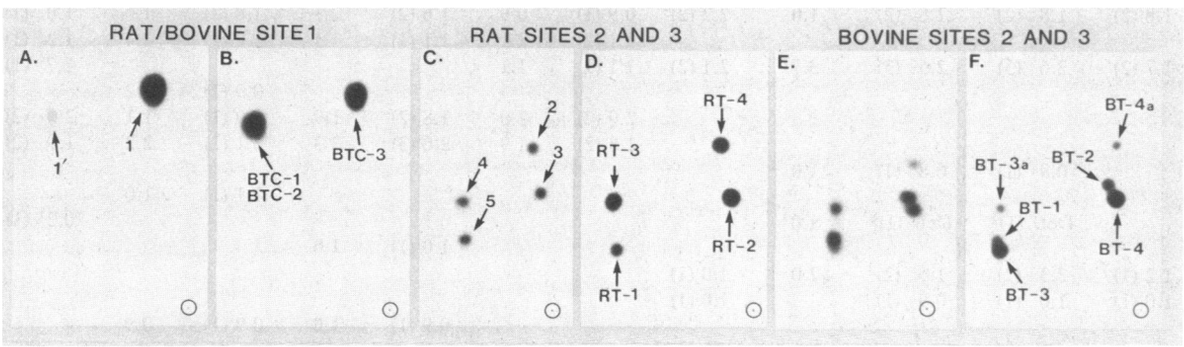

FIG. 1. Two-dimensional peptide mapping of rat and bovine synapsin I phosphorylation sites. (*A*, *C*, and *E*) Autoradiographs of phosphopeptides derived from limit tryptic/chymotryptic digestion of synapsin I. *A*, Bovine synapsin I phosphorylated with cAMP kinase; *C* and *E*, rat and bovine synapsin I, respectively, phosphorylated with Ca^{2+}/CaM kinase II. (*B*, *D*, and *F*) Autoradiographs of the purified phosphopeptides used in the sequence analysis. In *B*, the position of BTC-1 is also noted although not run on the same chromatograph. Electrophoresis was in the horizontal direction (cathode at the left). Ascending chromatography was in the vertical direction, using a solvent system of pyridine/acetic acid/water/1-butanol, 15:3:12:10 (vol/vol). ○, Origin.

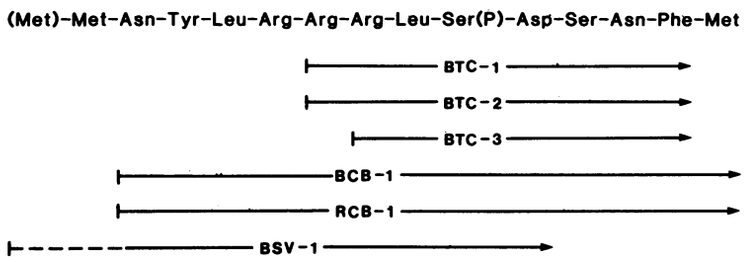

FIG. 2. Summary of sequence analysis around site 1. Sequences of rat and bovine peptides are indicated by the solid arrows. Results for peptide BSV-1 were determined after CNBr treatment; the position (---) of the methionine residue(s) was consistent with these results and with amino acid analysis.

mol of protein, was cleaved with CNBr, and the resultant phosphopeptide RCB-1 was purified as described for BCB-1. The sequence and amino acid composition of RCB-1 were identical to those of BCB-1 (Table 1 and Fig. 2).

Another peptide, BSV-1, derived from a *S. aureus* V8 protease digest of bovine synapsin I in which site 1 was not phosphorylated, proved to be related to the sequence surrounding this site. BSV-1 was purified by TSK-CM chromatography using 75–500 mM ammonium acetate (pH 4.6) followed by C_{18} HPLC in system A (17–20% CH_3CN in 60 min at 1 ml/min). The composition of BSV-1 was consistent with the site 1 sequence, with the presence of 1 or 2 additional methionine residues (Table 1). However, when sequencing was attempted, the NH_2 terminus of BSV-1 was found to be blocked. The filter was removed from the sequencer cartridge, suspended above a solution of CNBr [50 mg/ml in 70% (vol/vol) trifluoroacetic acid] for 36 hr in a 20-ml capped vial, and then returned to the sequencer. The following sequence for BSV-1 was then obtained: Asn-Tyr-Leu-Arg-Arg-Arg-Leu-Ser-Asp (Fig. 2). From these data we conclude that the methionine residue(s) was located at the NH_2 terminus of BSV-1, that the methionine at the NH_2 terminus of BSV-1 is blocked by an unknown moiety, and that the site 1 sequence shown in Fig. 2 is located at the NH_2 terminus of synapsin I. The failure to obtain NH_2-terminal sequence data with the native protein was consistent with these results (A.J.C., unpublished observations).

Identification of the Site 1 Serine Residue Phosphorylated by Ca^{2+}/CaM Kinase I. Since two serine residues were present in the site 1 phosphopeptide, we performed direct sequence analysis of ^{32}P-labeled phosphopeptides derived from bovine synapsin I that had been phosphorylated with Ca^{2+}/CaM kinase I. A 5-mg aliquot, phosphorylated to a stoichiometry of 0.9 mol of phosphate per mol of protein, was digested with endoproteinase Arg-C. After Sephadex G-75 chromatography, the major pool (79% of the recovered radioactivity) was digested with trypsin and chymotrypsin. Phosphopeptides were resolved by TSK-CM chromatography with 0–100 mM NaCl in 25 mM ammonium acetate (pH 4.6). Two peptides, which eluted at NaCl concentrations of 12 mM (BTC-3) and of 25 mM (BTC-2), contained 20% and 53% of the recovered radioactivity. Each peptide was purified using C_{18} HPLC in system A (5–35% CH_3CN in 30 min at 0.5 ml/min). Results of compositional and sequence analysis of BTC-2 were identical to those of BTC-1 (Table 1 and Fig. 2). The sequence of BTC-3 Arg-Leu-Ser(*P*)-Asp-Ser-Asn-Phe revealed it to be produced by tryptic cleavage at an alternative site within the sequence. BTC-3 migrates in the same position as peptide 1, the major phosphopeptide observed in limit digestion of synapsin I (Fig. 1 *A* and *B*). Manual Edman degradation of BTC-3 demonstrated that $^{32}P_i$ was released at cycle 3, confirming unequivocally that the serine residue phosphorylated by cAMP kinase is also phosphorylated by Ca^{2+}/CaM kinase I (data not shown).

Determination of the Amino Acid Sequence Around Sites 2 and 3 in Bovine and Rat Synapsin I. Aliquots of bovine (2.6 mg) and rat (1.7 mg) synapsin I were phosphorylated with Ca^{2+}/CaM kinase II to a stoichiometry of 1.7 and 1.8 mol of

Table 1. Amino acid composition of peptides derived from bovine and rat synapsin I

	Amino acid composition, mol of amino acid per mol of peptide												
	BTC-1	BCB-1	RCB-1	BSV-1*	BTC-2	RT-1	RT-2*	RT-3	RT-4*	BT-1	BT-2*	BT-3	BT-4*
Asx	2.2 (2)	1.5 (3)	1.8 (3)	2.1	2.0 (2)								
Glx						2.0 (2)	2.0	0.9 (1)	1.4	1.9 (2)	2.3	1.9 (2)	2.2
Ser	1.8 (2)	1.8 (2)	1.8 (2)	1.0	2.2 (2)	0.9 (1)	0.9	1.6 (2)	2.1	1.8 (2)	1.7	1.0 (1)	0.9
Gly						2.1 (2)	2.1	1.1 (1)	1.1	1.0 (1)	1.4	1.2 (1)	1.2
Arg	1.7 (2)	2.6 (3)	2.5 (3)	3.7†	2.1 (2)	1.1 (1)	1.1					1.7† (1)	1.5†
Thr										0.9 (1)	1.2		
Ala						2.0 (2)	2.0	1.8 (2)	1.9	1.0 (1)	1.1	2.0 (2)	2.0
Pro						1.8 (2)	1.9	2.6 (3)	2.3	2.1 (2)	2.2	1.9 (2)	1.9
Tyr		0.9 (1)	0.8 (1)	1.0									
Val										1.1 (1)	1.0		
Met		Det‡ (1)§	Det‡ (1)§	1.0†								0.2† (1)	0.2†
Ile								1.0 (1)	1.0				
Leu	1.2 (1)	2.3 (2)	1.9 (2)	2.0	1.0 (1)								
Phe	1.0 (1)	1.0 (1)	0.9 (1)		1.0 (1)								
Lys								0.9 (1)	1.0	0.9 (1)	0.9		

Column headings give peptide designations. Numbers in parentheses indicate the residues identified by sequence analysis.

*Peptide was not sequenced because of a blocked NH_2 terminus.

†A phenylthiocarbamoyl derivative of methionine sulfoxide was observed to co-elute with the phenylthiocarbamoyl derivative of arginine under the conditions employed (D. Atherton and A.J.C., unpublished observations).

‡Det, detected qualitatively as the phenylthiocarbamoyl derivative of homoserine.

§Identified as the phenylthiohydantoin derivative of homoserine; retention time was identical to that for the phenylthiohydantoin derivative of threonine.

phosphate per mol of protein, respectively, and then digested with chymotrypsin. The digests were chromatographed using C_4 HPLC in system A (12–26% CH_3CN in 28 min at 0.4 ml/min). Three major peaks, BC-1, BC-2, and BC-3, representing 50, 13, and 11% of the recovered radioactivity, were obtained for the rat synapsin I digest. These peptides, 20–25 kDa in size based on $NaDodSO_4$/PAGE, were pooled, digested with trypsin (enzyme/substrate weight ratio, 1:50), and chromatographed using C_{18} HPLC in system A (10–25% CH_3CN in 60 min at 1.0 ml/min). Four peaks, which eluted at 14.1 (RT-1), 14.6 (RT-2), 15.6 (RT-3), and 16.0% (RT-4) CH_3CN contained 23, 13, 24, and 15%, respectively, of the recovered radioactivity. Each peak was purified by C_8 HPLC in system B (0–20% CH_3CN in 20 min at 0.3 ml/min) followed by C_{18} HPLC in system A (12–30% CH_3CN in 18 min at 0.6 ml/min). Similar procedures resulted in the purification of the following four phosphopeptides derived from bovine synapsin I: BT-1, BT-2, BT-3, and BT-4.

Amino acid analysis revealed an interesting relationship among the peptides. The compositions of RT-1 and RT-2 were identical, as were the compositions of RT-3 and RT-4, of BT-1 and BT-2, and of BT-3 and BT-4 (Table 1). However, only RT-1, RT-3, BT-1, and BT-3 yielded sequence data; RT-2, RT-4, BT-2, and BT-4 were found to have blocked NH_2 termini. The sequence of RT-1 was Gln-Ala-Ser(*P*)-Ile-Ser-Gly-Pro-Ala-Pro-Pro-Lys; the sequence of RT-3 was Gln-Ala-Ser(*P*)-Gln-Ala-Gly-Pro-Gly-Pro-Arg. Homologous sequences were identified for the bovine peptides BT-1 [Gln-Thr-Ser(*P*)-Val-Ser-Gly-Gln-Ala-Pro-Pro-Lys] and BT-3 [Gln-Ala-Ser(*P*)-Gln-Ala-Gly-Pro-Met-Pro-Arg].

The presence of glutamine at the NH_2 termini of all four phosphopeptides suggested the possibility that cyclization of this residue to form a pyroglutamyl moiety during the purification and analysis of the peptides was responsible for the appearance, in each case, of a second peptide, identical in composition but with a blocked NH_2 terminus. The order of elution of the phosphopeptides from C_{18} HPLC supported this explanation. The retention time for RT-2, RT-4, BT-2, and BT-4 was greater than that for each related peptide possessing a free NH_2 terminus. The migration patterns of the purified phosphopeptides observed in peptide mapping experiments was also consistent with this explanation. Peptides RT-2, RT-4, BT-2, and BT-4 migrated at positions that were less basic and less polar than those for the related peptides. Minor phosphopeptides, BT-3a and BT-4a, were observed in the peptide maps of BT-3 and BT-4 and in the peptide maps of bovine synapsin I (Fig. 1 *E* and *F*). The appearance of BT-3a and BT-4a was variable and was likely due to the state of oxidation of the methionine residue present in the peptide. Final evidence to demonstrate the presence of a pyroglutamyl moiety at the NH_2 terminus was provided by the digestion of an aliquot of BT-4 with pyroglutamyl aminopeptidase (22). Approximately 25% of the starting material was converted to a different species, which eluted with a shorter retention time from C_{18} HPLC. Sequencing of this peptide was now possible and yielded the sequence Ala-Ser(*P*)-Glu-Ala-Gly-Pro-Met-Pro-Arg, identical to the sequence of BT-3, minus the cleaved NH_2-terminal pyroglutamyl residue.

The relative positions of sites 2 and 3 within the synapsin I molecule were established by sequence analysis of three larger bovine peptide fragments. The major 20-kDa chymotryptic fragment (BC-1) was isolated by Mono-S chromatography using 0–400 mM NaCl in 10 mM potassium phosphate (pH 6) followed by TSK-3000 SW chromatography and by C_4 HPLC in system A (16–26% CH_3CN in 40 min at 0.4 ml/min). An aliquot of BC-1 was digested with collagenase and then chromatographed using C_8 HPLC in system A (0–40% CH_3CN in 40 min at 0.4 ml/min). A phosphopeptide, BCC-1, which eluted at 17% CH_3CN, contained 44% of the recovered radioactivity. The peptide BVL-1 was derived by endoproteinase Lys-C digestion of a pool obtained after primary digestion of synapsin I with *S. aureus* V8 protease and Sephadex G-75 chromatography. BVL-1 was purified by C_{18} HPLC in system A (15–25% CH_3CN in 120 min at 1 ml/min) followed by TSK-CM chromatography using 0–500 mM NaCl in 50 mM ammonium acetate (pH 4.6). NH_2-terminal sequencing of BC-1, BCC-1, and BVL-1 yielded overlapping regions that identified 87 consecutive amino acids containing the sequences around sites 2 and 3 (Fig. 3). This region was localized to the COOH-terminal region of synapsin I, based on results with collagenase-derived fragments (6) and on the current finding that site 1 was located at the NH_2 terminus of the molecule. Based on their relative position in the primary structure, we designated the phosphorylated serine residue within the bovine sequence Gln-Thr-Ser(*P*)-Val-Ser and in the homologous rat sequence Gln-Ala-Ser(*P*)-Ile-Ser as site 2. The serine residue within the sequence Gln-Ala-Ser(*P*)-Gln-Ala, occurring in both bovine and rat synapsin I, was designated site 3. Thus, the sequences surrounding the four sites that are phosphorylated by Ca^{2+}/CaM kinase II, namely rat and bovine sites 2 and 3, exhibit a high degree of homology.

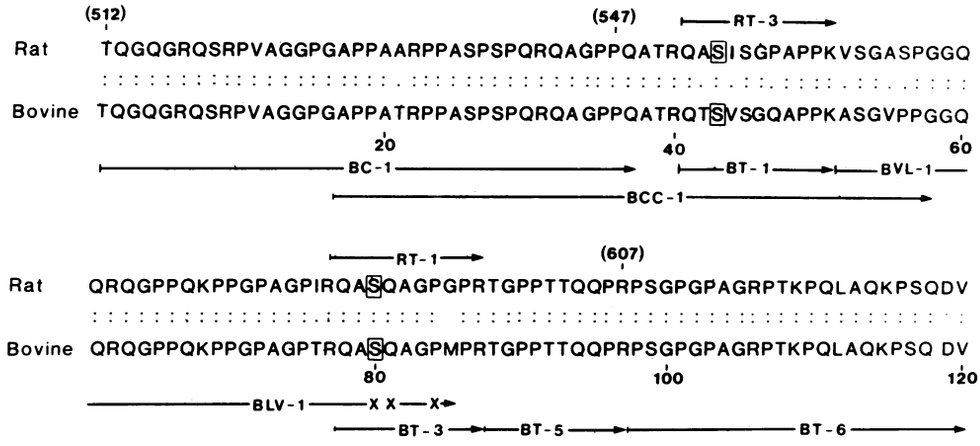

FIG. 3. Summary of sequence analyses around sites 2 and 3. Sequences of bovine peptides are indicated by the solid arrows. Unidentified residues are marked with an X. The sequence for the homologous region of rat synapsin I was derived by translation of our modification of the published pSyn5 nucleotide sequence (23). Sequences of the rat site 2 and site 3 phosphopeptides RT-1 and RT-3 are also shown. Exact homology between rat and bovine residues is indicated by a colon; conservative substitutions are indicated by a period. The phosphorylated serine residues are boxed. Numbers in parentheses refer to the position designated in the published pSyn5 amino acid sequence (23). Sequences are in the single-letter amino acid code.

A primary structure of synapsin I has been deduced (23) from the reported nucleotide sequence of the rat brain cDNA clone pSyn5. None of the phosphorylation sites identified in the present study by direct peptide sequencing were present within this deduced amino acid sequence, and none of the proposed phosphorylation sites were found in the present study. We have identified several factors that appear to account for these discrepancies. The failure to locate site 1 within the predicted pSyn5 amino acid sequence was due to the absence of the full-length protein coding sequence at the 5′ end of pSyn5. The base sequence AACTTC, located on the 5′ side of the proposed start site in pSyn5, together with the initiator codon ATG translate to the sequence Asn-Phe-Met, which is found at the COOH terminus of the site 1 phosphopeptide RCB-1. Thus, it appears that the 5′ end of pSyn5 lacks 33–36 nucleotides that encode for the NH_2-terminal sequence of synapsin I.

The failure to locate sites 2 and 3 within the predicted pSyn5 amino acid sequence was a consequence of three nucleotide sequencing errors, each involving the deletion of a cytidine residue, which resulted in frameshifts in the translation of the nucleotide sequence for 61 consecutive amino acids in the region surrounding the phosphorylation sites. Although the rat site 2 and 3 sequences were not present in the reported primary structure, translation of the pSyn5 nucleotide sequence in the two alternate reading frames revealed the presence of the RT-1 and RT-3 phosphopeptide sequences, with each reading frame containing one site. Based on the demonstrated homology between rat and bovine synapsin I, we were able to locate each frameshift in the pSyn5 translation by comparing (*i*) the sequences of the 87-amino acid bovine peptide described above and of two additional peptides (BT-5 and BT-6) found to be contiguous with this peptide with (*ii*) the amino acid sequences predicted by translating the pSyn5 base sequence in all three reading frames. Beginning at Thr-512, the pSyn5 rat protein sequence was identical to the NH_2-terminal region of the bovine peptide at 34 of the next 35 residues. Deletions occurred at nucleotides 1642–1645, 1724–1725, and 1827–1830. These deletions caused a shift from the correct reading frame into the second, then the third, and finally back into the original frame. Thus, the peptide sequence reported (23) from His-547 through Pro-607 is incorrect. With the inclusion of cytidine residues at the appropriate locations in the base sequence of pSyn5, the open reading frame was translated into an amino acid sequence that was identical to the bovine peptide sequence at 111 of 120 residues and contained the sequences of RT-1 and RT-3. A summary of these data is shown in Fig. 3, including our proposed modification of the rat synapsin I protein sequence for this region.

The phosphorylation sites in rat and bovine synapsin I possess the primary structural requirements described for two classes of protein kinases that have been studied in detail. The sequence around site 1 conforms to the well-characterized consensus sequence found in many substrates for cAMP kinase that is Arg-Arg-Xaa-Ser (24). The sequences around rat and bovine sites 2 and 3 conform to the consensus sequence Arg-Xaa-Yaa-Ser/Thr, which has been demonstrated to represent the minimal specificity determinant for Ca^{2+}/CaM kinase II (21), also known as the multifunctional calmodulin-dependent protein kinase (25). However, synapsin I is the best of the known substrates for these enzymes (5, 25), and other factors, such as higher-order structure, are obviously important in conferring to synapsin I its overall properties as a protein kinase substrate. The determination of the amino acid sequences surrounding the phosphorylation sites in synapsin I and the identification of their location within its primary structure should contribute to the further study of the phosphorylation-dependent regulation of synapsin I and its physiological significance.

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