Communication

Synapsin III, a Novel Synapsin with an Unusual Regulation by Ca\textsuperscript{2+}

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Synapsins I and II are synaptic vesicle proteins essential for normal Ca\textsuperscript{2+} regulation of neurotransmitter release. Synapsins are composed of combinations of common and variable sequences, with the central C-domain as the largest conserved domain. The C-domain is structurally homologous to ATPases, suggesting that synapsins function as ATP-dependent phosphotransfer enzymes. We have now identified an unanticipated third synapsin gene that is also expressed in brain. The product of this gene, synapsin IIIa, shares with synapsins Ia and Iia three conserved domains that are connected by variable sequences: the phosphorylated A-domain at the amino terminus, the large ATP-binding C-domain in the center, and the E-domain at the carboxyl terminus. Like other synapsins, synapsin IIIa binds ATP with high affinity and ADP with a lower affinity, consistent with a cycle of ATP binding and hydrolysis. ATP binding to the different synapsins is directly regulated by Ca\textsuperscript{2+} in a dramatically different fashion: Ca\textsuperscript{2+} activates ATP binding to synapsin I, has no effect on synapsin II, and inhibits synapsin III. Thus vertebrates express three distinct synapsins that utilize ATP but are specialized for different modes of direct Ca\textsuperscript{2+} regulation in synaptic function.

Synapsins are abundant phosphoproteins of synaptic vesicles (1). Two genes for synapsins were described, the synapsin I gene, which produces alternatively spliced transcripts encoding synapsins Ia and Iib (2). Sequence comparisons revealed that synapsins are composed of a mosaic of domains. All synapsins share a short amino-terminal domain (A-domain) that is phosphorylated by CaM kinase I and protein kinase A, a linker sequence (B-domain) that is rich in short-chain amino acids (proline/alanine/glycine/serine), and a large central domain (C-domain) that comprises approximately one-half of the total synapsin sequences. After the C-domain, different combinations of domains are observed (the D-, E-, F-, and G-domains). Interestingly, at the very carboxyl terminus synapsins Ia and Iia contain an additional short common domain of 50 residues (the E-domain) despite distinct sequences between the C- and E-domains (3). The E-domain is absent from synapsins Ib and IIb. A synapsin homologue was described in Drosophila in which the C- and E-domains are the only conserved domains, suggesting that these domains are responsible for the function of synapsins (3, 4).

Synapsins have been studied extensively both biochemically and genetically (5–15). Although a large number of synapsin functions have been proposed, their precise biological activity has proved elusive. Based on the analysis of mouse knockouts, currently the best defined role of synapsins exists in the regulation of synaptic vesicle function in mature nerve terminals (16–18). Synapsins I and II are phosphorylated at the amino terminus during stimulation, but the physiological consequences of this phosphorylation are unknown. Synapsins interact with a number of proteins with high affinity and stoichiometry (e.g. actin filaments, neurofilaments, microtubules, calmodulin, spectrin, annexin VI; Refs. 5–15); however, it seems unlikely that synapsins will bind to all of these proteins in vivo, and the biological significance of these interactions is unclear.

Recently the crystal structure of the C-domain of synapsin I was solved, giving unexpected insights into its function (19). The structure showed that the C-domain is an independently folding domain that forms a stable dimer. Data bank searches revealed that the C-domain is structurally closely related to five ATP-utilizing enzymes: glutathione synthetase, d-alanine: D-alanine ligase, biotin carboxylase o-chain, succinyl-CoA synthetase O-chain, and pyruvate,orthophosphate dikinase. More than 80% of the Ca carbon atoms of the synapsin I C-domain can be superimposed on those of glutathione synthetase or d-alanine:D-alanine ligase with a root mean square deviation of 0.32 nm, suggesting a close structural and evolutionary similarity between these enzymes and synapsins (19). The enzymes to which synapsin I is structurally related bind ATP and transfer phosphate from bound ATP to a substrate (20, 21). Synapsins also bind ATP with high affinity and ADP with a lower affinity, suggesting that synapsins also represent phosphotransfer enzymes (19, 22). Surprisingly, ATP binding to the C-domains of synapsins I and II was found to be differentially regulated: ATP binding to synapsin I required Ca\textsuperscript{2+} which was not necessary for ATP binding to synapsin II.

We now report that a third unanticipated synapsin gene is expressed in vertebrates. Synapsin IIIa is closely homologous to synapsins Ia and Iia, with the highest similarity observed in the A-, C-, and E-domains. As in the other synapsins, the C-domain of synapsin IIIa binds ATP with high affinity. Different from synapsins I and II, however, Ca\textsuperscript{2+} inhibits ATP binding to synapsin IIIa at micromolar concentrations. As a consequence, Ca\textsuperscript{2+} has distinct regulatory effects on the three synapsins: it activates ATP binding in synapsin I, the most abundant synapsin; it is without effect with synapsin II; and it inhibits ATP binding to synapsin IIIa, the least abundant synapsin. Our data suggest an unexpectedly direct and diverse regulatory role of Ca\textsuperscript{2+} in a family of synaptic vesicle phosphoproteins.

**EXPERIMENTAL PROCEDURES**

cDNA Cloning of Rat Synapsin III—Data bank searches with the rat synapsin I and II sequences (2) revealed that sequences from cosmids...
performed in 75 mM Hepes-NaOH, pH 7.0, 25 mM NaCl, and 4 mM
performed essentially as described above except that all steps were
ined on the same gel.
by SDS-polyacrylamide gel electrophoresis and Coomassie staining,
combinant proteins were purified using standard techniques, analyzed
rat synapsin II), and pGEXrSynIII-C (residues 89–399 of rat synapsin
pGEX constructs were used in the current study: pGEXrSynI-C (resi-
dues 110–420 of rat synapsin I), pGEXrSynII-C (residues 113–421 of

**RESULTS**

*Identification of Synapsin IIIa—* Data bank searches with
synapsin sequences uncovered exonic sequences from a novel,
unidentified synapsin gene on chromosome 22. These
sequences were highly homologous to those of synapsins I and II
and exhibited the same exon-intron structure as the synapsin I
gene (23). Nevertheless, two reasons led us to conclude that the
chromosome 22 gene encodes a novel gene, named here synap-
sin III. Firstly, its predicted sequences differ at several posi-
tions at which glutamate residues coordinate Ca2+
C-domain of synapsin I are marked by

**Domain Structure of Synapsins IIIa—** Alignment of the syn-
apsin Ia, IIa, and IIIa sequences reveals a similar domain
organization in all three synapsins. The sequence of the amino-
terminal A-domain is nearly identical between synapsins and
includes a phosphorylation site for CaM kinase I and CAMP-dependent protein kinase present in all
three synapsins is indicated by an

sequence that is similar to the “a” variants of synapsins I and II (Fig. 1).

**Structure and Regulation of Synapsin IIIa**

N104C7, N28H9, E86D10, and N80H12 from human chromosome
22q11.2-22q12-qter (accession numbers Z71183, Z80902, Z82181, and
Z82246) contained multiple exons of a gene highly homologous to syn-
apsins I and II. A 0.24-kilobase fragment of the corresponding cDNA
was cloned by PCR1 from human first-strand brain cDNA (obtained from
CLONTECH) and used as a probe for screening rat brain cDNA
libraries (2, 23). Multiple overlapping clones were isolated and se-
quenced. Data bank analyses were performed using DNA-Star and
BLAST softwares. The synapsin III sequence was submitted to Gen-
BankTM (accession number AF056704).

**Northern Blotting—** RNA blotting experiments were performed using
multiple rat tissue blots obtained from CLONTECH and a rat synapsin
III probe corresponding to amino acid residues 440–526.

**Construction of Expression Vectors and Expression of Recombinant
Proteins—** Synapsin expression vectors in pGEX-KG (24) with C-domain
sequences were obtained by PCR with oligonucleotide primers contain-
ing flanking restriction sites essentially as described (22). The following
pGEX constructs were used in the current study: pGEXrSynI-C (resi-
dues 110–420 of rat synapsin I), pGEXrSynII-C (residues 113–421 of
rat synapsin II), and pGEXrSynIII-C (residues 89–399 of rat synapsin
III), together with control plasmids described previously (22). All re-
combinate proteins were purified using standard techniques, analyzed
by SDS-polyacrylamide gel electrophoresis and Coomassie staining,
and quantified using known amounts of bovine serum albumin exam-
ined on the same gel.

**ATPγS Binding Measurement—** Purified GST-fusion proteins in-
mobilized on glutathione-agarose beads were washed 3× with buffer A
(50 mM Hepes-NaOH, pH 7.4, 25 mM NaCl) containing either 2 mM
EGTA or 2.1 mM calcium or 2 mM EDTA. Aliquots of the beads (5 pmol
of recombinant protein) were used in 0.1-ml binding assays containing
buffer A with 10 mM ATPγS and the indicated addition of nucleotides,
Ca2+, Mg2+, and/or chelator. After a 1-h incubation at room tempera-
ture, beads were washed 3× in the incubation buffer without ATPγS, and
the radioactivity bound to the beads was determined by scintilla-
tion counting. For determination of the Ca2+ concentration dependence
of ATP binding, Ca2+/EGTA buffers were used. Binding assays were
performed essentially as described above except that all steps were
performed in 75 mM Hepes-NaOH, pH 7.0, 25 mM NaCl, and 4 mM
EGTA. The concentration of free Ca2+ was calculated using the Chela-
tor program.

2 The abbreviations used are: PCR, polymerase chain reaction; GST,
glutathione S-transferase; EST, expressed sequence tags; ATPγS,
adenosine 5'-[γ-thio]triphosphate.
domain (the C-domain) is flanked by small amino- and carboxyl-terminal domains that are also very homologous. These conserved domains are connected to each other by linker sequences rich in prolines, glutamines, alanines, serines, and glycines, short side-chain amino acids characteristic of non-structured protein regions. Together with the crystallographic data characterizing the C-domain as an independently folding dimeric module, these data suggest that the central C-domain of synapsins is linked to the two other domains by flexible loops.

Tissue Distribution of Synapsin IIIa Expression—To test which tissues express synapsin IIIa, an RNA blot was probed with a specific probe. A single band corresponding to the right size was detected in brain, indicating that synapsin IIIa is expressed primarily in brain similar to other synapsins (Fig. 2).

ATP Binding by Synapsin III—The crystal structure of the C-domain of synapsin I and biochemical studies with the C-domains of synapsins I and II revealed that the C-domain constitutes an ATP-binding module related to several ATPases with functions as phosphotransfer enzymes (19, 22). The C-domain of synapsin III is highly homologous to that of the other synapsins, and most of the residues involved in ATP binding are conserved. This suggests that synapsin III may also be an ATP-utilizing protein. To test this, we measured ATPγ35S binding to the recombinant C-domain of synapsin III. ATPγ35S bound specifically and was displaced by ATP and, to a lesser degree, GTP (data not shown). To test the relative affinity of ATP and ADP, we performed displacement titrations of bound ATPγ35S with cold ATP or ADP. The half-maximal concentrations needed for competition were approximately 0.5 µM ATP and 12 µM ADP (Fig. 3), suggesting that as in other synapsins, ATP is bound much more tightly than ADP. The half-maximal displacement concentrations indicate that synapsin III is a high-affinity ATP-binding protein, although its ATP affinity is slightly lower than that of the other synapsins. The difference in affinity for ATP and ADP is consistent with a catalytic role of the C-domain in which ADP would be dissociated by cellular ATP after bound ATP was hydrolyzed. A catalytic role as an enzyme of unknown specificity for synapsin III is also supported by the fact that the sequence in synapsins that corresponds to the catalytic loop in the structurally related enzymes is highly conserved, including the position of a catalytically active lysine residue (underlined in Fig. 1).

Regulation of ATP Binding by Ca2+—Previous studies showed that ATPγS binding to the C-domain of synapsin I or to full-length synapsin I required Ca2+ whereas binding was Ca2+-independent for synapsin II (22). Surprisingly, Ca2+ inhibited ATPγS binding to the C-domain of synapsin III despite its high degree of sequence homology to synapsins I and II and despite their similar ATP affinity (data not shown). The inhibition of ATPγS binding to synapsin III was not an artifact caused by Ca2+-triggered proteolysis because analysis of pro-

![Fig. 2. Tissue distribution of synapsin III expression analyzed by RNA blotting. A blot containing poly(A)+-enriched RNA from the indicated rat tissues was hybridized with a 32P-labeled probe from synapsin IIIa. Numbers on the left indicate positions of size markers. The 2.0-kilobase signal observed in testis (asterisk) is probably an artifact because it is too small to encode a synapsin and is observed with many unrelated probes.](image)

![Fig. 3. Relative ATP and ADP affinities of the C-domain of synapsin III. ATPγ35S (10 nM) binding to immobilized GST-synapsin III or GST alone was carried out in the presence of increasing concentrations of free Ca2+ using Ca2+/EGTA buffers. Binding data were fitted to a single binding site, resulting in the half-maximal inhibition constants (EC50 values) shown adjacent to the binding curves. Data shown are means ± S.E. from triplicate determinations of a representative experiment repeated multiple times.](image)
teins after the ATP incubations showed that Ca\(^{2+}\) did not change the amount of protein left (data not shown). These data suggest that despite their close similarity, synapsins I, II, and III are specialized for different types of synaptic regulation. Synapsin I is activated by Ca\(^{2+}\), synapsin II is not affected by Ca\(^{2+}\), and synapsin III is inhibited by Ca\(^{2+}\).

To test whether the regulation of synapsins by Ca\(^{2+}\) occurred at physiologically meaningful concentrations, we measured the Ca\(^{2+}\) concentration dependence of ATP\(\gamma\)S binding (Fig. 4). All three synapsin C-domains were compared in the same experiment. Ca\(^{2+}\) had no effect on ATP binding to synapsin II but regulated ATP binding to synapsins I and III with Ca\(^{2+}\) concentration dependences that were mirror image patterns. Whereas Ca\(^{2+}\)-activated ATP binding to synapsin I with a half-maximal activation concentration (EC\(_{50}\)) of \(\sim 2 \mu M\) free Ca\(^{2+}\), it inhibited ATP binding to synapsin III with an almost identical EC\(_{50}\) of \(\sim 3 \mu M\) free Ca\(^{2+}\). These data demonstrate that synapsins are similar to each other in structure and ATP binding but diverge dramatically in regulation.

**DISCUSSION**

Synapsins I and II are abundant peripheral membrane proteins of synaptic vesicles that are phosphorylated in nerve terminals (reviewed in Refs. 1 and 25). Although synapsins bind to a number of proteins, the physiological functions of synapsins are unclear. Knockouts revealed that synapsins are essential for synaptic regulation but are not required for the establishment of synapses, clustering of vesicles, or long term synaptic plasticity (16, 17). The crystal structure of the C-domain of synapsin I uncovered an unexpected structural homology to a special class of ATPases (19). These ATPases transfer active phosphates to substrates and mediate ligations or syntheses of small molecules. In agreement with the possibility that synapsins belong to these ATPases, the C-domains of synapsins I and II bind ATP and, with much lower affinity, ADP. In addition, the flexible loop in the ATPases that is catalytically active is conserved in synapsins.

The current data suggests that the function of synapsins in nerve terminal regulation may be more complex and interesting than previously envisioned. With the discovery of synapsin III, we now show that the synapsin family is larger than anticipated. The sequence of synapsin III allowed a further definition of the domain structure of synapsins, reinforcing and refining the previous model (2). The sequence alignment confirms the role of the central C-domain of synapsins as their largest, conserved domain that presumably carries the activity of the protein. All C-domains of synapsins constitute high affinity ATP-binding modules that may function as phosphotransfer enzymes. In addition, the alignment demonstrates that the amino-terminal phosphorylation domain (the A-domain) is a fixture in synapsins. The fact that this phosphorylation domain is so highly conserved makes the necessity to elucidate the physiological importance of the amino-terminal phosphorylation site even more important. Finally, the structure also confirms the role of the carboxyl-terminal domain (the E-domain) as a constant part of synapsins, again of unknown function. The sequence alignment also highlights the high content of proline, glutamine, serine, and alanine residues in the two variable regions linking the three conserved domains. These variable regions are not conserved between synapsins, and the phosphorylation sites in synapsin I in these sequences are also not conserved. Nevertheless, these variable sequences are characterized by similar amino acid compositions in all synapsins.

Our most surprising result, however, was the divergence in Ca\(^{2+}\) regulation of synapsins. Analysis of the synapsin I and II knockouts suggested a physiological role for synapsins in short term regulation of neurotransmitter release which is Ca\(^{2+}\)-dependent (16, 17). Therefore a direct regulation of synapsins by Ca\(^{2+}\) would agree very well with their essential functions. However, the fact that the three synapsins, despite their high homology and similar ATP affinity, are differentially regulated was unexpected. Synapsin I is Ca\(^{2+}\)-activated, synapsin II Ca\(^{2+}\) independent, and synapsin III Ca\(^{2+}\)-inhibited. For synapsins I and III, Ca\(^{2+}\) acts at concentrations that are similar and physiologically relevant. These data suggest that whatever the enzymatic function may be that is being performed by synapsins in nerve terminals, this function responds differently to increases in nerve terminal Ca\(^{2+}\) during an action potential. Such a difference in Ca\(^{2+}\) regulation for members of a closely related family of proteins is unprecedented and suggests that the three synapsin isoforms specifically evolved to allow different types of synaptic Ca\(^{2+}\) regulation.

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