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Localization of Membrane Binding

Domains in Synapsin I

by

Mahmoud Salkhordeh, B. Sc. (Hons.)

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment
of the requirements for the degree of
Masters of Science

Department of Biology

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In partial fulfillment of the requirements for the degree of Masters of Science

Chair, Department of Biology

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Carleton University
2000

ABSTRACT

Communication between neurons is mediated by the controlled release of neurotransmitters. Several proteins are involved in the regulation of neurotransmitter release. The synapsins constitute a family of synaptic vesicle-associated phosphoproteins essential for regulating neurotransmitter release and synaptogenesis. The molecular mechanisms underlying the specific targeting of synapsin I to synaptic vesicles are thought to involve specific protein-protein interactions, while the high affinity binding to the synaptic vesicle membrane may involve both protein-protein and protein-lipid interactions. The highly hydrophobic NH₂-terminal region of the protein has been shown to bind with high affinity to the acidic phospholipids phosphatidylserine and phosphatidylinositol and to penetrate the hydrophobic core of the lipid bilayer.

There is currently some debate over the roles of the A and C domains of synapsin I in binding the protein to synaptic vesicles. To resolve the binding functions of these domains, fragments of synapsin Ia, which encode the amino acid residues 1-29 (domain A) and / or residues 110-420 (domain C) were expressed in *E. coli* and purified. Liposome binding assays revealed that both domains can bind to acidic phospholipid-containing liposomes, albeit less well than full length synapsin. These results imply that the A and C domain both contribute to the tight binding of synapsin I to membranes.

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LIST OF ABBREVIATIONS

BCA	Bichromic acid
CaMKII	Ca ²⁺ /Calmodulin Kinase II
DOPC (S)	Dioleoyl phosphotidyl choline (serine)
DTT	Dithiotheritol
ECL	Enhanced chemiluminescent
EDTA	Ethelynediamine tetraactic acid
GST	Glutathione S-transferase
IPTG	Isopropyl α -D-thiogalacto-pyranoside
LB	Liquid broth
PCR	Polymerase chain Reaction
PKA	Protein kinase A
PKC	Protein kinase C
SSPE	Sodium chloride sodium phosphate-EDTA
SV	Synaptic vesicle
SYN I	Synapsin I
TE	Tris-EDTA
U	Units

1.0 INTRODUCTION

Neurons communicate with each other predominantly through the controlled release of neurotransmitters from synaptic vesicles. Numerous proteins within presynaptic nerve terminals are thought to participate in the regulation of the synaptic vesicle life cycle (Figure 1.1). An important task in understanding neurotransmitter release is to define the roles of these proteins.

The synapsins are a family of proteins that are located at presynaptic nerve terminals, where they are bound to the cytoplasmic surface of small synaptic vesicles (Figure 1.1) (De Camilli *et al.* 1983; De Camilli *et al.* 1983). Synapsins were first identified almost 30 years ago, as major substrates for cAMP-dependent protein kinase (PKA) (Johnson *et al.* 1971). Synapsins are the most abundant proteins on synaptic vesicles and account for approximately 9% of the total vesicle-associated protein (Bahler and Greengard 1987; Benfenati *et al.* 1989).

Synapsin I interacts *in vitro* with lipid and protein components of synaptic vesicles and with various cytoskeletal proteins, including actin (Bahler and Greengard 1987; Benfenati *et al.* 1989; Benfenati *et al.* 1989). These and other studies have led to a model in which synapsin I, by tethering synaptic vesicles to each other and to an actin-based cytoskeletal meshwork, maintains a reserve pool of vesicles away from the active zone (Figure 1.1). Perturbation of synapsin I function in a variety of preparations led to a selective disruption of this reserve pool and to an increase in synaptic depression, suggesting that the synapsin I-dependent clustering of vesicles is required to sustain release of neurotransmitter in response to high levels of neuronal activity.

1.1 Synapsin isoforms

The synapsins are a multigene family and occur in three major forms in vertebrates (synapsin I, synapsin II, and synapsin III). The synapsin I gene produces RNA transcripts that are alternatively spliced to yield synapsins Ia and Ib. The mRNAs encoding synapsin Ia and Ib are identical in nucleotide sequence at the 5' end of the coding region. The difference is in the 3' end of the coding region, where the synapsin Ia mRNA contains a 38-nucleotide insertion that is absent in the synapsin Ib mRNA. The last intron of the synapsin I gene contains two splice-acceptor sites. The inclusion or exclusion of the 38-nucleotide insertion depends on which acceptor site is utilized. The 38-nucleotide insertion results in a frameshift in the nucleotide sequence producing two different carboxyl-terminal amino acid sequences for synapsin Ia and Ib (Sudhof *et al.* 1989; Sudhof 1990).

The synapsin II gene is expressed and spliced to yield synapsins IIa and IIb (Sudhof *et al.* 1989; Sudhof 1990). The synapsin III gene, expresses RNA which is spliced to produce transcripts which can encode at least six synapsin III isoforms (Porton *et al.* 1999).

In the rat, the mRNAs encode proteins of 706 and 668 amino acid residues for synapsins Ia and Ib, respectively (Sudhof *et al.* 1989). The apparent molecular weights of rat synapsins Ia and Ib on SDS-PAGE are 86,000 and 80,000, respectively (Sieghart *et al.* 1978).

DNA blotting revealed that synapsins IIa and IIb are also produced from a common synapsin II primary transcript by differential splicing (Chin *et al.* 1994; Li *et al.*

1995). In this case, the mRNAs encode proteins of 586 and 479 amino acid residues for synapsins IIa and IIb, respectively (Sudhof *et al.* 1989).

The synapsin III gene can give rise to up to six transcripts (synapsins IIIa-III f). This unexpectedly large number of transcripts may be due to gene size. The intron sizes within the synapsin genes vary largely. The synapsin III gene spans 400,000 nucleotides, whereas the synapsin I gene spans only 78,000 nucleotides. This difference may help to explain the large number of transcripts of the synapsin III gene. The probability that new exons and promoters exist within introns is increased in the larger intronic sequences in the synapsin III gene locus (Porton *et al.* 1999).

Synapsin III gene expression differs from expression of synapsins I and II in the number of isoforms and tissue-and developmental-specific expression. While transcripts derived from synapsins I and II are expressed predominantly in adult brain, synapsin III transcripts are expressed differently. Of the six transcripts, three (synapsins IIIa, IIIb, and IIIc) are expressed in fetal and adult brains, synapsin IIId is expressed in fetal brain, and two synapsins (IIIe and III f) are expressed only in non-neuronal tissue. These differences suggest that the synapsin III gene may function in ways that are distinct from synapsins I and II (Porton *et al.* 1999).

Figure 1.1

a) Cartoon illustrating various synaptic vesicle proteins and their interactions with the vesicle membrane and with other vesicle proteins. **b)** Role of synapsin I in the synaptic vesicle cycle. When an action potential reaches the nerve terminal, voltage-gated calcium channels open, allowing a rapid influx of calcium ions, which activate several processes. One such process is the fusion of synaptic vesicles with the plasma membrane, which releases neurotransmitter. Calcium also binds to calmodulin, which in turn activates several protein kinases such as CaM kinase II. CaM kinase II phosphorylates a variety of substrates including synapsin I (which is the best substrate for CaM kinase II so far). The phosphorylation of synapsin I by CaM kinase II occurs rapidly and to high stoichiometry during physiological activity of neurons. Phosphorylation by CaM kinase II causes a conformational change in the synapsin I molecule, and is associated with major changes in its biological properties. Upon phosphorylation of synapsin I, vesicles can be released from actin filaments and move from a non-releasable pool of vesicles into a releasable pool (Cheetham, *et al.*, unpublished).

a)

b)

1.2 Synapsin Expression and Localization

Both synapsins I, and II are highly concentrated at presynaptic nerve terminals, and are associated with the cytoplasmic surface of synaptic vesicles (De Camilli *et al.* 1983; De Camilli *et al.* 1983). The average concentration of synapsin I in the presynaptic nerve terminal is approximately 10-30 μM and it has been estimated that 10-30 synapsin I molecules are present on a single synaptic vesicle, suggesting that they cover a large portion of the synaptic vesicle surface (Schiebler *et al.* 1986; De Camilli *et al.* 1990). Synapsin III, the most recently discovered member of the synapsin family is present in human, mouse, rat, and *Xenopus* brain (Hosaka and Sudhof 1998; Kao *et al.* 1998). Synapsin III is also associated with synaptic vesicles, but at a much lower concentration than synapsins I and II (Kao *et al.* 1998).

1.3 Homodimerization and Heterodimerization of the Synapsins

Synapsins form homo-and heterodimers with variable efficiency. Hosaka and Sudhof, (1999) showed that all synapsins strongly homodimerize also, synapsin II strongly heterodimerize with synapsin I and III, but synapsin I and III interacted only weakly (Hosaka and Sudhof 1999). X-ray crystallography revealed that domain C of synapsin I dimerizes in the presence or absence of ATP (Figure 1.4) (Esser *et al.* 1998). The contact surface between the two subunits in the dimer is very large, leading to a strong interaction. They suggested that the synapsins coat the vesicle surface as dimers. Therefore, up to 13 synapsin dimers could be formed by the five different synapsins, suggesting that this family of synaptic vesicle proteins is functionally more variable than was previously thought.

1.4 Synapsin Domain Structure

Amino acid sequence alignments reveal that synapsins are composed of a mosaic of domains (Sudhof *et al.* 1989; Kao *et al.* 1999). The N-terminal and central domains are shared between all synapsins while the C-terminal domains are different between synapsins. All vertebrate synapsins contain a short amino-terminal domain (A-domain), which has a CaM kinase I and a PKA phosphorylation site (Czemik *et al.* 1987). C-terminal of the A domain, rich in short-chain amino acids (proline/ alanine/ glycine/ serine) linker sequence (B-domain) is found. Next, a large central domain that constitutes about one-half of the total synapsin amino acid sequence is located (C-domain). The C-domain is followed by different combinations of domains (the D, E, F, G, H, I, and J-domains). Synapsins Ia, IIa, and IIIa share a short common domain of 50 residues (E-domain) at their C-terminus. Synapsins Ib and IIb lack the E-domain (Figure 1.2) (Kao *et al.* 1999).

Domain C is the largest and most conserved domain in the synapsin family. Domain C is both hydrophobic (39% M, A, L, V, F, and W residues) and highly charged (27% D, E, K, and R residues (Sudhof *et al.* 1989). Specific regions of domain C are important for the high-affinity binding of synapsins to the lipid bilayer of synaptic vesicles (Benfenati *et al.* 1989; Benfenati *et al.* 1989; Cheetham *et al.* 2000). In addition, distinct regions within this domain are responsible for the binding of synapsins to actin filaments (Bahler and Greengard 1987; Bahler *et al.* 1989). Amino acid sequence analyses revealed over 50% identity between vertebrate and invertebrate synapsin C domains, implying a crucial role for this domain in many of the conserved functions of the synapsins (Hilfiker *et al.* 1999; Kao *et al.* 1999).

Domain A, which has a phosphorylation site for PKA, CaM kinase I, and CaM kinase IV, is highly conserved in vertebrates but not in invertebrates. Domain E is equally conserved, especially the last 18 amino acids, in both vertebrates and invertebrates (Kao *et al.* 1999). While the function of domain A has not been fully defined yet, some evidence indicates a specific role for domain E in the clustering of synaptic vesicles (Hilfiker *et al.* 1998). A synapsin homologue has also been described in *Drosophila* in which the C- and E-domains are the only conserved domains, also suggesting that these domains are important for the function of synapsins (Klagges *et al.* 1996). Analysis of the primary structure of synapsin III indicates that the most conserved domains are A, C, and E, when compared with either synapsin I or synapsin II (Kao *et al.* 1998).

1.5 Synapsin gene structure

In humans, the structures of the synapsin I and III genes are very similar: they contain 13 exons, with 12 introns in almost the same location (Kao *et al.* 1998). The first exon in synapsin I encodes domains A, B, and a small part of C, while the last exon encodes domain E. The synapsin II gene may have the same structure, since the positions of the first three introns are almost the same as those found in synapsins I and III. Synapsin IIIa contains exons 1-13, and is the largest protein isoform. Synapsins IIIb and IIIc lack exons 11, and exon 12 respectively, resulting in a reading frame shift, and early stop codon, and the absence of domain E. Synapsin IIId contains the first five exons and a previous unidentified exon resulting in a protein containing domains A, B, the first 148 amino acids of domain C, and 13 residues of new sequences. Synapsins IIIe and IIIf

contain no new sequence information and represent proteins containing the last 30 amino acids of domain C, domain J, and in the case of IIIe, domain E (Porton *et al.* 1999).

The similarity of intron locations in these synapsin genes suggests that they are originated by gene duplication from a common ancestor (Kao *et al.* 1999).

The exact chromosomal locations of the genes for human and mouse synapsins have been identified (Yang-Feng *et al.* 1986; Li *et al.* 1995). Human and mouse synapsin I map to the X chromosome, synapsin II maps to 3p25 and 6f, respectively, and synapsin III maps to 22q12.1 and 10, respectively.

Figure 1.2

A domain model for the synapsin family of proteins in humans. The A domain is shaded in dark pink, the B domain in green, the C domain in yellow, D domain in pink, E in orange, F in turquoise, G in brown, H in purple, I in dark green and J in light pink. Phosphorylation sites are indicated by the letter P. Green represents sites phosphorylated by the cAMP-dependent protein kinase, protein kinase A (PKA); blue sites are phosphorylated by mitogen-activated protein kinases (MAP kinases); and red sites are phosphorylated by calcium/calmodulin-dependent protein kinase II. The scale at the bottom of the figure represents amino acid residue number. (Cheetham *et al.*, unpublished).

1.6 Molecular Evolution of the Synapsin Family of Proteins

Invertebrates contain a single synapsin gene, while most vertebrates possess at least three synapsin genes. This difference in gene number suggests that gene duplication may have occurred when vertebrates branched from invertebrates. Only one synapsin gene exists in *C. elegans*, and so far, one in squid and one in *Drosophila*. Lamprey contains two synapsin genes. Higher vertebrates, such as frogs and mammals have three synapsin genes (Kao *et al.* 1999). From the observation that there is only one synapsin gene in the common ancestor of the higher vertebrates, Kao *et al.*, 1999 hypothesized that there were at least two duplication events: one duplication event would have separated synapsin III from the rest, and the other would have separated synapsin I and II. These duplication events would have occurred from 500 million years ago (the date of divergence of Agnathans from other chordates) to 400 million years ago (the date of divergence of Amphibia from other higher vertebrates) (Kao *et al.* 1999).

1.7 Synapsin I and Neurotransmitter release

Neurotransmitter release occurs by exocytosis, through which a quantum of transmitter stored in small synaptic vesicles is released. Synaptic vesicles are the key organelle in this process, and undergo a local cycle at the presynaptic terminal. A large number of the total synaptic vesicle within a presynaptic terminal, called the reserve pool, is clustered away from the active zone (Greengard *et al.* 1993). A small portion of vesicles, called the releasable pool, is docked to the plasma membrane. Some of the docked vesicles undergo a series of priming reactions, which make them competent for fusion. Upon stimulation, an action potential is produced and Ca^{2+} moves into the cell

through voltage-gated Ca^{2+} channels. In response to Ca^{2+} influx, some of the docked vesicles fuse with the plasma membrane and release their contents into the synaptic cleft. The vesicle is then retrieved from the plasma membrane by endocytosis and rejoins to the reserve pool (Heuser and Reese 1973; Takei *et al.* 1995).

Synaptic vesicle cycle regulation experiences plasticity. For example, one possible mechanism by which nerve cells regulate the amount of neurotransmitter release is thought to be by changing the dynamic equilibrium between the reserve and the releasable pool of vesicles at the nerve terminals. Therefore, proteins involved in tethering the synaptic vesicles in reserve pool are excellent candidates as regulators of synaptic transmission.

1.8 Other Roles of synapsins

Several experiments have revealed distinct roles for synapsin I and synapsin II during neuronal development. Synapsin I plays an important role in axonal elongation and branching in hippocampal neurons while synapsin II plays an important role in the initial elongation of undifferentiated processes (Chin *et al.* 1995; Ferreira *et al.* 2000). The high level of synapsin III expression during the early stages of development may indicate a distinct role for synapsin III, different from the other members of the synapsin family where the levels of synapsin I and synapsin II correlate with the extent of synapse formation (Ferreira *et al.* 2000). According to Kao *et al.* (1999) in both vertebrates and invertebrates, such as the squid giant axon, gold fish Mauthner neurons, and isolated mammalian synaptosomes, synapsins have been shown to regulate neurotransmitter release. Injection of exogenous dephospho-synapsin I into the various pre-synaptic nerve terminals led to blockage of neurotransmitter releases (Llinas *et al.* 1985). In fact,

exogenous synapsin I inhibited synaptic transmission by recruiting vesicles from the releasable pool back in to the reserve pool, without interfering in the release process itself (Llinas *et al.* 1985). Therefore, disruption of synapsin function may increase the number of vesicles available for release, leading to increase in neurotransmitter release.

Mice bearing a homozygous knockout in the gene for synapsin I, synapsin II or double knockouts exhibited abnormal neurotransmission. Mice lacking synapsins were viable but suffered from impaired presynaptic functions and a high incident of seizures. Biochemically, levels of synaptic vesicle proteins were reduced significantly in double knockouts, and vesicle numbers decreased by 50% in the double knockouts. Thus synapsins appear to be selectively required for the acceleration of synaptic vesicle traffic during repetitive stimulation at physiological frequencies (Rosahl *et al.* 1995). Based on these results, it was proposed that synapsins perform a regulatory function in increasing the supply of fusion-competent synaptic vesicles at the active zone during conditions of accelerated vesicle traffic. Synapsin function could occur either in the movement of vesicles to the active zone, their docking at the active zone, or their maturation after docking to a fusion-competent state (Rosahl *et al.* 1995).

Synapsin I is the most surface-active protein known to date (Ho *et al.* 1991). It can contribute to the maintenance of vesicle integrity and size uniformity, by stabilizing phospholipids in the bilayer arrangement. It also prevents random fusion events (Benfenati 1993; Benfenati *et al.* 1993). Some unusual physico-chemical properties of synapsin I may be responsible for features of the secretory process in neurons, such as: (a) the formation of synaptic vesicle clusters close to the active sites, which minimizes vesicle diffusion and prevents random fusion with the plasma

membrane (Hirokawa *et al.* 1989); (b) the very uniform size of synaptic vesicles (40-50 nm in diameter) (Valtorta *et al.* 1992); (c) the existence of a reserve pool of synaptic vesicles away from the active site (more than 95% of total vesicles in certain terminals) which are unavailable for neurotransmitter release (Greengard *et al.* 1993); (d) the decrease in the Ca^{2+} -independent, spontaneous fusion of the vesicles, induced by the exogenous synapsin I, with the presynaptic membrane (Llinas *et al.* 1985); (e) the dissociation of synapsin I from cytoskeleton, such as actin filaments, and synaptic vesicles upon phosphorylation by CaM kinase II (Schiebler *et al.* 1986; Sihra *et al.* 1989; Bahler *et al.* 1990).

From these studies a model was suggested that synapsin I tethers synaptic vesicles to an actin-based cytoskeletal meshwork and to each other, making a reserve pool of vesicles away from the fusion site. In support of this model, it has been shown that anti-synapsin antibodies injected into lamprey neurons, and that synapsin proteins injected into the giant axon of squid, caused pre-synaptic vesicle clusters to disappear (Pieribone *et al.* 1995; Hilfiker *et al.* 1998). There is some evidence that synapsins regulate the formation of new nerve terminals, both in vertebrates and invertebrates. For example, introduction of synapsin IIb in differentiated NG108-15 cells, a neuroblastoma X glioma hybrid clonal cell line, caused the formation of more nerve terminals in each cell, more synaptic vesicles, as well as synapse-like cell-cell connections (Han and Greengard 1994). In addition, introduction of synapsin I or II into embryonic *Xenopus* neurons increased electrophysiological features of synaptogenesis (Schaeffer *et al.* 1994). Whereas inhibition of synapsin II expression caused inhibited axon elongation and the failure to form or maintain synapses in hippocampal neurons (Ferreira *et al.* 1995). Also,

mice having a homozygous knockout of the synapsin genes (I, and II, or both) show abnormalities in synapse number and morphology of nerve terminals (Ferreira *et al.* 1998).

1.9 Interactions of Synapsins with Other Proteins

Synapsin I possesses at least two distinct actin-binding sites that allow synapsin I to bundle actin *in vitro* (Bahler *et al.* 1989). This formation of actin bundles is regulated by phosphorylation (Baines and Bennett 1986). There is no direct evidence available that synapsin III interacts with actin but its high degree of homology with the actin-binding domain of synapsin I suggests that synapsin III may also have a role in the organization of actin (Kao *et al.* 1998). A distinct region within the domain C is responsible for the binding of synapsin I to actin filaments. The affinity of synapsin-actin interaction is about 100-fold lower ($K_d = 1\text{-}2\ \mu\text{M}$) than the affinity of synapsin I for synaptic vesicles (Bahler *et al.* 1989; Benfenati *et al.* 1993).

Synapsin I also interacts with microtubules ($K_d = 5\ \mu\text{M}$) (Baines and Bennett 1986). Nerve terminals contain these protein components suggesting that these interactions might occur *in vivo* (Hilfiker *et al.* 1999). Although synapsins bind to a number of proteins, their functions have remained to be solved.

Synapsins are primarily localized on synaptic vesicles, as was shown by immunoelectronmicroscopy (Navone *et al.* 1984). Synapsins have one or more binding partners on the vesicle membrane that specifically target synapsins to vesicle membranes. So far, two protein kinases have been identified as synapsins' partners on the vesicles. Synapsin I was shown to interact to a vesicle-associated form of CaM kinase II (Benfenati *et al.* 1992) as well as a vesicle-associated form of c-src (Onofri *et al.* 2000).

Table 1.1

Synapsin isoforms identified in various species

Species	Isoforms	Size	Source	Reference:
Human	Ia Ib IIa IIb IIIa	627 aa 614 aa 718 aa	Human Human Human	(Sudhof <i>et al.</i> 1989; Sudhof 1990)
Rat	Ia Ib IIa IIb IIIa	704 aa 668 aa 586 aa 479 aa 750 aa	Rat brain Rat brain Norway rat Norway rat Norway rat	(Sudhof <i>et al.</i> 1989; Sudhof 1990)
Mouse	Ib IIb IIIa	1231 aa 796 aa 653 aa	House mouse House mouse House mouse	(Kao <i>et al.</i> 1999)
Bovine	Ia Ib	706 aa 760 aa	Cow brain Cow brain	(Sudhof <i>et al.</i> 1989; Sudhof 1990)
Squid				
<i>Drosophila</i>	I II	1320 aa 1120 aa	Fruit fly Fruit fly	(Klagges <i>et al.</i> 1996)
<i>C. elegans</i>	Ia	404 aa	<i>Caenorhabditis elegans</i>	(Kao <i>et al.</i> 1999)
<i>Canis familiaris</i>	I	1580 aa	Dog	(Kao <i>et al.</i> 1999)
<i>Xenopus laevis</i>	I IIa IIIa	428 aa 885 aa 684 aa	African clawed frog African clawed frog African Clawed frog	(Kao <i>et al.</i> 1999)
<i>Lampetra fluviatilis</i>	Ia Ib IIa IIb	702 aa 699 aa 732 aa 1020 aa	European river lamprey	(Kao <i>et al.</i> 1999)

Table 1.2 Synapsin binding proteins

Associated protein	Synapsin isoforms	Reference:
Actin filaments	Synapsin I	(Benfenati <i>et al.</i> 1989)
Microtubules	Synapsin I	(Baines and Bennett 1986)
Spectrin	Synapsin I	(Baines and Bennett 1985)
SH3 domain of Grb2	Synapsin I	(Onofri <i>et al.</i> 2000)
SH3 domain of c-Src	Synapsin I	(Onofri <i>et al.</i> 2000)
Phospholipase C	Synapsin I	(Onofri <i>et al.</i> 2000)
P85 subunit of phosphatidylinositol-3 kinase	Synapsin I	(Onofri <i>et al.</i> 2000)
SH3 domain of amphiphysins I/II	Synapsin I	(Onofri <i>et al.</i> 2000)
SH3 domain of Crk	Synapsin I	(Onofri <i>et al.</i> 2000)
NADPH oxidase factor p47phox	Synapsin I	(Onofri <i>et al.</i> 2000)
SH3 domain of p21 ras GTPase activating protein	Synapsin I	(Onofri <i>et al.</i> 2000)

1.10 Synapsins as enzymes

The crystal structure of the C-domain of synapsin I revealed that it is structurally similar to ATP-dependent synthetases, suggesting an enzymatic activity for synapsins (Esser *et al.* 1998). The crystal structure revealed that the C-domain independently folds to form a stable dimer. In addition, it showed that the C-domain is structurally closely related to five ATP-utilizing enzymes: glutathione synthetase, D-alanine:D-alanine ligase, biotin carboxylase α -actin, succinyl-CoA synthetase *beta*-chain, and pyruvate orthophosphate dikinase (Esser *et al.* 1998). More than 80% of the α -carbon atoms of the C-domain of synapsin I can be superimposed on those of glutathione synthetase or D-alanine : D-alanine ligase, indicating a close similarity between these enzymes and synapsins (Esser *et al.* 1998).

Synapsins also bind to ATP with high affinity and to ADP with a lower affinity, indicating that synapsins may represent phosphotransfer enzymes (Hosaka and Sudhof 1998). On the other hand, the studies have not yet identified an enzyme activity related to synapsins, which remains to be demonstrated. Purified synapsin I did not hydrolyze ATP at a detectable rate, indicating that synapsins are not constitutively active ATPases (Hilfiker *et al.* 1999). ATP hydrolysis might depend on the proper localization and substrate binding of synapsin. Also, the enzymatic activity might depend on the proper multimeric state of synapsins.

ATP binding to the C-domains of the synapsins is differentially regulated. Ca^{2+} is necessary for ATP binding to synapsin I. It is not required for ATP binding to synapsin II. Ca^{2+} inhibits ATP binding to synapsin IIIa at μM concentrations. As a result, Ca^{2+} increases ATP binding to synapsin I, but has no effect on ATP binding to synapsin II, and

inhibits ATP binding to synapsin iIIa, the least abundant synapsin isoform (Hosaka and Sudhof 1998; Hosaka and Sudhof 1998).

1.11 Synapsin Phosphorylation Sites

The phosphorylation sites have been best characterized for mammalian synapsin I. At least seven phosphorylation sites (sites 1-7) have been identified for synapsin I: site 1 (Ser 9) is a substrate for PKA, CaM kinase I, and CaM kinase IV (Czernik *et al.* 1987); sites 4-6 (Ser 62, Ser 67, and Ser 551) are substrates for MAP (Matsubara *et al.* 1996); and sites 7 (Ser 553) is a substrate for cdk5 (Matsubara *et al.* 1996). Site 6 is a substrate for cdk1 (Hall *et al.* 1990) cdk2, and cdk5 (Matsubara *et al.* 1996). The only phosphorylation site for synapsin II is Ser 10 in domain A, a substrate for PKA, CaM kinase I, and CaM kinase IV (Czernik *et al.* 1987). The phosphorylation site of synapsin III has not been fully characterized. The most probable site is site 1 in domain A (Kao *et al.* 1999).

The modulation of synaptic vesicle release by synapsins involves a common phosphorylation event. The phosphorylation site at the N-terminus in all synapsins is therefore a candidate for such a modulatory function. In fact, an unexpected biological role for the N-terminal phosphorylation site (site 1) was identified by showing that the A domain of synapsins posses a phospholipid-binding domain whose activity is regulated by phosphorylation (Hosaka *et al.* 1999). Surprisingly, the common phosphorylation site for PKA and CaM kinase I in synapsins, which triggers their dissociation from synaptic vesicles is located in the middle of the A domain, which mediates binding to phospholipids. DNA sequencing of the A domain showed that three positively charged arginine residues at the N-terminus precedes the phosphorylation site. The

phosphorylation of the serine residue may modulate the interaction of the A domain with phospholipid bilayer, therefore triggers the dissociation of the synapsin from phospholipid (Hosaka *et al.* 1999).

1.12 Synapsin-Phospholipid interactions

The binding of synapsin I to synaptic phospholipid vesicles is saturable, reversible and exhibits an absolute requirement of acidic phospholipids, namely phosphatidyl serine and / or phosphatidylinositol (Benfenati *et al.* 1989; Benfenati *et al.* 1989). This interaction occurs in two steps: an initial electrostatic interaction with the negatively charged head groups on the surface of the membrane, followed by penetration of the hydrophobic regions of the head domain of synapsin I into the hydrophobic core of the bilayer (Cheetham *et al.* 2000).

The mechanisms that attach synapsins to synaptic vesicles remained to be determined. So far, the most accepted hypothesis is that synapsins interact with vesicles through an interaction of its variable carboxyl-terminal domains with CaM kinase II, which also phosphorylates these domains (Benfenati *et al.* 1992). This interaction may mediate some of the association of synapsin I with vesicles, but it is not the major mechanism because there is not much CaM kinase II present on synaptic vesicles. Most cellular CaM kinase II is localized at postsynaptic densities, which do not contain synapsins. In addition, synapsin II does not bind CaM kinase II but is still targeted to synaptic vesicles. Furthermore, the abundance of synapsins in presynaptic nerve terminal suggests that they are not stably bound to any vesicle protein components since few other vesicle proteins are present at a similar concentration. Instead synapsins are targeted to

vesicles by a dynamic mechanism so that, once on synaptic vesicles, synapsins become attached to them by a nonspecific mechanism (Benfenati *et al.* 1993).

A study by Hosaka *et al.* (1999) discovered that the major synapsin binding to synaptic vesicles depends on domain A. This discovery suggests that this domain of synapsin besides the large central C-domain, attaches synapsins to synaptic vesicles. They suggest that the attachment of synapsins by the short N-terminal A domain places their large C domains onto the vesicle membrane, followed by the shorter variable domains. The important thing is that not only does a short 29 residue mediate the interaction but it also has a regulatory role in this interaction. Currently, there is no other similar short phospholipid-binding domain regulated by phosphorylation, indicating that this is a novel mechanism that needs a minimal sequence and phosphorylation at a single site to initiate the translocation of a large protein (Hosaka *et al.* 1999).

A major question from these studies is how synapsins are specifically targeted to synaptic vesicles. The protein phospholipid binding by the A domain only keeps synapsins on the vesicle after a specific targeting protein has placed them there. This interaction cannot explain synapsins targeting to synaptic vesicles since there is little specificity in the phospholipid-binding reaction. Therefore, synapsin targeting to synaptic vesicles involves a specific protein, whose nature and mechanism of action remains to be elucidated (Hosaka *et al.* 1999).

The synapsin-induced cross-linking of vesicles with each other is mediated either by the existence of multiple phospholipid binding sites in one synapsin molecule or by self-association of two synapsin molecules, each having a phospholipid-binding site. Some evidences suggest that both hypotheses may be true (Benfenati 1993; Benfenati *et*

al. 1993). For example, several residues predicted to form amphiphilic secondary structures with the potential to bind to phospholipid membranes exist within synapsins' head region (Ho *et al.* 1991). On the other hand, synapsin I has been shown to have a high tendency to self-associate via its head region (Hosaka and Sudhof 1999).

1.13 Question and hypothesis

In this study the question: which domains in synapsin I are required for binding to phospholipid membranes, was addressed. It was hypothesized that the A and C domain of synapsin I mediate binding to phospholipid membranes.

Figure 1.3

Location of the membrane penetrating CNBr fragments of synapsin I in the three-dimensional structure of the bovine synapsin I domain C and domain C dimer. The image of the structural model of domain C was generated from the synapsin I domain C coordinates (1AUV) downloaded from the Brookhaven protein database:

(<http://www.rcsb.org/pdb/cgi/explore.cgi?pdbId=1AUV>) and imported into RasMol

2.7.1.. The CNBr fragments identified using hydrophobic photoaffinity labeling are indicated in the figure in yellow (amino acids 166-192), green (amino acids 233-258) and purple (amino acids 278-327) (Cheetham *et al.* 2000).

2.0 MATERIALS AND METHODS

The pET15b-synapsin Ia (full length) construct was a kind gift from Dr. E. Horichi, The Rockefeller University, New York, NY. GST (Glutathione S-transferase) fusion proteins were produced by cloning the corresponding cDNA sequences of interest into an appropriate pGEX plasmid (Pharmacia Biotech). The proteins were then expressed as fusion proteins at the 3'-end with the GST protein. The methods for making fusion proteins included several steps which are described below. Synapsin I, purified from bovine brain, was provided by Dr. James J. Cheetham.

The genotypes of the bacteria used in this thesis are:

DH5 α F⁻, phi80dlacZdeltaM15, *recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17* (*r_K*⁻, *m_K*⁺), *supE44*, *deoR*, *relA*, *delta(lacZYA⁻argF)* U169, *lamda*⁻.

JM103 *endA1*, *sbcBC*, *thi-1*, *rspL* (*Str*^r), *delta(lac-pro)* (F⁻ *traD36*, *lacI^qZdeltaM15*, *proAB*⁻), *supE*, (*p₁*), (*r_K*⁺, *m_K*⁺, *r_{pi}*⁺, *m_{pi}*⁺).

BL21 (DE3) F⁻, *ompT*, (*lon*), *hsdS_B* (*r_B*⁻, *m_B*⁻; an *E. coli* B strain) with DE3, a lambda prophage carrying the T7 RNA polymerase gene.

2.1 Polymerase Chain Reaction (PCR)

A sequence of cDNA encoding residues 110-420 of rat synapsin Ia from the pET15b-synapsin Ia (0.2 μ g / μ L) construct (E. Horichi, The Rockefeller University, New York, NY) was amplified by polymerase chain reaction (PCR). The amplification of the DNA fragment was performed in an Amplitron II thermolyne apparatus, by adding the following reagents to a 0.2 mL thin-walled tube:

2 μ L 2 mM dNTP (Gibco BRL), 10 μ L of 10 X PCR buffer (Gibco BRL), 10 μ L

of each of the primers flanking the region to be amplified (synthesized by University of Ottawa, Molecular Biology Service), 0.5 μ L of the template DNA molecule 0.2 μ g / L (pET15b synapsin Ia), 3 μ L of 50 mM $MgCl_2$ (Gibco BRL), 0.5 μ L Taq DNA polymerase (500 U, 5 U / μ L) (Gibco BRL), and d.d H_2O to the final volume of 100 μ L.

The reaction was performed with a 95 °C, 5 minute pre-dwell temperature followed by incubation at three temperatures: strand denaturation at 94 °C, primer annealing at 50 °C, and primer extension at 72 °C, typically all for 1 minute. The reaction was stopped with a post-dwell temperature of 72 °C for 5 minutes (Sambrook *et al.* 1989). After PCR, aliquots of the mixture were loaded onto an agarose gel containing ethidium bromide and subjected to electrophoresis to detect amplified products. In some instances where the product was impure, the reaction was repeated with an increased annealing temperature (increasing the annealing temperature prevented the non-specific binding of the primers to the DNA templates). The PCR products were then purified.

2.2 Purification of PCR Fragments for Cloning

About 150 μ L of Direct Purification Buffer (50 mM KCL, 10mM Tris-HCL pH 8.8, 1.5 mM $MgCl_2$, 0.1% Triton X-100) and up to 300 μ L of PCR product were vortex mixed 3 times within one minute (Promega, Technical Bulletin). One mL of PCR Preps DNA Purification Resin (Promega) was added to the mixture, it was vortex mixed 3 times, then subjected to vacuum filtration. The sample was washed with 2 mL of 80% isopropanol, then centrifuged (10000 g for 2 minutes) to evaporate residual alcohol. DNA samples were solubilized in 50 μ L of TE buffer (10 mM Tris-HCL, pH 7.5, 1mM EDTA) and pelleted (Eppendorf centrifuge, 20 seconds). DNA was stored at -20° C.

2.3 Primer design

Two primers were used that flanked the ends of the DNA coding for amino acid residues 110-420 of rat synapsin Ia from the pET15b-synapsin Ia construct. The forward primer was, 5'-GCC AGT **CGA CTG CTG GTC ATC GA**-3' containing a restriction site for *Sal* I and the reverse primer was, 5'-CCC GCT **CGA GAG GCA GAG CCT GAG**-3' containing a restriction site for *Xho* I. The primers were designed using Oligo (Oligo Software Inc.).

2.4 Restriction Digestion

Restriction enzyme digests were performed by incubating double-stranded DNA with an appropriate amount of restriction enzyme, in buffers as recommended by the supplier, at the optimal temperature for those enzymes. Both PCR products and pGEX-4T-1 (500 µL / mL) cloning vector (Pharmacia) were cut with *Sal* I and *Xho* I restriction enzymes. Since these two enzymes are not compatible, (*Sal* I digests in buff. # 10, *Xho* I digests in buffer # 2 with 100% efficiency) (Gibco BRL), the digestion was performed on two successive days (first day with *Sal* I, and second day with *Xho* I restriction enzymes) followed by purification of the digested fragments. A typical restriction digestion contained the following reagents in a microcentrifuge tube: 8 µL of PCR product (0.3 µg / µL), 4 µL of buffer #2, (Gibco BRL), 26 µL of d.d H₂O, and 2 µL of *Sal* I (15 U / µL, Gibco BRL) resulting in a total volume of 40 µL. The reaction was mixed gently, and incubated for 4 hours at 37 °C. Aliquots of the digests were analyzed on agarose gels and remaining DNA was kept at -20 °C.

2. 5. Purification of restriction digest products (Sambrook *et al.* 1989).

To check the efficiency of digestion, aliquots of digested PCR mixtures were analyzed on agarose gels. The remainder of the reaction mixtures was purified and was concentrated by ethanol precipitation as follows: the reaction volume was increased from 40 μ L to 300 μ L by adding TE buffer then, 1 mL of 95 % ethanol containing 0.16 M Na acetate was added. The reaction mixture was kept at -20 °C overnight. It was then centrifuged (18,000 rpm for 15 minutes, 4 °C, Eppendorf centrifuge), the supernatant was removed completely. The DNA was washed with 70% ethanol, and was subjected to a vacuum for 30 minutes to remove residual ethanol. The pellet was resuspended in 25 μ L of TE buffer.

2.6 Ligation of PCR products into the cloning vector pGEX-4T-1

After digestion with *Sal* I and *Xho* I restriction enzymes, PCR products and pGEX-4T-1 plasmid contained staggered ends (Sambrook *et al.* 1989). A typical ligation product had the following composition. The reaction volume was 10 μ L: Two μ L of pGEX-4T-1 (500 μ L / mL) (Pharmacia), 6 μ L (0.6 μ g) of PCR product, 1 μ L ligase buffer (Gibco BRL), and 1 μ L d.d H₂O. The reaction mixture was gently mixed, centrifuged (Eppendorf centrifuge, 20 seconds, room temperature), kept at 60 °C for 1 minute to separate the double strand DNA, and was immediately placed in ice. Then 1 μ L of DNA ligase (100 U, 1U / μ L) was added to the mixture. The reaction mixture was kept at 4 °C for 12-17 hours.

2.7 Transformation of *E. coli* DH5 α bacteria

Transformation of *E. coli* DH5 α bacteria was done by electroporation (Sambrook *et al.* 1989). Competent cells (section 2.8) were thawed on ice. About 0.1 μ g (1 μ L-1.7 μ L of the ligation reaction) was added to the cells, mixed well, and transferred to an ice-cold electroporation chamber (0.2 cm, Invitrogen Inc.) and was kept on ice for 1 minute. Both the ice-cold applicator and cuvette were dried with tissue paper, and the chamber was quickly put in the applicator. A pulse of 2.5 kV was applied (Bio Rad *E. coli* Pulser), and the volume was brought to 1 mL by SOC medium in less than 5 seconds. The reaction mixture was incubated at 37 °C for 15 minutes with shaking. Dilutions were made (5 X, and 10 X) and the cells were spread on agar plates containing 50 μ g / mL ampicillin. The bacteria were grown at 37 °C overnight. To prepare SOC solution, the following reagents were mixed, dissolved in 980 mL of d.d H₂O pH 7.0 and autoclaved.

Tryptone	20 g / L
Yeast extract	5 g / L
NaCL (10mM	0.58 g / L
KCL (2.5 mM)	0.19 g / L

Once the mixture was cool, the following ingredients were filter sterilized, and added:

MgCl₂ (2M) 5 mL, MgSO₄ (2M) 5 mL, and Glucose (2M) 5mL.

2.8 Preparation of competent cells for electroporation

One liter of sterile LB medium (Nutrient Broth, 13 g /L d.d H₂O, Oxoid LTD) was inoculated with 10 mL of overnight culture of *E. coli* DH5 α . The culture was grown

at 37 °C with shaking until OD₆₀₀= 0.5-0.7 was reached. The cells were harvested by centrifuging at 5000 X g, 4 °C, 15 minutes (Sorval RC- 5B Refrigerated Superspeed Centrifuge). The cells were resuspended in 1 L of sterilized 10% glycerol, spun collected at 5000 X g, 4 °C, 15 minutes (Sambrook *et al.* 1989). The cells were washed with a smaller volume (200 mL) of 10 % glycerol at 5000 X g, 4 °C, 15 minutes. The next wash was done with 20 mL of 10% glycerol at 5000 X g, 4 °C, 20 minutes. Finally, the cells were resuspended in a total final volume of 4 mL of 10% glycerol, and 50 µL aliquots were dispensed into ice-cold eppendorf tubes. The cells were frozen at -80 °C quickly.

2.9 Identification of bacterial colonies that contained recombinant plasmids

About 30 randomly transformed bacterial colonies were picked and grown in small-scale cultures. Plasmid DNA was isolated from each culture and analyzed by gel electrophoresis. Bands with the expected molecular weight were analyzed by digestion with restriction enzymes followed by gel electrophoresis.

2.10 Plasmid purification

Plasmid purification was done using a small-scale or miniprep reaction. Ten mL of overnight culture cells were centrifuged at 6000 X g for 10 minutes (International clinical centrifuge, Adamon CO). The supernatant except, one mL was removed. The cell pellets were suspended, and transferred to a 1.5 mL Eppendorf tube, and spun at 15000 X g, for 3 minutes (Eppendorf centrifuge, 4 °C). The supernatant was completely removed, and the cells were resuspended in 300 µL of Cell Resuspension Solution (50 mM Tris, pH 7.5; 10 mM EDTA; 100 µg / mL RNase A, Promega). The cells were lysed

by adding 300 μ L of Cell Lysis Solution (0.2 M NaOH and 1% SDS, Promega), mixed by inverting the tube. Then 300 μ L of Neutralization Solution (1.32 M potassium acetate, Promega) was added, mixed by inverting the tube several times. The lysate was centrifuged (15000 $\times$ g, 4 $^{\circ}$ C, Eppendorf centrifuge, 5 minutes). A miniprep column was set by adding 1 mL of Wizard minipreps DNA Purification Resin (Promega), to each syringe barrel. The columns were attached onto a vacuum manifold. The lysate from each miniprep was transferred to the barrel of a Minicolumn / Syringe assembly containing the resin. The samples were subjected to vacuum until all the samples had passed through the column. The samples were washed with 2 mL of Column Wash Solution (80 mM potassium acetate, 8.3 mM Tris-HCL, pH 7.5, 40 μ M EDTA and 55% ethanol, Promega). The columns were then dried by continuing to draw a vacuum for 30 seconds after the solution had been pulled through the columns. The syringe barrels were transferred to Eppendorf tubes and centrifuged (10000 $\times$ g, 2 minutes) to remove the washing solution. The plasmids were recovered by adding 50 μ L of TE buffer and spinning 10000 $\times$ g, for 20 seconds, stored at -20° C before using.

2.11 Screening pGEX clones for fusion protein expression (Sambrook *et al.* 1989)

Expression of the GST fusion protein was induced with IPTG (Isopropyl β -D-Pyranoside, Sigma Chemical Company, St. Louis, MO). The crude protein extract was examined on a gel, and the fusion protein was also purified using GST-sepharose affinity chromatography.

2.12 Small scale expression and affinity purification of GST-fusion synapsin Ia C and A domains

A small-scale culture of bacteria containing the GST-fusion protein construct was grown (5 mL LB broth + 10 μ L of 100 mg / mL ampicillin, Sigma) at 37 °C for 12-15 hours. One mL of this culture was transferred into 50 mL LB broth containing ampicillin (50 mg, Sigma), was grown to $OD_{600} = 0.5-0.7$. Induction of GST-fusion protein was accomplished by adding 2 mM IPTG (Sigma). A time course of induction using was done by pelleting 1.5 mL of the culture at times 0, 0.5 hour, 1 hour, 1.5 hour, 2 hours and 2.5 hours. 1.5 mL cultures were pelleted, resuspended in 10 μ L of 5 x SDS-PAGE loading buffer pH 5, vortexed, run on SDS-PAGE gels.

2.13 Large scale expression and affinity purification of GST-fusion synapsin Ia C and A domains

A single colony of *E. coli* (BL21) bacteria, transformed with plasmid containing a coding region for glutathione S-transferase (GST) synC / synA fusion protein was inoculated into 5 mL LB broth (+ 5 mg / L ampicillin), grown at 37 °C for 12-15 hours (Orbital incubator, New Brunswick Scientific Inc.). It was transferred into 1L of LB broth containing 50 mg / L ampicillin and was incubated to OD_{600} of 0.5- 0.7. The culture was induced with 2 mM IPTG for 2-3 hours. The cells were harvested by centrifuging at (5000 rpm, 10 min, at 4 °C, in Sorval RC-5B Refrigerated Superspeed

Centrifuge) and suspended in 15 mL of ice cold 1 X PBS buffer (40 mM NaCL, 2.7 mM KCL, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 1mM EDTA, 1mM EGTA, and protease inhibitors one tablet / 50 mL of the buffer (Boehringer Mannheim). The bacterial cells were lysed by sonication (Virsonic 60, 12 times, 10-15 seconds each time, on ice). The insoluble fragments were pelleted out (20 minutes, 10000 rpm, Sorval RC-5B Refrigerated Superspeed Centrifuge, 4 °C). The supernatant containing the protein was added to 1 mL of pre-washed ice-cold glutathione sepharose 4 B bead (Amersham Biotech AB), and incubated for 1 hour at 4 °C. It was washed with 1 X PBS for three times, and the protein was eluted by adding 0.5 mL of glutathion elution buffer (15 mM glutathione reduced form, Sigma, in 50 mM Tris-HCL, pH 8). The bead was pelleted out (Eppendorf centrifuge, 4 °C), and the GST-fusion synA / synC protein was recovered, and 20 µL subjected to SDS-PAGE for analysis. The rest of the protein was dialyzed in (150 mM NaCL, 10 mM HEPES, 0.05 % Tween-20, pH 7.4, 12-17 hours, 4 °C), and stored at -20 °C. *E. coli* JM103 was also transformed with the plasmid containing the coding region for GST-synA, and the protein was purified as explained above.

2.14 Preparing the GST matrix

The 50% glutathione sepharose bead slurry suspended in water (Amersham) was washed with 10 X volume of ice cold 1 X PBS, by inverting the beads in a tube 5 times (Protocol Manual, Cheetham). The beads were centrifuged (6000 X g, 5 minutes, room temperature), the supernatant was discarded, and the pellet (beads) was kept on ice.

2.15 BCA Protein Assay (Protocol Manual, Cheetham)

A standard curve was prepared using BSA (Pierce albumin standard 1mg / mL in d.dH₂O) and d.d H₂O, to the final volume of 100 μ L (10-15 μ L of the GST-fusion protein was used). A working solution was prepared by mixing 50 parts Reagent A (BCA protein assay reagent A, (Pierce) with 1 part Reagent B (BCA protein assay reagent B). 2 mL of working solution was added to unknowns and standards followed by a brief vortex mixing. The solutions were incubated at 37 °C for 30 minutes, and cooled to room temperature before measuring the absorbances at 562 nm (Beckman instruments, Inc.) against dd H₂O. The concentrations of unknown proteins were calculated using linear regression of standards and the GST-fusion proteins.

2.16 Western Blotting

Purified proteins were separated by SDS-PAGE without coomassie blue staining. The proteins were electrophoretically transferred (Bio Rad, 0.3 A, 12-17 hours) to nitrocellulose paper (Optitran), where they are bound irreversibly (Sambrook *et al.* 1989). The proteins on the membrane were stained with 5 mL of Ponceau Red 0.5%, in 3% TCA, for 5 minutes with gentle shaking, and then rinsed quickly with d.d H₂O. The nitrocellulose sheet was fixed [10% acetic acid (v/v), 25% isopropyl alcohol (v/v), 15 minutes], rinsed 3 times with d.d H₂O. It was blocked with blocking solution (25 g of fat free skimmed milk / L in 1 X Tris- buffered saline containing 0.5% Tween 20, 60 minutes) with gentle shaking (Bellco). Then primary rabbit polyclonal Ig G antibody (G541, affinity purified, Santa Cruz Biotechnology, 100 μ g / mL, 500 X dilution) was

added for 90 minutes. The nitrocellulose membrane was washed 4 times (2 X 1 min, and 2 X 15 minutes each time) with blocking solution, incubated with secondary antibody (goat anti rabbit horseradish peroxidase (HRP), 1 / 1000 dilution) for 90 minutes in 1 X Tris-buffered saline containing 0.5% Tween 20, no dry milk). The sheet was washed 4 times (2 X 1 min, 2 X 15 minutes) with the same buffer. An additional two washings (5 minutes each time) were done with the same buffer, without Tween-20. The membrane was air dried, wrapped with plastic cling film, and then stored at 4 °C for Enhanced Chemiluminescent (ECL) analysis.

2.17 Enhanced Chemiluminescent (ECL) analysis (Amersham International)

ECL was performed in the dark room in the presence of a Kodak utility safety light. The nitrocellulose membrane containing the desired proteins was soaked in 10 mL of a reagent mixture of (5 mL Luminol / Enhancer Solution + 5 mL Stable Peroxide Solution) Super Signal West Pico (Biolynx INC), for 3 minutes with gentle shaking. The sheet was air dried, wrapped in saran wrap, and sandwiched against Kodak scientific imaging film in a spectroline cassette for 30-60 seconds. The film was placed in the developing solution (Kodak GBX developer and replenisher, 3 minutes), rinsed with tap water and fixed in fixation solution (Kodak GBX fixer and replenisher, 3 minutes).

2.18 Liposome preparation (Protocol Manual, Cheetham)

Phospholipids were dissolved in chloroform / methanol (2: 1, v/v). Three lipid films with compositions of 100% PC, 50: 50% PC / DOPS, 80 : 20 % PC / DOPS (Avanti Polar Lipids, Alabaster, AL), were made by drying the phospholipids on the test tube walls under a stream of dry nitrogen gas. The films were subjected to vacuum for 1.5

hours (Kimax, USA) to remove any traces of solvent. The films were covered with parafilm and stored at -20°C under nitrogen. Lipid films were hydrated in sucrose solution (91 mM sucrose, 10 mM HEPES, 1mM EDTA, pH 7.4). Lipids were freeze-thawed 5 times using liquid nitrogen and a 37°C water bath, and were vortex mixed each time. Lipids were extruded 11 times through two polycarbonate membranes (100 nm pore diameter, Avestin) to make unilamellar vesicles either manually, or using a Liposo-Fast-Pneumatic machine (Avestin Inc, Ottawa, ON). Liposomes were washed in buffer A (50 mM KCL, 10 mM HEPES, 1mM EDTA, pH 7.4, filter sterilized) and stored at 4°C .

2.19 Phosphate Assay (Protocol Manual, Cheetham)

A series of standards were made in pyrex tubes, ranging from 0 to 100 mM from 1 mM potassium phosphate (KH_2PO_4) (final volume of 100 μL with d.d H_2O). Five μL of liposome samples were used, and the final volumes of the standards and samples were made up to 100 μL with d.d H_2O . Thirty μL of 10% magnesium nitrate [$\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ in 95% ethanol] was added and the tubes were heated over a Bunsen burner with gentle shaking until all fumes had disappeared. When the tubes reached at room temperature, 0.3 mL of 0.5 M HCL was added to each tubes, and vortexed vigorously. The tubes were capped with marbles, boiled for 10 minutes, and cooled to room temperature. Then 0.7 mL of a working solution [5 mL of solution A (10% w/v ascorbic acid in d.d H_2O) + 30 mL of solution B (42% w/v ammonium molybdate, 4 H_2O in 1N d.d H_2O)] was added, vortexed, and was incubated for 20 minutes at 45°C . The absorbances at 820 nm were

measured using Beckman spectrophotometer and the phosphate concentrations were calculated using d.d H₂O as blank and a linear regression of the standard and unknowns.

2.20 Liposome Binding Assay A (Protocol Manual, Cheetham)

The volumes of buffer, liposomes, and proteins for the assay were calculated and the final volume was adjusted to 50 μ L. The appropriate volume of liposomes were added to the binding buffer A (50 mM KCL, 20 mM HEPES, 1mM EDTA, pH 7.4, filter sterilized), and vortexed. The calculated amount of proteins (GST-synC / GST-synA, or synapsin Ia) were added, vortexed, and were incubated on ice for 15 minutes. The mixtures were spun at 15000 X g for 30 minutes, 4 °C. The supernatants were separated, and the pellets (10 μ L) were suspended in 30 μ L of binding buffer A and 10 μ L of 10% Triton X-100. The supernatants were mixed with 10 μ L of Triton X-100 and all tubes were vortexed. To determine bound protein, both supernatants and pellets were subjected to quantitative dot immunoblotting followed by ECL.

2.21 Liposome Binding Assay B

This assay was performed essentially as described by (Hosaka *et al.*, 1999). Liposomes were prepared as explained previously (section 2.18) except that no sucrose solution was used to hydrate the lipid films. Instead, binding buffer A (50 mM KCL, 10 mM HEPES, 1mM EDTA, pH 7.4, filter sterilized) was used. About 25 μ g of desired protein was bound to 10 μ L of pre-washed glutathione sepharose 4B beads (Amersham). Both liposomes and binding buffer A were added to the beads containing the GST-fusion proteins (GST itself was used as the negative control). The binding solution was incubated on ice for 15 minutes, spun at 1000 X g, 4 °C, and supernatant was discarded.

The beads were washed once with buffer A, and the beads containing the GST-fusion proteins were subjected to fluorescent spectrophotometry (Hitachi fluorescence spectrophotometer) to detect liposomes bound to the proteins.

2.22 Quantitative Dot Immunoblotting (Reinhard *et al.* 1984)

A nitrocellulose sheet (Optitran) was cut, soaked for 5 minutes with dd H₂O, and air-dried before use. Appropriate volumes of protein-liposome mixtures from both supernatant and pellet fractions were spotted on the sheet. (spots were <1 Cm in diameter) air dried and fixed for 15 minutes with a solution of (10% acetic acid v/v, 25% isopropyl alcohol v/v). The membranes were rinsed in dd H₂O (3 times) and the rest of the experiment was performed exactly as described in section 2.16, Western Blotting, from blocking of the nitrocellulose sheet to detection by ECL.

3.0 RESULTS

3.1 Cloning of synapsin I C domain

A segment of pET15b-synapsin Ia cDNA which encoded domain C of rat synapsin Ia (amino acids 110-420) was amplified by PCR (Figure. 1a). Sharp bands were obtained on an agarose gel at the predicted molecular weights (960 bp) (Figure 3.1b). The amplified sequence was ligated into the expression vector pGEX-4T-1, which also encodes glutathione-S-transferase (GST). The PCR product and the pGEX-4T-1 vector were digested with *Sal* I, and *Xho* I restriction enzymes (Figure 3.2), and were subjected to ligation. *E. coli* DH5 α was transformed with the ligation products using electroporation. Plasmid DNA was isolated from several colonies grown on ampicillin plates. DNA was subjected to agarose gel electrophoresis (Figure 3.3) to identify the plasmids carrying the insert. Only those bands with the expected molecular weight, about 6 kb (pGEX-4T-1 = 4950 bp, and PCR domain C product = 960 bp) were further analyzed to test for the presence of the insert in the vector DNA.

A second set of PCR reactions was performed using plasmids identified by restriction analysis as the templates and using the primers used for the initial cloning. PCR products with the same molecular weight as the insert were obtained indicating that the clones contained the insert (coding region for synC) (Figure 3.4). The cloned DNA was sequenced (University of Ottawa DNA Sequencing Service), and the presence of an open reading frame coding for synC was confirmed (Appendix 1).

3.2. Expression of GST-synC fusion protein

Following confirmation of the correct clone, *E. coli* BL 21 was transformed with the plasmid (Figure 3.5). In GST-synC construct, the N-terminus of synC was fused to the C-terminus of GST. Induction of bacteria with IPTG was predicted to express a fusion protein with a molecular weight of approximately 62 kDa. To establish the best induction time, a time course for GST-synC fusion protein expression was performed (Figure 3.6). A 2-hour induction period was concluded to be optimal for GST-synC expression.

A single colony of *E. coli* BL21 containing plasmid with the coding region for GST-synC was grown overnight. The cells were induced for 2 hours and were collected. The cells were ruptured using sonication, and the soluble fusion protein was affinity purified using glutathione sepharose beads. Beads with bound GST-synC were washed, and the fusion protein was eluted with reduced glutathione elution buffer, and analyzed using SDS-PAGE (Figures 3.7a, and 3.7b).

SDS-PAGE analysis indicated that the recombinant protein had the predicted molecular weight of GST-synC, in agreement with previous studies (Wang *et al.*, 1997). Protein purification without inclusion of protease inhibitors also resulted in a single band using SDS-PAGE. Therefore, proteolysis of the fusion protein was not a major concern in this purification. A standard curve was prepared using the BCA protein assay (Pierce albumin standard 1 mg / mL in d.d H₂ O). The concentration of the fusion protein was calculated to be 11 mg / mL.

3.3. Liposome binding assays

The final volume of each binding assay was 50 μ l. On average, the ratio of protein to lipid used in the assays was approximately 1 / 350 (mol / mol). Full-length bovine synapsin Ia (2 mg / mL) was used as the positive control (Figure 3.8a). The full-length synapsin Ia did not bind to liposomes made of pure PC, (almost all protein was in the supernatant), but it bound to liposomes that contained PS (protein was present in the pellet fragment). As the amount of acidic phospholipid (PS) increased, more binding occurred (almost all protein sedimented into the pellet fragment). GST was used as a negative control. GST alone did not bind to liposomes regardless of the charge of the liposomes (Figure 3. 8b).

GST-synC binding to various compositions of liposomes was examined (Figure 3.8c). Compared to whole length of synapsin Ia, synC bound to liposomes containing acidic phospholipids (50% PS) but not to the same extent as full length synapsin Ia (only a small amount of synC is sedimented in to the pellet, but almost all synapsin Ia was sedimented when mixed liposomes were used).

To narrow down the synapsin Ia domains that specifically bind to liposomes, two more regions of domain C were cloned. The first clone codes residues of 278-327 of synapsin Ia that is believed to include coding region for α -helix-1, as a fusion protein (Figure 3. 9a). The GST- α -helix-1 protein (32 kDa) was expressed, and the presence of α -helix-1 region was confirmed by using an antibody against synC, followed by detection with ECL (Figure 3.9b). The second clone codes for residues 116-276, a subset of synC in which α -helix-1 was excluded (GST- $\Delta\alpha$ -helix-1). The processes of cloning of $\Delta\alpha$ -

helix-1 were: PCR products Figure 10a, digestion Figure 10b, and ligation products Figure 10c. The cloning of the coding regions coding for GST- α -helix-1 and GST- $\Delta\alpha$ -helix-1 were stopped at this stage, without any further analysis. Instead, the coding region for domain A of rat synapsin Ia was cloned, expressed, and used for liposome-binding assay.

3.4. Cloning of the residues 1-29 of rat synapsin Ia that codes for synA

A new paper was published by Hosaka *et al* (1999) that assigned domain A of synapsins (residues 1-29) as the specific binding region to acidic liposomes. Therefore, it was decided to express the A domain of synapsin Ia (synA) and to compare its binding to liposomes with synC. Primers were designed such that the PCR product was flanked with *Bam*H I and *Eco*R I restriction enzyme sites. The primers in this reaction were: forward, 5'-CCG CGC **GGA TCC** CAT ATG AAC TAC-3' containing *Bam*H I restriction site, and reverse, 5'-CGG CGG **GAA TTC** TTG CGG GCG CT-3' containing *Eco*R I restriction site. The PCR condition was: 95 °C / 5 minutes pre-dwell; 94 °C / 1 minute denaturation; 58 °C / 1 minute annealing; 72 °C / 1 minute extension; 30 reaction cycles; and 72 °C / 5 minutes post-dwell. The PCR products and ligation products are shown in Figures 3.11a, and 3.11b respectively. Two more tests were performed (double digestion, Figure 3.12a, and digestion with *Pst* I restriction enzyme, Figure 3.12b) as well as DNA sequencing to confirm the presence of coding region for synA.

3.5 Expression of GST-synA fusion protein

The bacterium *E. coli* JM103 was transformed with the ligation products by electroporation. The bacteria was grown overnight, was induced with 2 mM IPTG for 2 hours, to allow expression of GST-synA. The cells were ruptured, and the soluble fusion protein was purified by affinity to glutathione agarose beads followed by elution from washed beads due to competition with free reduced glutathione. When purified protein was screened on polyacrylamide gel, two bands were observed (Figure 3.13, lane 1). The larger band (31 kDa) was the whole length of GST-synA, and the smaller band (27 kDa) was the degradation products, corresponding only the GST. At first, it was assumed that the degradation might be due to the number of sonications, as a result, the number of sonications was reduced. However, still two bands at the same sizes were observed (Figure 3. 13, lane 2). Therefore, it was concluded that *E. coli* JM103 was not a proper expression bacteria for GST-synA. It was decided then to express synA fusion protein in *E. coli* BL21. The purified fusion protein was screened on a polyacrylamide gel, and a single band at about 31 kDa (Figure 3. 14) was observed when *E. coli* BL21 was used.

3. 6 Protein-Liposome Binding Assays

Both GST-synA, and GST-synC were bound to glutathione sepharose 4 B beads and spun, then liposomes with various compositions were added to the proteins. GST alone was used as the negative control. The experiments were performed in the absence of a positive control, because GST-synapsin Ia fusion protein was not available. Bindings was detected and measured by fluorescence spectrophotometry.

The binding of synC to liposomes was compared to the binding of synA to liposomes. When GST was used, the protein did not bind to liposomes, regardless of liposome charges. Both synC and synA, however, bound to acidic liposomes almost to the same extent (Figure 3. 15). This experiment was done as previously described by Hosaka *et al* 1999, but different results were obtained. In their experiment, synC alone did not bind to acidic liposomes in contrast with this experiment. Therefore, the regions in synapsin Ia, targeting the acidic liposomes are both domains A / and C according to this experiment.

Figure 3.1. Agarose gels showing amplifications of a section of pET15b synIa vector that codes for domain C of rat synapsin Ia (amino acids 110-420). **a)** Lane 1. 1 kb linear DNA ladder; lane 2, a PCR using d.d H₂O as the negative control; lanes 3-5. PCR products with different amounts of DNA templates (pET15b synIa) to optimize the reactions. The PCR condition was: 95 °C / 5 min pre-dwell; 94 °C / 1 min denaturation, 50 °C / 1 min annealing; 72 °C / 1 min extension; 30 reaction cycles; and 72 °C / 5 min post-dwell. **b)** Lane 1. 1kb linear DNA ladder; lanes 2-3. PCRs with different amounts of the DNA templates. The same PCR reaction condition was done except that the annealing temperature was increased to 60 °C and the TE buffer was autoclaved before use. The PCR products contain restriction sites for *Sal* I and *Xho* I restriction enzymes.

Figure 3.2. An agarose gel showing restriction digestions (*Sall*, and *XhoI*) of cloning vector pGEX-4T-1, and PCR product. Lane **1**, 1 kb linear DNA ladder; lane **2**, restriction digestion of the PCR products; lane **3**, restriction digestion of pGEX-4T-1 vector; lane **4**, undigested circular pGEX-4T-1 vector; and lane **5**, circular supercoiled DNA ladder.

Figure 3.3. An agarose gel showing the transformation of bacteria *E. coli* DH5 α transformed with the ligation products using electroporation. Out of 19 randomly chosen putative bacterial transformants, 3 (lanes **9**, **10**, and **14**) contained DNAs with the expected molecular weights. Lane **20** is the circular supercoiled DNA ladder. The rest of the colonies contained only the pGEX-4T-1 vectors (backgrounds).

Figure 3.4. Agarose gels showing tests to check if the coding region for synC is inserted in previous 3 DNAs (lanes **9**, **10**, and **14**). **a)** Double PCR using one of these clones as the template. pET15b-synIa vector was also used as the template for amplifications (as the positive control). The reaction was done with the annealing temperature of 58 °C. Lane **1**, 1kb linear DNA ladder; lane **2**, PCR products using the clone (**#9**) as the template; lane **3**, PCR products using pET-15b-syn Ia as the template.**b)** Double restriction digestion (*Xho* I and *Sal* I) of clone **#9**. Lane **1**, 1 kb linear DNA ladder; lane **2**, double restriction digestion of clone **#9**.

PCR

a)

Double digestion

b)

Figure 3.5. Agarose gel showing the transformation of bacteria *E. coli* BL21 with clone containing the coding region for synC by electroporation. *E. coli* BL21 was used as an expression bacteria. Lanes **1-4**, DNAs extracted from *E. coli* BL21; and lane **5**, DNA extracted from bacteria DH₅α (as the positive control); lane **6** is the circular supercoiled DNA ladder.

Figure 3.6. A 12% Commassie blue stained polyacrylamide gel electrophoresis of a time course induction of GST-synC fusion protein showing the expression of the protein (about 62 kDa) after induction. Lane **1**, high molecular weights markers; lane **2**, 0 hour induction; lane **3**, 0.5 hour; lane **4**, 1 hour; lane **5**, 1.5 hours; and lane **6**, 2 hours after induction.

Figure 3.7. Commassie blue stained polyacrylamide gels showing the purified recombinant GST-synC fusion protein. **a)** Lane **1**, affinity purified GST-synC; lane **2**, second wash of the eluate; lane **3**, first wash; and lane **4**, high molecular weights markers. **b)** The molecular weight of the GST-synC was further verified using two different molecular weights markers. Lane **1**, high molecular weights markers; lane **2**, purified GST-synC fusion protein; and lane **3**, low molecular weights markers.

a)

b)

Figure 3.8. Protein-Liposome assays detected by chemiluminescent (ECL). **a)** The full length of bovine synapsin Ia (as the positive control) was bound to various compositions of liposomes, and the protein was detected in both supernatant and pellet fragments. Lane 1, 100% PC; lane 2, 80% PC / 20% PS; and lane 3, 50% PC / 50% PS. As the amount of PS (negatively charged phospholipids) increased, more proteins were detected in the pellet fraction indicating more binding occurred. **b)** The binding of GST (as the negative control) to various liposome compositions were compared. Lane 1, 100% PC; lane 2, 50% PC / 50% PS. The pattern shows that GST does not bind to the liposomes, regardless of the liposome's charge (almost all the proteins are in the supernatant fractions). **c)** The whole synapsin Ia was compared to synapsin C in terms of binding to various liposome compositions. Lanes 1 and 3, 100% PC; lanes 2 and 4, 50% PC / 50% PS. The pattern suggests that synapsin C binds to the acidic liposomes but nowhere to the same extent as whole synapsin Ia.

**Positive control,
whole Synapsin Ia**

**100
%
PC**

**80 / 20
PC / PS**

**50 / 50
PC / PS**

Supernatants

Pellets

a)

**Negative control,
GST alone**

**100
%
PC**

**50 / 50
PC / PS**

Supernatants

Pellets

b)

**Whole synapsin Ia
versus Synapsin C**

Synapsin Ia

Synapsin C

**100
%
PC**

**50 / 50
PC /
PS**

**100
%
PC**

**50 / 50
PC / PS**

Supernatants

Pellets

c)

Figure 3.9. Commassie blue stained polyacrylamide gel followed by ECL detection of the purified recombinant GST fusion protein (residues 278-327) (α -helix-1) of rat synapsin Ia. **a)** Lane 1, purified recombinant GST fusion protein (α -helix-1); lane 2, low molecular weights markers; lane 3, second wash of the eluates; lane 4, first wash of the eluates; and lane 5, cell lysates. The molecular weight of the GST- α -helix-1 was 32 kDa. **b)** A dot blot showing the expression of GST- α -helix-1. Primary antibody was against synapsin Ia, and not GST. Synapsin Ia was used as the positive control. The primers for amplification of this region were:

Forward: 5'-CAC GGA TCC ATG GGC AAG GTC AAG-3'

Reverse: 5'-CCC GAA TTC TGA TGT CCT CAT GTA-3'

a)

b)

Figure 3.10. Agarose gels showing the steps of cloning of residues (116-276) of rat synapsin Ia coding for a region of synC in which α -helix-1 had been deleted ($\Delta\alpha$ -helix-1). **a)** Lane 1, linear 1 kb DNA ladder; lane 2-4, amplification products of residues 116-277 that codes for $\Delta\alpha$ -helix-1. The PCR condition was: 95 °C / 5 minutes pre-dwell; 94 °C / 1 minute denaturation; 50 °C / 1 minute annealing; 72 °C / 1 minute extension; 30 reaction cycles; and 72 °C / 5 minutes post-dwell. **b)** The restriction digestion (*Sal* I, *Xho* I) of the PCR products coding for $\Delta\alpha$ -helix-1 and the pGEX-4T-1 vector. Lane 1, linear 1 kb DNA ladder; lane 2, restriction digestion of the PCR products; lane 3, circular pGEX-4T-1 vector; and lane 4, restriction digestion of pGEX-4T-1 vector. **c)** Plasmid DNA isolated from bacteria *E. coli* DH₅ α transformed with ligation products. The expected molecular weights (plasmids containing the inserts) are shown by arrows. The circular supercoiled DNA ladder is the last lane.

Figure 3.11. Agarose gels showing the PCR products and transformation of bacteria *E. coli* DH₅ α with the ligation products. **a)** Amplification of a section of pET15b vector that codes for domain A of rat synapsin Ia (residues 1-29). The PCR condition was: 95 °C / 5 minutes pre-dwell; 95 °C / 1 minute denaturation; 58 °C / 1 minute annealing; 72 °C / 1 minute extension; 30 reaction cycles; and 94 °C / 5 minutes post-dwell. Lane **1**, 100 bp linear DNA ladder; lanes **2-3**, PCR products; and lane **4**, primers. **b)** Plasmid DNA isolated from bacteria *E. coli* DH₅ α transformed with the ligation products. The expected molecular weight (plasmid containing the insert) is shown by an arrow. The last column is the circular supercoiled DNA ladder.

Forward primer: 5'-CCG CGC **GGA TCC** CAT ATG AAC TAC-3'

Reverse primer: 5'-CGG CGG **GAA TTC** TTG CGG GCG CT-3'

Figure 3.12. Agarose gels showing the restriction analysis to test if the clone contains the region coding for synA protein. **a)** The double digestion (*EcoR* I, *BamH* I) of the clone. Since the ratio of the pGEX-4T-1 to the PCR products is about 50 to 1 respectively, it is hard to see a band at 100 bp corresponding to the region coding for domain A of synapsin Ia. Lane 1, double digestion (*EcoR* I, *BamH* I) of the expected clone; lane 2, 100 bp linear DNA ladder. **b)** A restriction analysis of the clone using *Pst* I restriction enzyme. *Pst* I only cuts circular pGEX-4T-1 vector once, resulting a linear band at 4970 bp. However, it cuts the clone (the pGEX-4T-1 vector + the coding region for synA) twice resulting two bands at 4 kb, and 1 kb. I, and II, are the *Pst* I digestion of pGEX-4T-1 and the expected clone respectively using DNAMAN computer software. III, is the actual restriction digestion of pGEX-4T-1 (lane 2), and the expected clone (lane 3). Lane 1 is the 1 kb linear DNA ladder.

a)

b)

Figure 3.13. A commassie blue stained polyacrylamide gel showing the purified recombinant GST-synA fusion protein. The fusion protein was expressed in bacteria *E. coli* JM103. Lane **1-2**, purified GST-synA fusion proteins; lane **3**, protein molecular weight markers (Kleidoscope prestained standard). The JM103 bacteria are not good expression bacteria for GST-fusion protein since the major band is GST (27 kDa) rather than GST-synA (31 kDa). Decreasing the number of sonications from 13 times (lane **1**), to 8 times (lane **2**), did not prevent protein degradation.

Figure 3.14. A commassie blue stained polyacrylamide gel showing the purified GST-synA fusion protein expressed in bacteria *E. coli* BL21. Lane **1**, purified GST protein (27 kDa); lane **2**, purified GST-synA fusion protein (31 kDa); and lane **3**, low molecular weight protein markers. *E. coli* BL21 is a good expression bacteria for GST-fusion proteins since there is no degradation of GST-syn A protein (31 kDa).

Figure 3.15. Protein-liposome binding assays comparing the ratio of binding of the three proteins (GST, GST-synA, and GST-synC) with liposomes of various lipid compositions. About 25 µg of each protein was bound to 10 µL of pre-washed glutathione sepharose 4 B beads, and liposomes containing 0.1 mol % Octadecyl rhodamine (Molecular Probes, Eugene, OR) were added to the solution. The beads were washed, and fluorescence spectroscopy was used to detect any liposomes bound to the proteins. Both synA, and synC bound to acidic liposomes (50% PC / 50% PS) but not to 100% PC. GST did not bind to the liposomes regardless of liposome charge. The bar graphs, which indicate the amount of fluorescent liposomes bound to protein, are the average of three measurements. * indicate a significant difference from the control with a P-value of <0.005.

4.0 DISCUSSION

Synapsin I is a vesicle-associated phosphoprotein found in neurons (DeCamilli., and Greengard, 1986). It's head domain contains a serine residue that is phosphorylated by cAMP-dependent protein kinase or calcium / calmodulin-dependent protein kinase I (site 1) and a tail domain contains two serine residues that are phosphorylated by calcium / calmodulin-dependent protein kinase II (sites 2 and 3) (Czernik *et al.*, 1987). Schiebler and co-workers partially characterized the association of synapsin I with highly purified brain small synaptic vesicles (Schiebler *et al.*, 1986). Huttner *et al.*, 1983, and showed that purified dephosphorylated synapsin I bound to synapsin I-depleted synaptic vesicles with high affinity ($K_d = 10 \text{ nM}$ at 40 mM NaCl) and saturability ($B_{\text{max}} = 800 \text{ fmol} / \mu\text{g}$ protein). It was observed that an increase in the ionic strength of the medium, or phosphorylating the tail domain, causes a decrease in binding affinity (Huttner *et al.*, 1983).

Dephosphorylated synapsin I also binds to F-actin, forming bundles (Bahler and Greengard, 1987). Phosphorylation of synapsin I on the tail site leads to a decrease in both bundling activity and binding to F-actin (Bahler and Greengard 1987). Synapsin I inhibits neurotransmitter release either by anchoring vesicles to the cytoskeleton or by preventing the fusion of vesicles with the presynaptic membrane. It is therefore, very important to further understand the nature of the interaction between synapsin I and small synaptic vesicles. Synapsin I interacts with synaptic vesicles via two sites; its head domain is involved in phospholipid binding and bilayer penetration, whereas its tail domain binds to other vesicle proteins (Benfenati *et al.*, 1989).

In this study two segments of pET15b-syn Ia vector which encode domain C (amino acids 110-420) and domain A (amino acids 1-29) of rat synapsin Ia were cloned using the polymerase chain reaction. These amplified sequences were ligated into the pGEX-4T-1 expression vector, and were transformed into the bacteria DH5 α . The plasmid DNA was isolated from the bacteria and the DNA with expected molecular weight was further analyzed by restriction digestion and DNA sequencing. *E. coli* DH5 α was transformed with the correct clone to express the GST-synapsin domain C protein, however, full-length protein was not expressed by this strain. Since *E. coli* DH5 α was not a suitable bacterium for expression of the GST-fusion protein, *E. coli* BL21 (DE3) was transformed to express the GST fusion proteins. The purified protein was used for protein-liposome binding assays and the assays were done in the presence of a positive control (bovine synapsin Ia) and a negative control (GST).

4.1 Expression of synapsin C (residues 110-420 of rat synapsin Ia)

The cloning of the C domain was proceeded by amplification of the section of the pET15b-synapsin Ia vector which encoded rat synapsin Ia (residues 110-420) using PCR (Figure 3.1a). Since there were amplifications at sites other than residues 110-420, PCR conditions had to be adjusted in order to obtain a clean and sharp band. The molecular weight of the PCR product (960 bp) was evaluated by electrophoresis on an agarose gel in the presence of a standard linear DNA ladder (Figure 3.1b).

The primers were designed such that restriction enzymes *Sal* I, and *Xho* I flanked the PCR products. After obtaining the desired PCR products, the products and the expression vector (pGEX-4T-1) were digested by *Sal* I and *Xho* I restriction enzymes. Figure 3.2 shows the restriction analysis of these constructs. Both linear and circular

DNA ladders were used to confirm the digestion of the DNA. The digested PCR products were ligated into the pGEX-4T-1 vector and introduced into bacteria DH5 α by transformation. The transformants were identified by choosing bacterial colonies and isolating their plasmid DNA. Some of the bacteria DH5 α colonies screened contained the correct molecular weight plasmids (Figure 3.3).

To check whether these bacteria contained the plasmids with the correct insert, a second round of PCR was performed using plasmids with the correct molecular weights as templates. PCR products with the expected molecular weight (about 960 bp) were obtained, which indicated that the plasmids contain the insert, as is shown in Figure 3.4a. As a further confirmation, the plasmid DNA of the clone was double digested with *Sa*II and *Xho*I restriction enzymes. The appearance of two bands at 4970 bp (corresponding to the linearized pGEX-4T-1 vector) and 960 bp (corresponding to the coding region for synC) is another indication of the presence of the insert in the plasmid (Figure 3. 4b). Finally, the DNA was sequenced and the appropriate insert sequence was confirmed to be in the plasmid.

Bacteria *E. coli* BL21 (DE3) was transformed with appropriate clones to express GST-synC fusion protein (figure 3.5). Foreign proteins expressed in *E. coli* are sometimes insoluble, and a useful aspect of GST-fusion proteins is that they are wholly or partially soluble. A series of pGEX-4T-1 vectors are available, that simplify the purification of foreign polypeptides expressed as a fusion with the C-terminal with Sj26, a 26-kDa glutathione S-transferase (GST) produced in *E. coli*. GST is encoded by parasitic helminth *Schistosoma Japonicum* (Marston,1986). The fusion proteins are soluble in aqueous solutions in the majority of cases. Affinity chromatography on

immobilized glutathione can be used to purify the fusion proteins from crude bacterial lysates under non-denaturing conditions (Marston, 1986).

The pGEX-4T-1 vector contains an open reading frame that encodes GST, followed by specific restriction endonuclease sites for *Bam*H I, *Eco*R I, *Sma* I, *Sal* I, *Xho* I, and *Not* I, followed by stop codons. The *lac* repressor (product of the *lac* I gene) binds to the P_{tac} promoter, repressing expression of GST fusion protein. Upon induction with IPTG, the lactose analog isopropyl- β -D-thiogalactoside, derepression occurs and GST fusion protein is expressed. Induced bacterial cultures are then allowed to express fusion proteins for several hours, after which time cells are harvested and then lysed by mild sonication. Fusion proteins can be purified from bacterial lysates by affinity chromatography using Glutathione Sepharose 4B beads (Pharmacia Biotech). One of the reasons that pGEX-4T-1 vector is used, is because its polylinkers contain protease cleavage sites so that the cloned protein can be released from the GST by thrombin. The GST itself and / or any undigested fusion protein by thrombin can be removed by adsorption on glutathione-agarose.

In cases where proteolytic degradation is of concern, it may be desirable to use a protease-deficient (e.g. *lon*⁻, *omp*⁻) bacteria host. Bacteria *E. coli* BL21 which is a protease-deficient host was an appropriate choice to express the whole length of these proteins. The proteolytic degradation of the fusion proteins expressed in this experiment was not a concern since both synA₁ and synC were not degraded.

E. coli BL21 transformed with the clone containing the coding region for GST-synC was grown overnight, and was induced with 2 mM IPTG. A time course induction of the GST-fusion protein was performed to optimize the induction time

(Figure 3.6). Induction of the bacteria for 2 hours resulted in a good yield of fusion protein for GST-synC. The induction time may vary when using another strain (e.g. *E. coli* JM103) transformed with the same vector. A very faint band at zero induction time (at 62 kDa) is due to the presence of the strong P_{lac} promoter, which is repressed by *lac* repressor. To diminish this basal expression level, it is suggested that the bacteria be grown in the presence of 2% glucose (Pharmacia P-L Biochemicals Inc).

GST-synC was purified by incubating the soluble material from lysed cells with glutathione agarose beads, followed by elution from washed beads due to competition with free glutathione, and was screened for expression of the expected fusion protein by 16% SDS-polyacrylamide gel (Figure 3.7a). The molecular weight of 62 kDa (GST = 27 kDa, synC = 35 kDa) was assigned to GST-synC (Figures 3.7a, 3.7b). The protein yield was evaluated to be 1 μ g / mL by using the standard BCA assay.

4.2 Protein-Liposome Assays

Synapsin I is a major synaptic vesicle-associated phosphoprotein. The investigation of possible interactions between synC and phospholipid vesicles was undertaken for several reasons. Synapsin I was found to specifically associate with high affinity to small synaptic vesicles. It was also found to form stable monolayers at an air-water interface and to possess a very high surface activity, which may be due to the presence of amphiphilic secondary structures in the molecules (Benfenati *et al.*, 1989). Therefore, identifying synapsin Ia residues that target the phospholipid bilayers is very important.

The interaction of purified synC with phospholipid membranes was investigated and this interaction was found to depend on phospholipid composition. Bovine synapsin

Ia and GST proteins were used as the positive and negative controls respectively. No interaction between synapsin Ia with phospholipid bilayers was observed when pure phosphatidylcholine was used, but interactions were present when a mixture of phosphatidylcholine / phosphatidylserine was used. The maximum interaction occurred when the concentration of phosphatidylserine was the highest. (Figure 3.8a). This result is in agreement with previous studies: when [125 I]-TID labeled liposomes that mimic the phospholipid composition of purified small synaptic vesicles (PC / PE / PS / Chol 40: 32: 12: 5: 10, wt/wt) (Benfenati et al., 1989) were used, significant labeling by [125 I]-TID was observed. Increasing the percentage of anionic lipid in the membrane from 20% PS to 50 % PS, increased the labeling of synapsin by [125 I]-TID. However, due to negligible binding to PC or PE, synapsin I was not labeled when liposomes lacking anionic phospholipids were used (Cheetham *et al.*, 2001).

SynC was also tested for binding. When pure phosphatidylcholine (100% PC) liposomes were incubated with GST-synC, almost all the protein was present in the supernatant, suggesting that synC did not bind to phosphatidylcholine. When mixed phospholipid (50%PC / 50% PS) liposomes were incubated with the protein most of the protein was present in the supernatant, but, a small amount was in the pellet, indicating that some binding had occurred. Compared to synapsin Ia phospholipid binding, synC binds to the acidic phospholipids, but not to the same extent as the intact protein (Figure 3.8c). When mixed phospholipid (PC / PS) liposomes or pure 100% PC liposomes were incubated with GST, almost all the GST protein was present in the supernatants, suggesting that GST does not bind to the phospholipids, regardless of the charges (Figure 3.8b).

So far, it is clear that synapsin Ia and / or synC binds to PS that has a net negative charge, but not to PC with a net positive charge. This interaction is not merely because of the net surface negative charges. In fact PC liposomes negatively charged by inclusion of 10% (wt/wt) dicetyl phosphate bind synapsin I to a low extent and with a low affinity (Benfenati *et al.*, 1989). The interaction therefore, is suggested to be initiated by a somewhat specific surface electrostatic attraction followed by the penetration of the hydrophobic regions of the head domain of syn I into the hydrophobic core of the bilayer (Benfenati *et al.*, 1989).

To narrow down the syn Ia region that is known to specifically binds to negatively charged liposomes, two more clones were constructed, each involving a region of rat synC. The first clone encoded amino acid residues 278-327 which are believed to include α -helix 1 (Figure 3.9a). This protein was confirmed as a piece of synC, by using an antibody against synC, followed by detection with ECL (Figure 3.9b). The second clone was constructed in a manner that residues encoding α -helix-1 were excluded (residues 116-277). The PCR, digestion, and ligation products are shown in Figures 3.10a, 3.10b, and 3.10c respectively. This clone was not further characterized.

4.3 Expression of synapsin A (residues 1-29 of rat synapsin Ia)

Synapsins are found on synaptic vesicles, which they coat as peripheral membrane proteins, and they constitute one of the most abundant neuronal PKA substrates. Synapsins contain a short N-terminal A domain that is highly conserved, a variable B domain and a large, central C domain, which is also highly conserved. Following the C domain, synapsins contain smaller C-terminal regions that differ among various synapsins (Esser *et al.*, 1998).

So far, the most accepted hypothesis has been that synapsin I binds to vesicles through an interaction of its variable C-terminal domains with CaM kinase II, which also phosphorylates these domains (Benfenati *et al.*, 1992). Hosaka *et al.*, 1999 suggested that, although this interaction may mediate some of the association of synapsin I with vesicles, it is unlikely to represent the major mechanism. They reasoned that very little CaM kinase II is present on synaptic vesicle, and also that most CaM kinase II is localized to structures that do not contain synapsin. Furthermore, they reasoned that synapsin II does not bind CaM kinase II but is still targeted to synaptic vesicles. Instead of the C-domain, they suggested that the A domain of synapsins constitutes a phospholipid-binding domain whose activity is regulated by phosphorylation. In an effort to test this, they showed that only the A domain, alone or in combination with the B and C domains, exhibited a strong interaction with phospholipids. The B and C domains, in contrast, did not bind. (Hosaka *et al.*, 1999).

To determine which domains of synapsin Ia were responsible for lipid binding, we cloned the residues (1-29), which code for domain A of rat syn Ia from pET15b vector. Primers were designed in a way that the PCR products were flanked with EcoR I and BamH I restriction sites. *E. coli* DH5 α was transformed with the ligation products. The PCR products, plasmids isolated from *E. coli* DH5 α transformed with ligation products are shown in Figures 3.11a, and 3.11b, respectively.

To express the GST-synA protein, *E. coli* JM103 was transformed with the clone containing the coding region for GST-synA, and the protein was purified. Polyacrylamide gel electrophoresis analysis showed two bands for purified GST-synA. the minor product, about 31 kDa, was assumed to be GST-synA, and the major product,

about 27 kDa, was assumed to be only GST (Figure 3.13, lane 1). When the number of sonications was decreased from 13 times (lane 1), to 8 times (lane 2), two bands still were produced at the respective sizes. Therefore, it is concluded that bacteria *E. coli* JM103 is not a good choice to express GST-synA fusion protein, instead, bacteria *E. coli* BL21 was used to express GST-synA. GST-synA was purified, and subjected to electrophoresis, and was observed as a single band at about 31 kDa, another indication that *E. coli* BL21 is a good expression system for GST-fusion proteins (Figure 3.14).

4.4 Protein-Liposome Assays

The purified GST-synA and GST-synC proteins were used for protein-liposome binding assay. Both GST-synA, and GST-synC were bound to pre-washed glutathione sepharose 4 B beads before addition of liposomes with various compositions. GST itself was used as the negative control. It would be advantageous if GST-synapsin Ia, as a positive control, was available so that the binding of GST-synA and / or GST-synC to liposomes could be compared to it. The GST-fusion proteins were subjected to fluorescent spectrophotometry, and their binding to liposomes was measured. This procedure was performed essentially as was described by Hosaka *et al.*, 1999, however, the result was somehow different. They showed that only the A domain, alone or in combination with the B and C domains, exhibited a strong interaction with acidic phospholipids. The B and C domains did not bind to the liposomes. In our experiments however, both the A and C domains individually interacted with acidic phospholipids. The fact that both domains individually interact with acidic liposomes may explain the stronger interaction of synapsin Ia than synC or syn A alone (Figure 3. 8c).

Hosaka and co-workers assumed that CaMKII is required for synapsin-vesicle binding. If so, they reasoned that synapsin II which does not have CaMKII binding sites should not bind to liposomes. For this reason they used synapsin IIa which does not contain a CaMKII binding site, and reasoned that phosphorylation of synapsin IIa with PKA dissociated the protein from liposomes. Although, CaMKII is involved in binding of synapsin Ia (through interaction with C-terminus of synapsin Ia), it is not required for liposome binding since *in vitro* synapsin Ia-liposome binding did not require CaMKII.

Benfenati and coworkers (1989) showed that the stoichiometry of the interaction was 1 mol of synapsin I / 900 mol of acidic phospholipids. The stoichiometry of the binding in this experiment was fairly low, about 1 mol / 350 mol protein / phospholipids. This can be explained since multiple freezing and thawing (the proteins were kept at -20°C and were used for several times) led to increased adsorption of synapsins to tube walls, hence the effective concentration of protein may have been lower than was estimated in the protein assays.

The present data are in agreement with previous findings regarding synapsin-membrane interactions. Synapsin I was found to have a high degree of surface activity and a high limiting surface area in amphiphilic environments suggesting that it may coat a significant portion of the SV surface. These properties are likely attributable to the presence of amphiphilic secondary structures within the NH_2 -terminal region of the protein.

It has been shown that, upon binding to phospholipid bilayers, synapsin I induces vesicle clustering and stabilizes the bilayer structure by inhibiting the transition from the stable lamellar phase to the inverted hexagonal phase induced by temperature or Ca^{2+}

(Benfenati, *et al.*, 1993). This stabilization may be a consequence of the insertion of synapsin I into the bilayer that may reduce the tendency of the vesicle membrane to experience phospholipid packing defects or to form concave surfaces, the occurrence of which is facilitated by the high intrinsic curvature of SV and by their relative enrichment in non-bilayer structure-preferring phospholipids such as PE.

The specific targeting of synapsin I to SV cannot be fully explained by synapsin I-phospholipid interactions, but is likely to involve specific, currently unidentified protein-protein interaction. However, pure phospholipid vesicles represent a useful model system to study the lipid interactions of synapsin I with the membrane of SV. The synapsin-phospholipid interaction may play an important functional role in clustering SV and in preserving their remarkably uniform shape and size. The observation that amino acid sequences in both domains A and C of synapsin I bind to the lipid membrane provides a molecular basis for the high-affinity binding of synapsin I to SV and the resulting stabilization of the vesicle membrane structure.

Figure 4.1

An updated model for the interaction of synapsin I with phospholipid bilayers. In this model binding of synapsin I to the phospholipid membrane is mediated by both the C-domain (yellow) and the A-domain (pink). Upon phosphorylation at site 1, the A-domain dissociates from the membrane. The C-domain also mediates binding of synapsin I to actin filament (Cheetham *et al.*, unpublished).

4.5 Possible Improvements

The coding regions for synapsin C and synapsin A were amplified and cloned from rat synapsin Ia but full length synapsin Ia was purified from bovine brain. Since there is a 98% amino acid identity between rat and bovine synC, and a 100% amino acid identity between rat and bovine synA amino acid sequences (Sudhof *et al.*, 1989) it was assumed that there should not be any considerable differences between these proteins in terms of phospholipid binding. Expression of recombinant full length synapsin Ia would be useful, but has not yet been achieved.

A protein-liposome binding assay requires a pure protein since any impurities may interfere with the binding. In the case of GST-synA, it was assumed that the protein was pure however, the presence of other proteins (Figure 3.14) in the solution might have affected the lipid binding interaction. All polyacrylamide gels in this experiment were stained with coomassie blue. Improving the staining procedure using methods such as silver staining would be more sensitive to the presence of other proteins.

At the N-terminus of domain A, three arginine residues that might be important for binding, precede the phosphorylation site. These residues could be mutated to check their involvement in binding. These mutations can be done simply by designing primers at the N-terminus that substitute the arginine residues with other non-charged amino acids (e.g. alanine). The mutated synA-liposome binding can be compared to the wild type synA.

Two other clones were constructed that code for amino acid residues 116-277, and amino acid residues 278-327 of rat synapsin Ia. Due to time constraints, these clones

were not analyzed. The analysis of the phospholipid binding properties of these proteins is recommended.

5.0 CONCLUSIONS

The cloning and expression of domain A and domain C of rat synapsin Ia were successfully performed and their interactions with phospholipid membranes were analyzed. The protein-phospholipid interaction was performed in the presence of a positive control (bovine synapsin Ia) and a negative control (GST). It was shown that both the A and C domains of synapsin Ia are capable of binding to liposomes containing acidic phospholipids such as phosphatidylserine. Maximum binding was in the presence of 50 mol % PS. The involvement of both A and C domains of synapsin I in phospholipid binding helps to explain the tight binding of full length synapsin I to phospholipid membranes.

6.0 APPENDIX

6.1 The amino acid sequence of pET15b vector that contains the coding region of rat synapsin Ia. The primary structure of rat synapsin Ia is shown in bold.

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1      FLKTKGPRDT PIFIG*CHDN NGFLDVRWHF SGKCARNPYL FIFLNTFKYV
51     SAHETITLIN ASIILKKEEY EYSTFPCRPY SLFCGILPSC FCSPRNAGES
101    KRC*RSVGCT SGLHRTGSQQ R*DP*EFSR RTFSNDEHF* SSAMWRGIIP
151    C*RRARATRS PHTLFSE*LG *VLTSHRKAS YGWHDSKRIM QCCHNHE**H
201    CGQLTSDNDR RTEGANRFFA QHGGSCNSP* SLGTGAE*SH TKRRA*HHDA
251    CSNGNMVAQT INWRTTYSSF PATINRLDGG G*SCRRTSAL GPSGWLVC*
301    *IWSR*AWVS RYHCSTGARW *ALPYRSYLH DGESGNYG*T K*TDR*DRCL
351    TD*ALVTVRP SLLIYTLD*F KTSFLI*KDL GEDPF**SHD QNPLT*VFVP
401    LSVRPRRKDQ RIFLRSFFSA RNLLLANKKT TATSGGLFAG SRATNSFSEG
451    NWLQQSADTK YCPSSVAVVR PPLQELCSTA YIPRSANPVT SGCCQWR*VV
501    SYRVGLKTIV TG*GAAVGLN GGFVHTAQLG ANDLHRTEIP TA*AMRKRHA
551    SRREKGGQVS GKRQGRNRRR HEGASRGKRL VSL*SCRVSP PLT*ASIFVM
601    LVRGAEPMEK RQQRGLFTVP GLLLAFCSHV LSCVIP*FCG *PYRL*VS*
651    YRSQPNDRA QRVSERGSGR APDAVFSPYA SVRYFTPHIW CTLSTICSDA
701    A*LSQYTLRY RYVTGSWLRP DTRQHPLTRP DGLVCSRHPL TDKL*PSPGA
751    ACVRGFHRHH RNARGSCGKA HQRGREAIHR CLPVHPRPAR *VSPEALMSG
801    F**SGPC*GR FFPVWSLMPP CKGDFCSWG* *YR*NERGCS RYGLLMMNMP
851    GYWNVVRVNN WRYGCGGTRE KSLRVNASAS LIQM*VFHRV ASSILRCRSG
901    T*WCRAL TSA FPDFTKHGNR RPFMLLLRSQ TFCSSSRFTF ARVSVIHSAN
951    Q*GNPASLAG SSTTGARSCA PVARTQRCPR CAACGCWRWR TRWICSAGKW
1001   FAHSQFSARI DWLQFLEW*I R*GAAGFHS GRGGPAPCTA TQGEADKV*
1051   GGAYNPCQPV PCARRGGINR RDDQRSSDRS *AGKSRRSL KLSLMVVIYL
1101   PGQHGLQRGH PDAAGSEKNH NGEHPASRR ERQQDVAQRV GRHAGDNGLL
1151   LAETFGGTS DEGLSEGVQD SEYRKRQADH RRAPAKAVLA ENDPERCRHL
1201   SYELHDKEDS HKCGDDSHAP RPPEGADWVE GSQGHRSRSL CLMSELTIN
1251   CVALTARFPV GKPVPVPAALM NRPTRGERRF AYWAPGWFFF SPVRRATADC
1301   PSPPGPERVA ASGPRWFAPA GENPV*WWLT AGYNMSCLRY RRIPLPRYPH
1351   QRAARTR*WR ALRPAPSDRW QPASQWERC PHSFAFWFVEN RTWHSSRLPV
1401   PLSAEFDCE* DIYASQPDAD APRQNLMGPL TARFAGDPMR PDAPRPVAYR
1451   LHGRK*YC*W VSGQRHQEIT PEH*CRQLPQ QWHPGHPADS **SAH*RVAR
1501   EDCAPPLYRL RRRFVLPSTP PRWHPVDRRE I*SPRQFATA RAGPDWRWQR
1551   QSATTVCPPV VVPRGWECNS APPSPLPLFP AFSQKRGWPG SPRGKRSDKR
1601   HRHTLRHRIT LLVSHSPP*I DSLPGAIMPY RERFCAIRWC PGSRRSPLCD
1651   SCIRKQPSSR LRPLSTAAAR NGACKEMAPN SPPATGPATI PTPKQALMSP
1701   KWRARSSPSV MSAI*APATA PVAPVMPATM RPA*RIEISI PRN*YDSL*G
1751   NCERITIP* K*FCLTLRRR YTMGSSHHH HHSSGLVPRG SHMNYLRRRL
1801   SDSNFMANLP NGYMTDLQRP QPPPPPPSAA SPGATPGSAA ASAERASTAA
1851   PVASPAAPSP GSSGGGGFTS SLSNAVKQTT AAAAATFSEQ VGGGSGGAGR
1901   GGAAARVLLV IDEPHTDWAK YFKGKKIHGE IDIKVEQAEF SDLNLVAHAN
1951   GGFSVDMENV RNGVKVVRSL KPDFVLIRQH AFSMARNGDY RSLVIGLQYA
2001   GIPSVNSLHS VYNFCDKPVV FAQMVRHLKK LGTEEFPLID QTFYPNHKEM
2051   LSSTTYFVVV KMGHAHSGMG KVKVDNQHDF QDIASVVALT KTYATAEPFI
2101   DAKYDVRVQK IGQNYKAYMR TSVSGMWKTN TGSAMLEQIA MSDRYKLVVD
2151   TCSEIFGGLD ICAVEALHGK DGRDHIEVV GSSMPLIGDH QDEDKQLIVE
2201   LVVNKMTQAL PRQRDASGR GSHSQTPSPG ALPLGRQTSQ QPAGPPAQQR
2251   PPPGGGPPQP GPGFQRQGGP LQQRPPPGGQ QHLISGLGPPA GSPLPQRLPS
2301   PTAAPQQSAS QATPMTQQGQ RQSRPVAGGP GAPPAARPPA SPSPQRQAGP
2351   PQATRQASIS GPAPPKVSGA SPGQQQRQGP PQKPPGPGAG IRQASQAGPG
2401   PRTGPPTTQQ PRPSGPGPAG RPTKPQLAQK PSQDVPPPII AAAGGPPHPQ
2451   LNKSQSLTNA FNLPEPAPPR PSLSQDEVKA ETIRSLRKSF ASLPSD*HLG
2501   *EPPEFCRYP SHWRPLESRI RLLTKPERKL SWLLPPLSNN *HNPLGPLNG
2551   S*GVFC*KEE LYPDIPQEAR QYRHNQAYAY SIQGDGAEDD DERIVRFHTR
2601   CLTALAI*L* *TTALKLIDD KLSNMR

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6.2 Translation of pgexfor.rtf(1-590)

Universal code

Total amino acid number: 191, MW=9436

Max ORF: 3-284, 94 AA, MW=1280

The primary structure of rat synA is shown in bold.

CTGAGTTCCGCGTGGATCCCATATGAACTACCTG
L S S A W I P Y E L P

CGGCGCCGCGCTGTCTGTPARGACAGCAACTTCATGGCCAATCTGCCTAATGGGTATATGACA
A A P P V X X Q Q L H G Q S A * W V Y D

GACCTGCAPARGCGCCCCGCCAGAATTCGCGCGGATCCCATATGAACTACCTGCGGCGCC
R P A X A P A R I P R G S H **M N Y L R R**

SPARCCTGTCTGGACAGCAACTTCATGGCCAATCTGCCTAATGGGTATATGACAGPARACC
R X L S D S N F M A N L P N G Y M T X X

TGCAGCGCCCGGCAAGAATTCGCGGGTCTGACTCGAGCGGCGGCATCGPARTGACTGACTG
L Q R P A R I P G S T R A A A S X * L T

ACGATCTGCCTCGCGCGTTTCGGTGATGACGGTGAAAACRPARNNNRANNVAAANARNNV
D D L P R A F R * * R * K X X X X X X X

6.3 Translation of jc9-leditcontig.rtf (1-1242)

Universal code

Total amino acid number: 410, MW=40204

Max ORF: 144-1205, 354 AA, MW=39321

The primary structure of rat synC is shown in bold.

TGAATGTATACGACTCACTATAGGCGATTGGCCCTCTAGATGCATGCTCGAGCGGCCGCC
 X N V Y D S L * A I G P L D A C S S G R

AGTGTGATGGATATCTGCAGAATTCGCCCTGCCAGTCGACTGCTGGTCATCGACGAGCCG
 Q C D G Y L Q N S P C Q S T A G H R R A

CACACCGACTGGCAAAATACTTCAAAGGGAAGAAGATCCATGGAGAAATTGACATTAAAG
 A H R L A K Y F K G K K I H G E I D I K

TAGAGCAAGCTGAATTCTCCGATCTCAATCTTGTGGCTCATGCCAATGGTGGATTCTCCG
 V E Q A E F S D L N L V A H A N G G F S

TGGACATGGAAGTTCTTCGGAATGGGGTCAAAGTTGTGAGGTCTCTGAAGCCAGACTTTG
 V D M E V L R N G V K V V R S L K P D F

TGCTGATCCGCCAGCATGCCCTTCAGCATGGCACGTAATGGAGACTACCGCAGTTTGGTCA
 V L I R Q H A F S M A R N G D Y R S L V

TTGGGCTGCAGTATGCTGGGATCCCCAGTGTTAACTCTTTGCATTCTGTCTACAACTTTT
 I G L Q Y A G I P S V N S L H S V Y N F

GTGACAAACCTGGGTGTTTGCCCGAGATGGTTCGACTACACAAGAAGCTTGGAAACAGAGG
 C D K P W V F A Q M V R L H K K L G T E

AATTCCTCTGATTGATCAGACTTTCTATCCCAATCATAAAGAGATGCTCAGCAGCACAA
 E F P L I D Q T F Y P N H K E M L S S T

CATACCCTGTAGTTGTGAAGATGGGCCACGCACACTCTGGGATGGGCAAGGTCAAGGTAG
 T Y P V V V K M G H A H S G M G K V K V

ACAACCAACATGACTTCCAGGATATTGCAAGTGTTGTGGCACTGACTAAGACATATGCCA
 D N Q H D F Q D I A S V V A L T K T Y A

CTGCTGAGCCCTTCATTGATGCTAAATACGATGTGCGTGTCCAGAAGATTGGGCAGAACT
 T A E P F I D A K Y D V R V Q K I G Q N

ACAAGGCCTACATGAGGACATCAGTGTCAAGGAACTGGAAGACCAATACAGGCTCTGCTA
 Y K A Y M R T S V S G N W K T N T G S A

TGCTTGAGCAGATTGCTATGTCTGACAGGTACAAGTTGTGGGTAGACACGTGCTCAGAGA
 M L E Q I A M S D R Y K L W V D T C S E

TTTTTGGGGGACTTGACATCTGCGCAGTGGGAAGCACTGCATGGCAAGGACGGAAGGGATC
 I F G G L D I C A V E A L H G K D G R D

ACATTATTGAGGTGGTGGGCTCCTCCATGCCACTCATTGGGGATCACCAGGATGAAGACA
 H I I E V V G S S M P L I G D H Q D E D

AGCAGCTCATCGTGGAACTTGTGGTCAACAAGATGACTCAGGCTCTGCCTCTCGAGCGGG
 K Q L I V E L V V N K M T Q A L P L E R

AAGGGCGAATTCCAGCACACTGGCGGCCGTTACTAGTGGATCCGAGCTCGGTACCAAGCT
 E G R I P A H W R P L L V D P S S V P S

TGGCGTAATCATGGTCATAGCTG
 L A * S W S *

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