Identification of synapsin I peptides that insert into lipid membranes

James J. CHEETHAM*[1], Sabine HILFIKER*[2], Fabio BENFENATI†[3], Thomas WEBER‡, Paul GREENGARD* and Andrew J. CZERNIK*

*Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University, 1230 York Avenue, New York, NY 10021, U.S.A., †Department of Experimental Medicine, Section of Human Physiology, University of Genova, Viale Benedetto XV, 3, I-16132 Genova, Italy, and ‡Cellular Biochemistry and Biophysics Program, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021, U.S.A.

The synapsins constitute a family of synaptic vesicle-associated phosphoproteins essential for regulating neurotransmitter release and synaptogenesis. The molecular mechanisms underlying the selective 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 N-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. To precisely identify the domains of synapsin I which mediate the interaction with lipids, synapsin I was bound to liposomes containing the membrane-directed carbene-generating reagent 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine and subjected to photolysis. Isolation and N-terminal amino acid sequencing of ^{125}I-labelled synapsin I peptides derived from CNBr cleavage indicated that three distinct regions in the highly conserved domain C of synapsin I insert into the hydrophobic core of the phospholipid bilayer. The boundaries of the regions encompass residues 166–192, 233–258 and 278–327 of bovine synapsin I. These regions are surface-exposed in the crystal structure of domain C of bovine synapsin I and are evolutionarily conserved among isoforms across species. The present data offer a molecular explanation for the high-affinity binding of synapsin I to phospholipid bilayers and synaptic vesicles.

Key words: hydrophobic labelling, phospholipid, synaptic vesicle.

INTRODUCTION

Synapsins are a family of neuron-specific, synaptic vesicle (SV)-associated phosphoproteins which are implicated in the regulation of neurotransmitter release and synapse formation [1–3]. The vertebrate synapsin family comprises at least three genes (synapsins I, II and III), and alternative splicing gives rise in neurons to at least five distinct protein isoforms (synapsins Ia, Ib, IIa, IIb and IIIa). Large parts of the N-terminal regions (comprising domains A, B and C) of all synapsins are highly conserved, whereas the C-terminal regions are divergent [4–7].

Synapsin I binds to SVs and to cytoskeletal elements such as actin *in vitro* [8–11]. The synapsins bind to both actin monomers and filaments and are both necessary and sufficient for the reversible attachment of SVs to actin filaments [12–14]. These and other observations have led to a model in which the synapsins tether SVs to each other and/or to cytoskeletal components in the presynaptic nerve terminal, thereby regulating the availability of SVs for exocytosis [2]. This model is supported by a multitude of studies *in vivo*, which show that synapsins are essential for the maintenance of a reserve pool of SVs that is used during times of high synaptic activity [15–22].

The binding of synapsin I to SVs has been characterized in great detail. The binding displays very high affinity (K_d = 10 nM) and saturability, and is mediated by interactions with both protein and phospholipid components [11,23]. While the C-terminal region of synapsin I has been shown to bind specifically to protein components of the vesicle, including an SV-associated form of calcium/calmodulin-dependent protein kinase II [24], the N-terminal region is known to interact with acidic phospholipids of the cytoplasmic leaflet of the vesicle [25].

The interaction of synapsin I with pure phospholipid vesicles displays an affinity and saturability similar to its binding to SVs, and involves both electrostatic and hydrophobic interactions with the surface and the core of the bilayer, respectively [23]. It is envisioned that, after an initial specific targeting step, the binding of synapsin I to the SV membrane is initiated by close contact mediated by an electrostatic surface interaction with acidic phospholipids [25].

Phospholipid binding of synapsin I induces a conformational change in the protein, with an increase in the α-helix content [26]. This conformational change is thought to be followed by insertion of the N-terminal region of synapsin I into the hydrophobic core of the membrane [23,25–27]. Studies using the non-perturbing technique of fluorescence resonance energy transfer have demonstrated that, at the steady state, the hydrophobic interaction is the more prominent of the interactions that account for the binding of synapsin I to phospholipid bilayers [27].

Recently, the phospholipid interaction of synapsin I has been proposed to be mediated by domain A, a short ($\approx$ 30 residues) conserved domain at the N-terminus of the protein [28]. However, these studies were performed using recombinant domains as fusion proteins, an approach that can give rise to artifacts due to incorrect folding of individual domains, and phospholipid-

Abbreviations used: NTCB, 2-nitro-5-thiocyanobenzoic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SV, synaptic vesicle; [^{125}I]TID, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine; [^{125}I]TID-PC/16, 1-palmitoyl-2-[9-[2′-[^{125}I]iodo-4′-(trifluoromethyldiazirinyl)-benzyloxycarbonyl]-nonaoyl]-*sn*-glycero-3-phosphocholine.

[1] Present address: Department of Biology, Carleton University, 1125 Colonel By Drive, Ottawa, ON, Canada K1S 5B6.
[2] Present address: University of Manchester, School of Biological Sciences, 2.205 Stopford Building, Oxford Road, Manchester M13 9PT, U.K.
[3] To whom correspondence should be addressed (e-mail benfenat@csita.unige.it).

binding experiments were performed in the presence of detergent, which eliminates most hydrophobic interactions.

In the present study, using native, full-length synapsin I, we have identified specific regions in synapsin I that insert into phospholipid membranes, using a combination of hydrophobic photoaffinity labelling with the membrane-directed carbene-generating reagent 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)-diazirine ([^{125}I]TID) and protein microsequencing techniques. We identified three regions within domain C, the largest and most conserved domain within the synapsin family, which penetrate the lipid bilayer. These three regions are evolutionarily conserved amongst synapsins from different species, are all surface-exposed and contain regions of amphipathic nature in the crystal structure, suggesting that they are essential domains for the high-affinity binding of synapsin I to SVs.

MATERIALS AND METHODS

Materials

Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) purified from bovine brain were obtained from Avanti Polar Lipids (Alabaster, AL, U.S.A.). The purity of phospholipids was verified using TLC. Dicetylphosphate, stearylamine, cholesterol, octyl glucoside and 2-nitro-5-thiocyanobenzoic acid (NTCB) were purchased from Sigma (St. Louis, MO, U.S.A.); [^{125}I]TID (specific activity 10 Ci/mmol) and [^{14}C]PC (specific activity 0.1 Ci/mmol) were purchased from Amersham (Arlington Heights, IL, U.S.A.). 1-Palmitoyl-2-[9-[2′-[^{125}I]iodo-4′-(trifluoromethyldiazirinyl)-benzyloxycarbonyl]-nonaoyl]-*sn*-glycero-3-phosphocholine ([^{125}I]TID-PC/16) was synthesized as described previously and purified by TLC to a specific activity of 2000 Ci/mmol [29]. Nitrocellulose membranes (0.2 μm pore size) were from Schleicher & Schuell (Dessel, Germany); PVDF membranes were from Millipore (Bedford, MA, U.S.A.). All other chemicals were of reagent grade.

Protein and SV purification

Synapsin I was purified from bovine brain under non-denaturing conditions as described previously [8,11] and stored in 200 mM NaCl/25 mM Tris/HCl, pH 7.4 (synapsin buffer) at −80 °C. Purified synapsin I was cleaved at cysteine residues (Cys^{223}, Cys^{360} and/or Cys^{370}) with NTCB as described previously [25,30]. The total NTCB digest was dialysed against synapsin buffer containing decreasing concentrations of urea (from 4 to 0 M). The C-terminal fragment of synapsin I generated by NTCB cleavage (molecular masses of 39–40 and 35–36 kDa for the synapsin Ia and Ib isoforms, respectively) was purified to homogeneity by Sephadex G-150 gel filtration followed by Mono S cation-exchange chromatography [30] and stored in synapsin buffer at −80 °C. The integral SV protein synaptophysin was extracted from rat forebrain membrane preparations, purified by affinity chromatography (monoclonal antibody 7.1 column [31]) and stored in PBS/0.1 % octyl glucoside. Synaptic vesicles were purified from rat neocortex through the step of controlled-pore glass chromatography and stored in 300 mM glycine/5 mM Hepes, pH 7.4 (buffer A). Depletion of endogenous SV-bound synapsin I was performed by dilution in 200 mM NaCl as described previously [10].

Preparation of phospholipid vesicles

Phospholipids and cholesterol were dissolved in 2:1 (v/v) chloroform/methanol, stored at −20 °C in the dark under argon and used within 1 month. Aliquots of phospholipid solutions were added to Pyrex tubes (13 × 100 mm) to obtain the following liposome compositions: pure PC liposomes; phospholipid liposomes mimicking the phospholipid composition of SV (mixed phospholipid liposomes, 45 % PC/35 % PE/14 % PS/6 % PI, with values given as percentages of the total phospholipid [25]); and PC liposomes containing various amounts of either positively or negatively charged phospholipids (stearylamine, dicetylphosphate, PS or PI) as indicated. To all phospholipid mixtures, cholesterol was added to 1/10 of the total phospholipid weight. Samples were dried under a stream of argon and subsequently held under vacuum for 2 h in a rotary evaporator to remove the last traces of solvent. Lipid films were suspended in 0.5–1 ml of buffer A containing 40 mM KCl/1 mM EDTA by vigorous mixing with a tube vortex. Large unilamellar vesicles were prepared by freeze–thawing the lipid suspension five times, followed by extrusion through two stacked polycarbonate membranes (100 nm pore diameter) for a total of 11 times using the LiposoFast Basic extrusion device (Avestin, Ottawa, ON, Canada). Liposome size was verified using electron microscopy with negative staining [32].

For binding assays, liposomes were labelled with a trace amount of [^{14}C]PC added to the phospholipid mixture before evaporation and, for quantitative high-speed sedimentation assays, liposomes were loaded with 10 % sucrose added to buffer A during vesiculation [25]. Synaptophysin was reconstituted into PC vesicles by dissolving the dried phospholipid with the purified protein in buffer A containing 3 % octylglucoside (detergent : lipid ratio > 10) followed by detergent removal by gel filtration (1.5 × 20 cm Sephadex G-50 column) or dialysis against buffer A as described previously [23]. The amount of synaptophysin incorporated into the bilayer ranged from 5 to 15 ng/μg of PC.

Synapsin I binding assays

The interactions between synapsin I and phospholipid vesicles of various compositions and the extent of liposome aggregation were analysed following two distinct procedures.

High-speed sedimentation assays

The binding of synapsin I to sucrose-loaded phospholipid vesicles (15–25 μg of phospholipids) was carried out in buffer A containing 40 mM NaCl and 0.1 mg/ml BSA, as described previously [25]. Synapsin I was added at concentrations ranging from 5 to 150 nM and samples (100 μl) were incubated on ice for 60 min. After the incubation, samples were layered on to a sucrose cushion (5 % sucrose in buffer A) and subjected to ultracentrifugation at 200000 ***g*** for 30 min to separate bound from free synapsin I. Pellets were resuspended in 40 μl of PBS/1.4 % (v/v) Triton X-100 and spotted on to nitrocellulose sheets for quantification of the synapsin by dot immunobinding as described previously [33]. The liposome recovery in the pellets was determined by liquid-scintillation counting of [^{14}C]PC and used to correct the amounts of synapsin I bound to the vesicles.

Non-equilibrium sucrose-density-gradient separations

Synapsin I (20 μg; 300 nM total concentration) was incubated with either pure PC or mixed phospholipid liposomes (2.5 mg of phospholipid) for 60 min at 4 °C in buffer A containing 40 mM NaCl and 0.1 mg/ml BSA (0.8 ml final volume). Samples were loaded on to a continuous 5–24 % (w/v) 10 ml sucrose gradient in buffer A and centrifuged at 200000 ***g*** for 2 h in a Beckman SW41 rotor. After the run, sucrose gradients were fractionated

into 0.5 ml fractions and analysed for sucrose concentration, [^{14}C]PC radioactivity and synapsin I immunoreactivity as described above.

Hydrophobic photoaffinity labelling

Hydrophobic photolabelling was carried out by using two carbene-generating membrane-directed reagents ([^{125}I]TID and [^{125}I]TID-PC/16), which have been employed previously to selectively label the membrane-embedded domains of proteins [23,25,29,34–36]. Liposomes of various phospholipid compositions (200 μg of phospholipid), PC vesicles into which synaptophysin was reconstituted (200 μg of phospholipid) or purified SVs (100 μg of total protein) were incubated in the absence or presence of synapsin I (240 nM final concentration), prespun total NTCB digest of synapsin I (resulting from 20 μg of synapsin I) or purified synapsin Ia/Ib C-terminal fragments (500 nM) in 500 μl of buffer A/40 mM NaCl in Pyrex test tubes (13 × 100 mm) for 60 min at 4 °C to achieve maximum binding. Occasionally, BSA (50 μg) was added to the incubation mixture to prevent non-specific binding. At the end of the incubation, 1–2 μCi of [^{125}I]TID in ethanol was added (final ethanol concentration $< 0.5\,\%$) and the samples were incubated under an argon atmosphere for 30 min at room temperature in the dark. Samples were photolysed for 2–5 min at room temperature with a 100 W near-UV lamp (maximum emission, 360 nm) equipped with a circulating cold water bath and a saturated $CuSO_4$ filter to screen out IR and short-wave UV light, respectively. After photolysis, samples were transferred to microcentrifuge tubes, and three volumes of chloroform/methanol (1:2, v/v) were added to remove phospholipids and excess [^{125}I]TID [37]. Samples were incubated for 30 min at room temperature and spun in a microcentrifuge for 5 min at maximal speed. Pellets containing [^{125}I]TID-labelled proteins were dried in a rotary evaporator for 30 min and subjected to SDS/PAGE or to CNBr digestion. In the latter case, hydrophobic labelling samples contained 100 μg of synapsin I and 5 mg of phospholipid vesicles. To ascertain that protein labelling with [^{125}I]TID was representative of a true hydrophobic interaction with the core of the phospholipid bilayer, liposomes containing 1 μCi of the PC analogue [^{125}I]TID-PC/16 [29] were also prepared. The reagent, bearing the photoactivatable diazirine moiety at the end of the aliphatic chain, was added to the phospholipid mixture to become permanently incorporated into the phospholipid bilayer during vesiculation. After binding of synapsin I, hydrophobic labelling experiments with [^{125}I]TID-PC/16-containing liposomes were performed as described above.

CNBr cleavage of ^{125}I-labelled synapsin I, and isolation and N-terminal amino acid sequence determination of ^{125}I-labelled synapsin I fragments

After recovery of the ^{125}I-labelled synapsin I, the sample was extracted with chloroform/methanol (2:1, v/v) three times to remove any non-covalently bound [^{125}I]TID- and [^{125}I]TID-labelled phospholipids. After chloroform/methanol extraction, the ^{125}I-labelled synapsin I was solubilized in 50 μl of 70 % formic acid, to which 0.1 mg of solid CNBr was added, followed by vigorous mixing. The sample was incubated overnight in the dark at room temperature under a nitrogen atmosphere. After cleavage, 1 ml of water was added and the sample was dried in a vacuum evaporator before resolubilization in 20 % formic acid. CNBr-derived peptides were separated from undigested synapsin I by reversed-phase HPLC using a diphenyl column (Vydac, 0.46 × 25 cm) and eluted with a 20–95 % gradient of acetonitrile in 0.1 % trifluoroacetic acid. Peptide absorbance was monitored at 214 nm. Fractions (0.5 ml) were collected and monitored for ^{125}I with a gamma counter. Radioactive fractions were dried down in a rotary evaporator and subjected to SDS/PAGE using 10–20 % polyacrylamide gradient gels (Novex, San Diego, CA, U.S.A.) using a Tris/Tricine buffer system for separation [38]. The resolved peptides were transferred to PVDF membranes and localized by autoradiography. Selected ^{125}I-labelled peptides were excised from the blot and subjected to automated N-terminal amino acid sequencing as described previously [39,40].

Miscellaneous techniques

Protein concentrations were determined using the BCA assay (Pierce, Rockford, IL, U.S.A.) or the Bradford assay (Bio-Rad, Milan, Italy), using BSA as standard. SDS/PAGE was performed as described in [41]. After electrophoresis, gels containing ^{125}I-labelled proteins were stained, destained, dried and exposed to Kodak X-OMAT films at −80 °C with an intensifying screen. Electrophoretic transfer to PVDF membranes was carried out as described in [42]. The immunolabelling of nitrocellulose sheets was performed using anti-synapsin I polyclonal antibodies followed by [^{125}I]Protein A overlay. Immunoprecipitation of synapsin I and synaptophysin from [^{125}I]TID-labelled purified SVs was carried out as described previously [23]. Saturation binding curves were analysed using the non-linear fitting procedure of Sigmaplot 3.0 (Jandel Co., San Rafael, CA, U.S.A.).

To determine the location of the labelled CNBr fragments, the co-ordinates for the three-dimensional X-ray crystallographic structure of domain C of synapsin I (1AUV) were downloaded from the Brookhaven Protein Database (http://www.rcsb.org/pdb/cgi/explore.cgi?pdbId = 1AUV) and imported into WebLab Viewer 2.0 (Molecular Simulations). Sequence alignments were carried out using Clustal X [43]. Secondary-structure and solvent-accessibility predictions were obtained from http://www.cmpharm.ucsf.edu/~nomi/nnpredict.html.

RESULTS

Phospholipid specificity of the synapsin I binding to liposomes

The binding of synapsin I to liposomes is known to consist of both electrostatic and hydrophobic interactions [25]. To address the electrostatic nature of synapsin I binding to phospholipid vesicles, we generated saturation curves of synapsin I binding to sucrose-loaded liposomes following high-speed sedimentation (Figure 1A). No significant synapsin I binding was found with zwitterionic (pure PC) or positively charged (PC/stearylamine) liposomes, indicating that the high surface activity of synapsin I [44] is not sufficient to ensure binding, which suggests that an electrostatic surface interaction of synapsin I with liposomes is mediated via negatively charged phospholipids. Synapsin I binding to negatively charged phospholipids showed a binding preference among various negatively charged headgroups. While it was not possible to detect saturable binding of synapsin I to negatively charged liposomes containing dicetylphosphate, saturable binding was observed to occur with mixed phospholipid liposomes containing an equal amount of PS/PI (14 % PS/6 % PI). Binding of synapsin I to PC liposomes containing either PS or PI revealed that the two acidic phospholipids were equally effective in binding synapsin I, and that the amount of synapsin I bound at saturation paralleled their concentrations in the phospholipid bilayer. The data suggest that an electrostatic surface interaction is essential for synapsin I binding to liposomes

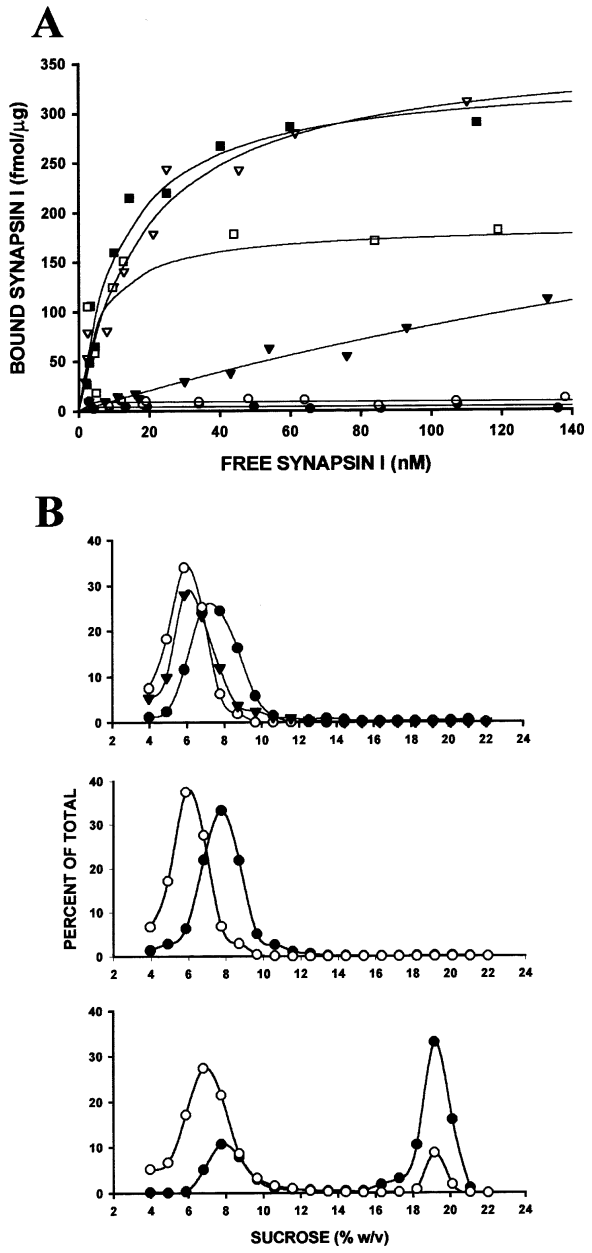

Figure 1 Specificity of synapsin I binding to phospholipid vesicles

(**A**) Sucrose-loaded phospholipid vesicles composed of 100% PC (○); 45% PC/35% PE/14% PS/6% PI (mixed phospholipid liposomes, □); 45% PC/35% PE/20% stearylamine (●); 45% PC/35% PE/20% dicetylphosphate (▼); 50% PC/50% PS (▽) or 50% PC/50% PI (■) were incubated with increasing concentrations of synapsin I in standard assay buffer with 40 mM NaCl equivalent (20 μg of phospholipid/sample; 5–150 nM synapsin I). Bound synapsin I was separated by ultracentrifugation through a 5% (w/v) sucrose cushion, quantified by dot immunobinding, corrected for vesicle recovery and expressed in fmol/μg of phospholipid. The corresponding saturation curves were fitted using a non-linear regression procedure following the simple model of one ligand, one non-cooperative binding site (mixed phospholipid liposomes, K_d 9$\pm$4 nM, B_{max} 185$\pm$22 fmol/μg of phospholipid; 50% PC/50% PS, K_d 14$\pm$5 nM, B_{max} 360$\pm$26 fmol/μg of phospholipid; 50% PC/50% PI, K_d 12$\pm$3 nM, B_{max} 336$\pm$20 fmol/μg of phospholipid; 45% PC/35% PE/20% dicetylphosphate, K_d $>$300 nM). No significant binding was observed with 100% PC or 45% PC/35% PE/20% stearylamine liposomes. (**B**) The binding of synapsin I to phospholipid vesicles and the subsequent synapsin I-induced vesicle aggregation were evaluated by non-equilibrium sucrose-density-gradient centrifugation. Pure PC or mixed phospholipid (45% PC/35% PE/14% PS/6% PI) liposomes labelled with a trace amount of [^{14}C]PC (2.5 mg of phospholipid) were incubated either alone or in combination with synapsin I (20 μg) for 60 min at 4 °C as described in the Materials and methods section. Samples were loaded on to a continuous 4–22% (w/v) sucrose gradient and centrifuged at 200000 ***g*** for 2 h. Gradients were fractionated into 0.5 ml fractions and analysed for sucrose concentration, indicated on the *x*-axis, as well as for [^{14}C]PC radioactivity and synapsin I immunoreactivity, indicated on the *y*-axes as percentages of the total amount loaded on to the gradient. The top panel shows the sedimentation patterns of synapsin I (●), PC liposomes (○) and mixed liposomes (▼) incubated alone. The sedimentation patterns of synapsin I (●) and liposomes (○) following incubation of synapsin I with either pure PC liposomes or mixed liposomes are shown in the middle and bottom panels, respectively.

and that the binding displays some degree of phospholipid specificity with preference for PS and PI.

Synapsin I binding to phospholipid vesicles was reported to lead to liposome aggregation attributable to synapsin–lipid and synapsin–synapsin interactions [45,46], and can thus be measured using non-equilibrium sucrose-density-gradient centrifugation. Pure PC or mixed phospholipid vesicles in the absence of synapsin I were restricted to the upper part of the sucrose gradient (4–8% sucrose), as was synapsin I alone (Figure 1B, top panel). The migration pattern was unchanged when synapsin I was incubated with pure PC vesicles (Figure 1B, middle panel). On the other hand, distinct peaks of phospholipid and synapsin appeared in the lower part of the gradient (17–21% sucrose) when synapsin I was incubated with vesicles containing a mixture of phospholipids that mimicked the phospholipid composition of SVs (Figure 1B, bottom panel), indicating that specific binding of synapsin I was accompanied by liposome aggregation. Under the specific conditions used (synapsin:phospholipid molar ratio = 1:13000; estimated stoichiometry at saturation = 1 mol of synapsin I/900 mol of acidic phospholipid [25]), 62% of total synapsin I was bound to mixed phospholipid vesicles (Figure 1B, bottom panel).

Hydrophobic labelling of synapsin I bound to liposomes and SVs using [^{125}I]TID

To address whether the interaction of synapsin I with liposomes or with the phospholipid component of purified SVs involves the penetration of the protein into the hydrophobic core of the membrane, hydrophobic photoactivatable lipid probes known to selectively label the membrane-embedded domains of integral membrane proteins were used [23,25,34,35,47]. When synaptophysin, an SV protein containing four transmembrane regions, was reconstituted into pure PC vesicles containing 1 μCi of [^{125}I]TID, it was markedly labelled with ^{125}I upon photolysis (Figure 2A, left-hand panel). When synapsin I was incubated with [^{125}I]TID-labelled pure PC vesicles, no significant synapsin labelling was observed, demonstrating the failure of synapsin I to bind to PC liposomes. In contrast, labelling of synapsin I occurred after incubation with liposomes containing acidic phospholipids (PS and PI) and mimicking the composition of the SV membrane. Increasing the amounts of PS resulted in a progressive increase in the incorporation of the radioactive label into the synapsin I molecule (Figure 2A, left-hand panel). These results confirm the hypothesis that the association of synapsin I with liposomes, which is initiated by an electrostatic interaction with anionic phospholipids, is followed by a direct penetration of selected regions of the molecule into the hydrophobic core of the membrane [25,27].

To ensure that interactions between hydrophobic regions in synapsin I and the hydrophobic core of the membrane also occur in SVs, [^{125}I]TID labelling was performed using intact SVs purified from rat brain (Figure 2A, right-hand panel). The total labelling pattern indicated that several protein bands, which primarily represent known integral membrane proteins, were labelled by [^{125}I]TID. Immunoprecipitation of synaptophysin and synapsin I showed that both proteins were labelled,

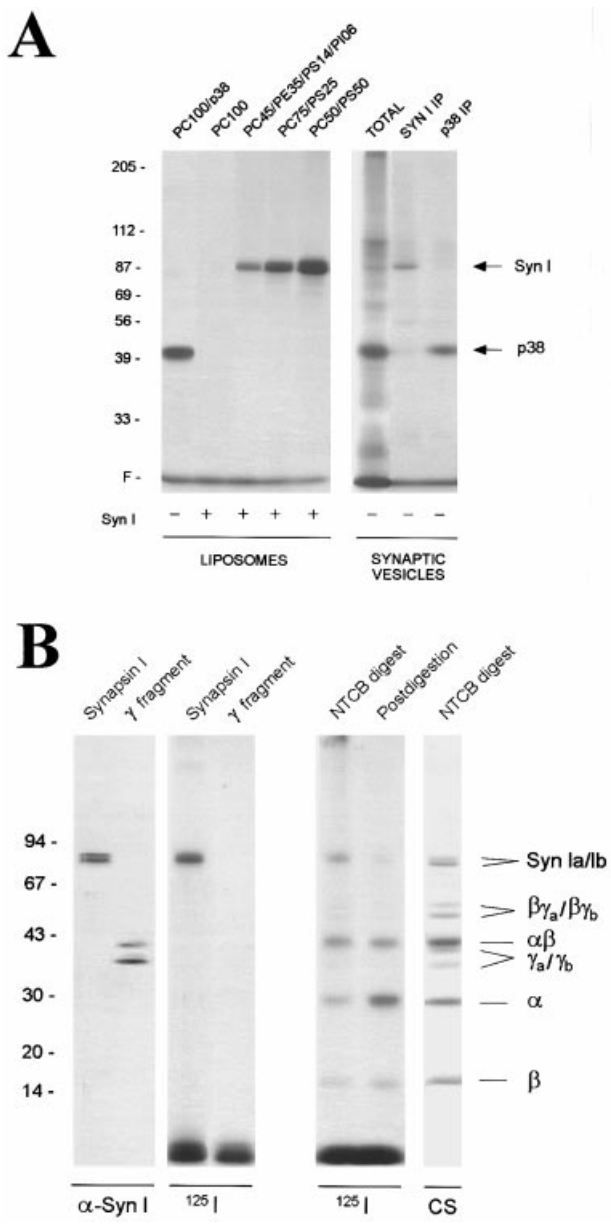

Figure 2 Characterization of the hydrophobic labelling of synapsin I

(**A**) Hydrophobic labelling of synapsin I and synaptophysin in phospholipid vesicles and purified SVs. Left-hand panel: purified bovine brain synapsin I (Syn I, 10 μg/sample) was incubated in standard assay buffer with pure PC liposomes (100% PC), mixed phospholipid liposomes (45% PC/35% PE/14% PS/6% PI) or PC liposomes containing increasing amounts of PS (75% PC/25% PS and 50% PC/50% PS) (200 μg of phospholipid/sample). After incubation for 60 min on ice, phospholipid membranes were labelled by the addition of a trace amount of [^{125}I]TID (1 μCi/sample) and the samples irradiated with UV light, delipidated and subjected to SDS/PAGE and autoradiography, as described in the Materials and methods section. Synaptophysin containing PC liposomes (PC100/p38) were also subjected to the same labelling procedure. Right-hand panel: purified, untreated SVs (TOTAL) were labelled with [^{125}I]TID, photolysed and delipidated as described above. Aliquots of the samples were solubilized and subjected to immunoprecipitation for synapsin I (SYN I IP; polyclonal antibody G93) and synaptophysin (p38 IP; monoclonal antibody 7.1). Molecular-mass markers are shown on the left in kDa. F, dye front. (**B**) Specificity of the [^{125}I]TID hydrophobic labelling of synapsin I. Sucrose-loaded mixed phospholipid liposomes (45% PC/35% PE/14% PS/6% PI; 200 μg/sample) were incubated with either synapsin I (10 μg/sample), purified synapsin I C-terminal fragment (γ fragment; 20 μg/sample) or with a total NTCB digest obtained from cleavage of 30 μg of synapsin I that had been cleared previously by ultracentrifugation to remove fragment aggregates. After incubation for 60 min on ice, liposomes were labelled with [^{125}I]TID, photolysed and sedimented by ultracentrifugation. The pellets were resuspended in assay buffer, delipidated and subjected to SDS/PAGE on 7–15% gradient gels followed by autoradiography (^{125}I) and immunoblotting with antibodies specific for the C-terminal region of synapsin I (α-Syn I). In one sample, the liposome-bound, [^{125}I]TID-labelled synapsin I was subjected to NTCB cleavage (postdigestion). The Coomassie Brilliant Blue stain of the NTCB digest (CS) shows the presence of three fragments spanning the synapsin I molecule, namely fragments α (residues 1–222), β (residues 224–359/369) and γ_a/γ_b (residues 361/371–704 and 361/371–668 for synapsins Ia and Ib, respectively), together with undigested synapsin I (Syn I) and two additional fragments ($\alpha\beta$ and $\beta\gamma$) resulting from partial cleavage. Molecular-mass standards are shown on the left in kDa.

suggesting that synapsin I interacts in a similar fashion with phospholipids of intact SVs as well.

The labelling specificity of [^{125}I]TID for membrane-penetrating domains of proteins was previously demonstrated in human erythrocyte membranes in which none of the peripheral membrane proteins incorporated significant amounts of radioactivity [34]. Under our assay conditions, the specificity and topographical precision of the hydrophobic labelling with [^{125}I]TID was ensured in several ways. First, the absence of detectable synapsin I labelling observed with pure PC vesicles (Figure 2A, left-hand panel) represents an internal control and rules out the possibility that weak extramembrane interactions between the reactive label and potential hydrophobic pockets in the synapsin I molecule, or non-specific interactions between synapsin I and the labelled bilayer, account for the labelling of synapsin by [^{125}I]TID. Second, BSA, which coats the surface of liposomes [48,49] and was occasionally included in the reaction mixture, was not labelled by [^{125}I]TID and nor did it affect the efficiency of synapsin I labelling (results not shown). Third, the strongly basic C-terminal region of synapsin I (γ_a/γ_b fragments, corresponding to residues 361/371–704 and 361/371–668 of synapsins Ia and Ib, respectively; where the alternative residue numbers indicate that cleavage of fragments can occur at either or both closely spaced cysteine residues, Cys^{360} and Cys^{370}, and thereby the mentioned peptides can vary in length), which binds weakly to negatively charged liposomes containing acidic phospholipids [25], was not labelled by [^{125}I]TID when it was incubated with liposomes containing acidic phospholipids either as purified fragment (Figure 2B, left-hand panel) or as a mixture of NTCB-generated fragments of synapsin I. Moreover, when intact synapsin I was first labelled with [^{125}I]TID upon liposome binding and subsequently subjected to cysteine-specific cleavage, the C-terminal fragment remained unlabelled and the labelling was specifically confined to the N-terminal and central fragments (fragments α and β, corresponding to residues 1–222 and 224–359/369, respectively; Figure 2B, right-hand panel).

Finally, hydrophobic labelling experiments using [^{125}I]TID-PC/16-labelled liposomes were carried out to confirm that protein labelling with [^{125}I]TID reflected a true hydrophobic interaction with the core of the phospholipid bilayer. The reactive moiety of [^{125}I]TID-PC/16 is linked covalently to the end of the phospholipid acyl chain most distal to the lipid headgroup and resides within the hydrophobic core of the lipid bilayer. This probe labels amino acid residues that penetrate deeply into the bilayer and is much less prone than TID to bind to hydrophobic domains of proteins located outside the bilayer [29]. Using [^{125}I]TID-PC/16-labelled pure PC and mixed phospholipid liposomes, synapsin I was labelled in an anionic phospholipid-dependent manner, as observed with TID (results not shown).

Specific peptide domains involved in synapsin I interactions with the hydrophobic core of the membrane

To identify the domains involved in the interaction with the hydrophobic core of the membrane, synapsin I was labelled with [^{125}I]TID, extracted with chloroform/methanol to remove any

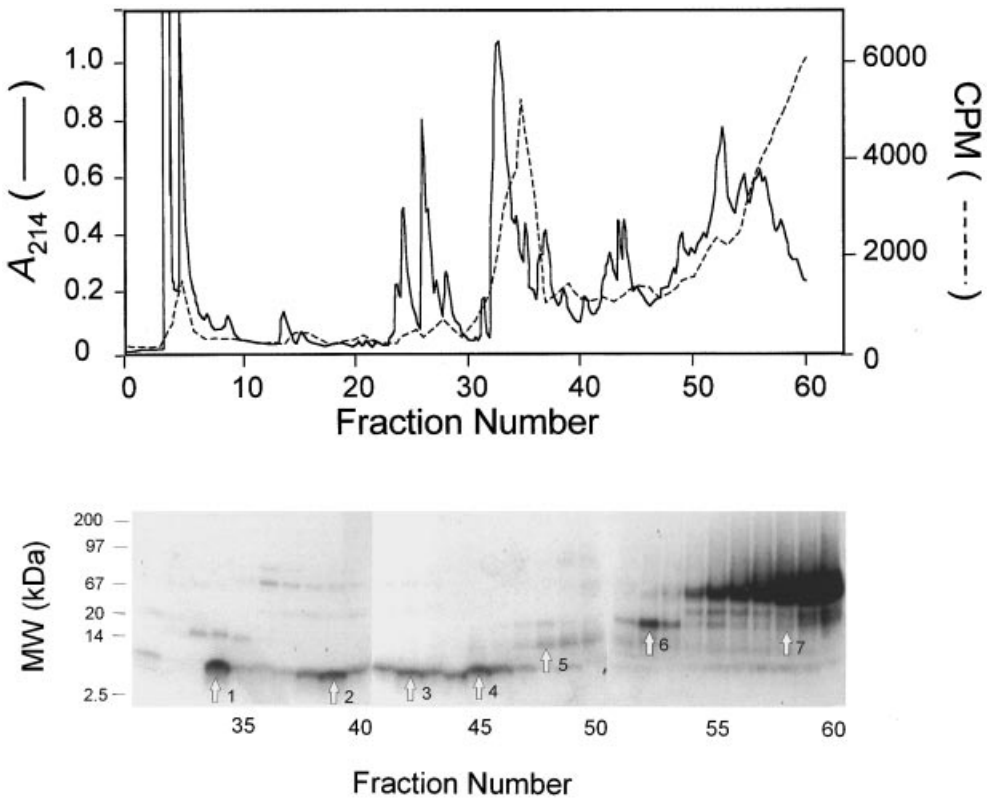

Figure 3 Purification of ^{125}I-labelled CNBr peptide fragments of synapsin I

Top panel: HPLC elution profile of CNBr fragments from a diphenyl column. Absorbance was measured at 214 nm (solid line). Fractions (0.5 ml) were collected and counted for ^{125}I with a gamma counter (broken line). Bottom panel: autoradiography of ^{125}I-labelled peptide fragments separated by SDS/PAGE on 10–20% polyacrylamide gradient gels run with a Tris/Tricine buffer system and transferred electrophoretically to PVDF membranes. Arrows 1–6 indicate the ^{125}I-labelled fragments that were excised from the membrane and subjected to N-terminal amino acid sequencing. Arrow 7 indicates undigested ^{125}I-labelled synapsin I. Molecular-mass markers are shown on the left in kDa.

non-covalently bound radiolabel and subjected to methionine-specific cleavage with CNBr. After cleavage, the resulting peptides were separated by reversed-phase HPLC as described in the Materials and methods section. As indicated by the HPLC profile (Figure 3, top panel), the majority of the radioactivity was recovered in two broad regions encompassing fractions 33–40 and 50–60, and the overall recovery was approx. 80–90%. Peptides in fractions 30–60 were resolved by SDS/PAGE with a Tris/Tricine buffer system, transferred electrophoretically to PVDF membranes and localized by autoradiography (Figure 3, bottom panel). Whereas fractions 54–60 contained significant amounts of undigested synapsin I (arrow 7 in Figure 3, bottom panel), several labelled peptides of various sizes were resolved in fractions 30–53.

Owing to the limited size resolution of the gels, we could not positively identify the peptides based on their apparent molecular masses. To identify the individually labelled peptides, six clearly resolved radioactive bands (arrows 1–6 in Figure 3, bottom panel) were excised from the membrane and subjected to N-terminal amino acid sequencing (Table 1). The N-terminal sequence of fragment 1 corresponded to residues 278–287, suggesting that fragment 1 represented amino acids 278–327 (assuming that CNBr cleavage occurred only after Met^{327}), with a predicted molecular mass of 5.7 kDa, consistent with its migration in the Tris/Tricine SDS/PAGE (Figure 3, bottom panel). By analogy, fragments 2 and 3 were found to correspond to the CNBr cleavage fragment encompassing residues 233–258 with a predicted molecular mass of 3.3 kDa (Table 1). Fragment 4 corresponded to the CNBr cleavage fragment encompassing residues 166–192, with a predicted molecular mass of 3.2 kDa (Table 1). Fragment 5 had the same N-terminal sequence as fragment 4, but migrated as a much larger peptide, indicating incomplete CNBr digestion (Table 1 and Figure 3, bottom panel). Similarly, fragment 6 had the same N-terminal sequence as fragment 1, but migrated as a larger peptide and thus

Table 1 Identification of synapsin I peptides labelled from the hydrophobic core of the lipid membrane

CNBr digestion of ^{125}I-labelled synapsin I peptides and identification of peptides using N-terminal amino acid sequencing. Sequencing was stopped after 8–10 cycles, which provided sufficient information to identify the peptides. The larger peptides (fragments 5 and 6) resulted from incomplete CNBr digestion, and their exact size could not be determined due to the limited size resolution of the Tris/Tricine gels.

Fragment	N-terminal sequence	Residues	Predicted molecular mass (kDa)
1	GKVKVDNQHD...	278–327	5.7
2	VRLHKKLGTE...	233–258	3.3
3	VRLHKKLGTE...	233–258	3.3
4	EVLRNGVK...	166–192	3.2
5	EVLRNGVK...	166–?	?
6	GKVKVDNQHD...	278–?	?

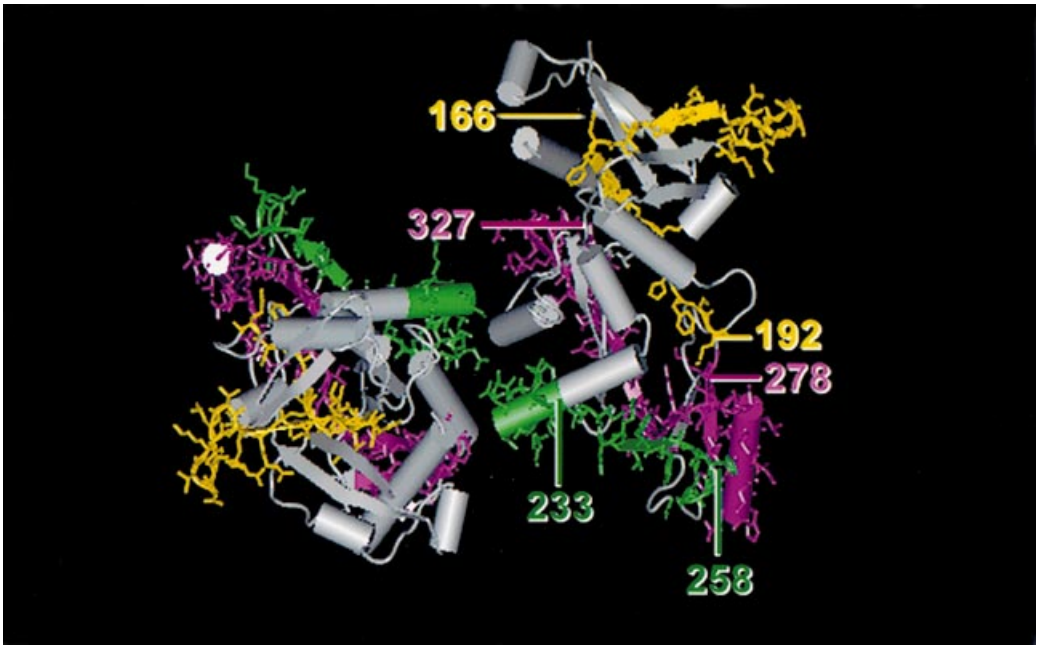

Figure 4 Location of the membrane-penetrating CNBr fragments of synapsin I in the three-dimensional structure of the bovine synapsin I domain C and domain C dimer

The image of the structural model of domain C was generated from the synapsin I domain C co-ordinates (1AUV) downloaded from the Brookhaven Protein Database (see text for details) and imported into WebLab Viewer 2.0. The CNBr fragments identified using hydrophobic photoaffinity labelling are indicated in yellow (amino acids 166–192), green (amino acids 233–258) and purple (amino acids 278–327). The first and last amino acids for each fragment are numbered to indicate orientation of the fragments within the context of the entire domain. Amino acid side chains for the three identified fragments are indicated with sticks.

represented a partial CNBr cleavage fragment (Table 1 and Figure 3, bottom panel).

These data indicate that three regions of synapsin I, encompassing amino acids 278–327, 233–258 and 166–192, were labelled with [^{125}I]TID, and thus were penetrating the hydrophobic core of the lipid membrane, in agreement with the results obtained from cysteine-specific cleavage of synapsin I (Figure 2B). Furthermore, since most of the radioactive label was associated with fragment 1 (Figure 3), the major lipid-binding region of synapsin I appears to be associated with the region bordered by residues 278–327.

Structural features and evolutionary conservation of synapsin I peptide domains which interact with the hydrophobic core of the membrane

The three synapsin I regions that interact with the hydrophobic core of the membrane all reside in domain C, the largest and most conserved structural domain among synapsins. The recently solved three-dimensional structure of domain C of bovine synapsin I [50] allowed us to locate the lipid-binding regions in the three-dimensional context of the entire domain C (Figure 4). The major labelled fragment encompassing amino acids 278–327 contains an amphipathic α-helix (amino acids 285–296), which lies on the outside of the synapsin I domain C structure and does not interact with the rest of this domain. The fragment encompassing amino acids 233–258 is surface-exposed as well, oriented on the same side of the molecule as amino acids 278–327, and contains the C-terminal part of an additional amphipathic α-helix (amino acids 225–239) that may interact with the membrane. Finally, the fragment encompassing amino acids 166–192 forms a loop on the surface of domain C that may fold over to contact the membrane upon binding. This may account for some of the conformational change observed upon synapsin I binding to phospholipid membranes [26]. Comparison of the identified fragments with the three-dimensional structure of the synapsin domain C dimer (Figure 4) confirmed that the three regions are all surface-exposed and generate a lipid-binding surface on one side of the molecule that does not overlap with the dimerization surface. In particular, the two 278–327 fragments (presumably representing the major lipid-binding domain) contributed by both monomers lie on opposite sides with respect to the dimerization axis. In addition, parts of fragment 233–258 and fragment 166–192 lie on the same side as fragment 278–327, forming a lipid-binding surface that is parallel to the dimerization axis. Thus, each synapsin monomer would be predicted to bind one liposome and liposome aggregation would occur by dimerization of synapsins, with the dimer axis away from the lipid-binding surface.

The study of the evolutionary conservation of the three membrane-penetrating sequences in domain C of synapsin from different mammalian and invertebrate species [6] showed that these regions are highly conserved (Figure 5). After sequence alignment of all known synapsin isoforms, from *Caenorhabditis elegans* to human, the mean identity for each amino acid position in bovine synapsin Ia was $53 \pm 21\,\%$, $52 \pm 18\,\%$ and $69 \pm 22\,\%$ for the 166–192, 233–258 and 278–327 peptides, respectively (results not shown). These values are significantly different from the overall mean identity of bovine synapsin Ia ($32 \pm 29\,\%$, $P < 0.005$), suggesting that the three identified, highly conserved regions within domain C may mediate the interaction of other synapsins with phospholipid membranes.

Secondary-structure predictions of the three identified membrane-penetrating regions are in good agreement with the

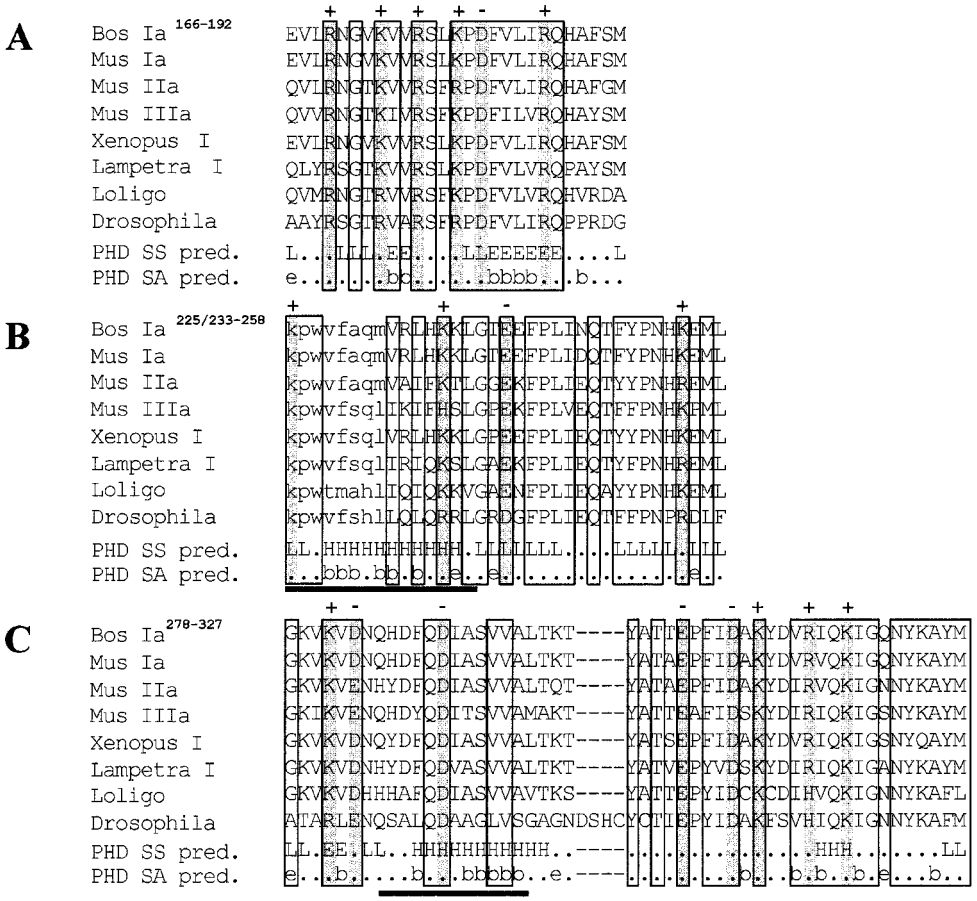

Figure 5 Evolutionary conservation of the membrane-penetrating CNBr fragments of synapsin I

The CNBr fragments identified using hydrophobic photoaffinity labelling of bovine (*Bos*) synapsin I (**A**, amino acids 166–192; **B**, amino acids 233–258; **C**, amino acids 278–327) were aligned with the corresponding sequences of mouse (*Mus*) synapsins Ia, IIa and IIIa, *Xenopus* synapsin I, lamprey (*Lampetra*) synapsin I, squid (*Loligo pealei*) and *Drosophila* synapsin homologues [6] using Clustal W 1.7 (http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.html). Identical amino acid residues are boxed, and conserved charged residues are shaded in grey, with the type of charge indicated on top. Secondary-structure predictions (PHD SS pred.) indicate the subset of the PHDsec prediction for all residues with an expected average accuracy $> 82\%$. H, helical; L, loop; E, β-sheet; a dot means no prediction made. Solvent-accessibility predictions (PHD SA pred.) indicate the subset of the PHDacc prediction for all residues with an expected average correlation > 0.69. Three states are used: b, 0–9%; i, 9–36%; e, 36–100%. Thick bars indicate the amino acid residues which form α-helical structures according to the three-dimensional structure of the entire domain C.

solved three-dimensional structure of the entire domain C (Figure 5) and, together with solvent accessibility predictions and distribution of conserved charged residues, suggest that the hydrophobic side of the amphipathic α-helices in fragment 233–258 (amino acids 225–239) and fragment 278–327 (amino acids 285–296) might interact with the hydrophobic core of the lipid membrane (Figures 5B and 5C). Fragment 166–192 may undergo a conformational change to adopt an amphipathic α-helical conformation, which would penetrate the lipid bilayer in a fashion similar to the other fragments. Alternatively, its solvent-inaccessible region (amino acids 182–185) might loop into the bilayer, with charges on either side neutralizing each other and/or the negatively charged phospholipid headgroups (Figure 5A).

DISCUSSION

In the present study, we show that the interaction of synapsin I with phospholipid membranes involves both electrostatic and hydrophobic interactions. The electrostatic interactions of synapsin I with liposomes display specificity for anionic phospholipids such as PS and PI and are followed by a penetration of part of the synapsin I molecule into the hydrophobic core of the lipid membrane. The boundaries of the regions within synapsin I which penetrate the lipid bilayer have been mapped to amino acids 166–192, 233–258 and 278–327, with the latter segment comprising the major lipid-interacting portion of the molecule. All these regions lie within domain C of the synapsin molecule (amino acids 113–428 in bovine synapsin I), are surface-exposed and conserved amongst synapsins from different species. These results are in complete agreement with the labelling pattern of synapsin I fragments generated by cysteine-specific cleavage after binding to liposomes of identical phospholipid composition in which the label incorporation occurred in the synapsin $I^{1–223}$ and synapsin $I^{223–360/370}$ fragments that include most of domain C [25,30].

The anionic phospholipid dependency of synapsin I binding to liposomes suggests that binding is initiated by an electrostatic surface interaction between positively charged residues of synapsin I and negatively charged phospholipids, whose affinity depends on the size and type of the headgroup. This ionic interaction may be mediated by domain C, or by other domains

within the N-terminal region of synapsin I, such as domain A [25,28]. This is also suggested by the fact that the synapsin $I^{1–223}$ fragment encompassing domains A and B and the N-terminal part of domain C binds with high affinity to mixed phospholipid liposomes and is as effective as holosynapsin I in competitively inhibiting the binding of ^{125}I-labelled synapsin I [25].

The electrostatic interaction with the charged surface of the bilayer is followed by a penetration of synapsin I into the hydrophobic core of the lipid membrane. The regions responsible for this hydrophobic interaction have been mapped to domain C, suggesting that domain C comprises the major lipid-binding domain of synapsin I. The hydrophobic interaction appears considerably stronger than the simple electrostatic interaction [27] and cannot be modulated effectively by site-specific phosphorylation [25]. These observations suggest that the modulation of phospholipid binding after phosphorylation of synapsin I by cAMP-dependent protein kinase recently found in phospholipid-detergent micelles [28] may be of limited physiological significance.

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 [44]. These properties are most likely attributable to the presence of amphiphilic secondary structures within the N-terminal region of the protein that we have confirmed to be present within the stretches of synapsin I domain C that interact with the hydrophobic core of the membrane. Whereas we could not identify the precise amino acid residues that are covalently labelled by [^{125}I]TID and thus penetrate the lipid bilayer, several studies using model peptides or proteins suggest that membrane-penetrating sequences adopt an amphipathic α-helical structure or a loop structure that dips into the bilayer [51–55]. Based on these findings, we suggest that the amphipathic α-helical structures in fragments 233–258 and 278–327 are oriented parallel to the bilayer surface and penetrate the core of the bilayer with the hydrophobic residues and that fragment 166–192 might either adopt an amphipathic α-helical structure upon lipid binding or loop into the bilayer with its hydrophobic residues.

It has been shown that, upon binding to phospholipid bilayers, synapsin I induces vesicle clustering and stabilizes the membrane structure by inhibiting the transition from the stable lamellar phase to the inverted hexagonal phase induced by temperature or Ca^{2+} [45]. This stabilization may be a consequence of the insertion of the three identified regions 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 SVs and by their relative enrichment in non-bilayer structure-preferring phospholipids such as PE. Moreover, the location of the lipid-binding surface formed by the identified fragments in the three-dimensional structure of synapsin domain C exposed on one side of the molecule and distinct from the dimerization plane offers a molecular explanation both for the high-affinity interaction with phospholipid bilayers and for the clustering of adjacent vesicles that is likely to be attributable to the formation of either homo- or hetero-dimers of synapsin molecules [45,46].

The specific targeting of synapsin I to SVs cannot be explained fully by synapsin I–phospholipid interactions, but is likely to involve a 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 membranes of SVs in molecular terms. The synapsin–phospholipid interaction may play an important functional role in clustering SVs and in preserving their remarkably uniform shape and size. The observation that several amino acid sequences in domain C of synapsin I penetrate into the lipid membrane provides a molecular explanation for the high-affinity binding of synapsin I to SVs and the resulting stabilization of the vesicle membrane structure.

We gratefully acknowledge the expert assistance of Joseph Fernandez, Protein/DNA Technology Center, The Rockefeller University, New York, NY, U.S.A. This work was supported by: a National Science and Engineering Research Council (NSERC) Research Grant (to J.J.C.); Consiglio Nazionale delle Ricerche, Telethon (n.1131), Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST) Cofin98 and Fischer Foundation for Alzheimer's Disease grants (to F.B.); and United States Public Health Service (USPHS) grants MH 39327 and AG 15072 (to P.G.).

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Received 3 July 2000/25 October 2000; accepted 4 December 2000