Isoform-specific, Calcium-regulated Interaction of the Synaptic Vesicle Proteins SV2 and Synaptotagmin*

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The identification and functional characterization of proteins localized to synaptic vesicles has contributed significantly to our understanding of neurotransmission. Studies of synaptic vesicle protein interactions have both led to the identification of novel synaptic proteins and suggested hypotheses of protein function. Synaptic vesicle protein 2 (SV2), is an integral membrane glycoprotein present in all synaptic vesicles. There are two characterized isoforms, SV2A and SV2B. Despite their homology to transporter proteins, the function of the SV2s remains unknown. In an effort to determine SV2 function and identify cofactors required for SV2 activity, we examined the protein interactions of SV2 using a combination of cross-linking, immunoprecipitation, and recombinant protein affinity chromatography. We report that SV2 is a large protein complex that contains the synaptic vesicle protein synaptotagmin. The interaction between SV2 and synaptotagmin is direct, specific to SV2A, and inhibited by calcium with an EC50 of approximately 10 μM. Interaction is mediated by the cytoplasmic amino terminus of SV2A and the C2B domain of synaptotagmin. Our observations suggest a regulatory relationship between these two proteins.

Formation and dissociation of protein complexes in the synapse mediate and regulate the events of the synaptic vesicle cycle. The identification of synaptic protein interactions has both suggested hypotheses for the role each protein plays in neurotransmission and has provided evidence for mechanistic models of synaptic vesicle docking, priming, and fusion (1).

Synaptic vesicle protein 2 (SV2) is a membrane glycoprotein present in all synaptic vesicles and regulated secretory vesicles of endocrine cells (2). Two isoforms of SV2, encoded by separate genes, have been characterized; SV2A and SV2B (3–6). The SV2 cDNAs predict 12 transmembrane domain proteins that have significant sequence homology to the major facilitator superfamily of transporters (3, 4, 7). This family of small molecule transporters include the vesicular transporters of amines and acetylcholine (7). Based on this homology, the SV2s were initially hypothesized to be vesicular neurotransmitter transporters, with each isoform transporting a specific neurotransmitter. However, the expression of SV2A and SV2B does not correlate with neurotransmitter phenotype (8), therefore it is not likely that they serve this function.

A current hypothesis of SV2 function is that it transports a constituent of synaptic vesicles other than neurotransmitters. However, attempts to demonstrate transport activity in SV2-expressing fibroblasts have been inconclusive, perhaps due to the absence of a cofactor required for SV2 function. Alternatively, the structural similarity of transporters and channels (9, 10) suggests that SV2 is either a vesicular ion channel or a component of the proposed proteinosacous fusion pore which mediates neurotransmitter release.

To distinguish between these hypotheses of SV2 function, and to identify potential cofactors or regulators of SV2 activity, we examined the interaction of SV2 with other synaptic proteins. SV2 has previously been reported to co-immunoprecipitate with synaptic vesicle proteins, including synaptophysin and synaptotagmin (11). However, SV2’s interactions varied with the detergent used for solubilization, suggesting that detergents alter protein interactions. In order to circumvent problems associated with detergent solubilization, we have employed a combination of cross-linking, immunoprecipitation, and recombinant protein affinity chromatography to identify proteins that interact with SV2. We report that SV2 interacts with the synaptic vesicle protein synaptotagmin. This interaction is mediated by the cytosolic amino terminus of SV2 and the second protein kinase C homology (C2B) domain of synaptotagmin. The interaction is isoform specific (SV2A but not SV2B interacts with synaptotagmin I) and is inhibited by calcium. These observations suggest that SV2 and synaptotagmin are cofactors in a calcium-regulated process and that the isoform-specificity of their interaction provides a mechanism for fine-tuning synaptic function.

EXPERIMENTAL PROCEDURES

Materials—Cross-linking reagents were obtained from Pierce, protein A-Sepharose from Pharmacia Biotech Inc., glutathione-agarose from Sigma, and detergents from Boehringer-Mannheim. Antibodies directed against synaptotagmin I were the generous gift of Richard Scheller, Stanford University, Synaptotagmin I-glutathione S-transferase (GST) fusion constructs were the generous gifts of Ken Miller and Richard Scheller, Stanford University and Zuhong Sheng, University of Washington. Anti-synaptophysin antibody was purchased from Boehringer-Mannheim.

Crude Synaptosome Preparations—Synaptosomes were prepared as described by Huttner et al. (12). Briefly, fresh rat brains were homogenized 10 strokes using a glass-Teflon homogenizer with a 0.004–0.006-inch clearance in 10 mM Hepes, 0.3 M sucrose. The homogenate was centrifuged at 1000 × g for 10 min to remove nuclei and disrupted cells, and the resulting supernatant was centrifuged for 13 min at 25,000 × g. The resulting pellet contains a crude synaptosome preparation which was washed once with Hepes-buffered saline (HBS) (10

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† The abbreviations used are: SV2, synaptic vesicle protein 2; DSP, dithiobis(succinimidyl propionate); HBS, Hepes-buffered saline; IB, incubation buffer; PAGE, polyacrylamide gel electrophoresis.

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mm HEPES, 142 mM NaCl, 2.4 mM KCl, 1 mM MgCl₂, 5 mM d-glucose, 0.1 mM EDTA, or HEPES incubation buffer (50 mM HEPES, 5 mM NaCl, 50 mM KCl, 95 mM KOAc, pH 7.5) and resuspended in the same buffer. The protein content was assayed using either the Pierce BCA or Bio-Rad protein quantification reagents. Synaptosomes were used immediately after preparation.

**Synaptic Vesicle Extract Preparation**—Crude synaptic vesicles were isolated from hypothetically ruptured synaptosomes as described previously (12). Briefly, synaptosomes were hypotonicily lysed by a 1:9 dilution in water with rehomogenization. Heavy membranes (including plasma membrane) were removed by centrifuging 15,000 rpm in a Beckman 528 rotor for 16 min. Synaptic vesicles were isolated by centrifuging at 28,000 rpm in the same rotor for 4 h. Synaptic vesicle proteins were solubilized in 20 mM KOAc, 2% Triton X-100 for 1 h at 4°C, washed five times in 1 ml of cold IB, after centrifuging at 19,000 g for 16 min. Soluble material was removed by centrifugation, at 19,000 × g for 30 min, and the protein concentration was determined using the Pierce BCA protein assay reagent.

**Cross-linking of Synaptic Proteins**—Crude synaptosome proteins were suspended in IB at room temperature at a final protein concentration of either 2 mg/ml (Fig. 1) or 5 mg/ml (Fig. 2). A 10% volume of water, Me₆SO, or 25 mM dithiobis(succinimidyl propionate) (DSP) in Me₆SO (final concentration 2.5 mM) was added, and the mixtures were incubated with gentle agitation at room temperature for 30 min. Reactions were terminated with the addition of 150 mM Tris to quench the cross-linking reagent, after which proteins were solubilized in 1% SDS for 2 h. Insoluble material was removed by centrifugation, at 19,000 × g for 15 min. Detergent extracts were then diluted in SDS-PAGE sample buffer and used in Western analyses or used in immunoprecipitations.

**Immunoprecipitations**—500 µg of total cross-linked or uncleaved synaptosomal protein was diluted in 1 ml of HKA (10 mM HEPES, 140 mM NaCl, 1 mM MgCl₂, 0.1 mM EDTA) with 0.1% bovine serum albumin, 0.1% gelatin, and 2% Triton X-100. Buffer or a polyclonal antibody directed against the cytoplasmic domain of synaptotagmin I was added and mixtures were incubated with gentle agitation for 1 h at 4°C. Protein A Sepharose was prepared by swelling in water, washing with HKA, and blocking with 0.1% bovine serum albumin and 0.1% gelatin in HKA at 4°C for 2 h. Antibody-protein complexes were isolated using 30 µl of 50% (v/v) protein A-Sepharose beads added to the reactions and incubated for another hour at 4°C. Beads were washed three times in HKA with 2% Triton X-100, resuspended in 20 µl of SDS-PAGE sample buffer with 2-mercaptoethanol (which cleaves the cross-linking reagent), incubated for 1 h at room temperature, after which the eluate was collected. Eluates were heated to 65°C for 2 min, separated by SDS-PAGE (13), and subjected to Western analysis.

**GST-SV2 Fusion Protein Construction**—cDNAs containing the amino-terminal regions of SV2A and SV2B were amplified by polymerase chain reaction using primers containing restriction enzyme sites compatible with subcloning into the expression vector PGEX-KT (14). All cDNAs were amplified from either ECL or 125I-labeled secondary antibody. 125I-Labeled samples were blotted onto nitrocellulose. Fusion peptides were visualized by Ponceau staining, and SV2 was visualized by Western analysis utilizing either ECL or 125I-labeled secondary antibody. 125I-Labeled samples were quantified by PhosphorImager analysis.

**RESULTS**

**Treatment of Synaptosomes with the Cross-linking Reagent DSP Produces a Large SV2 Protein Complex**—To identify in situ interactions of SV2 with other synaptic proteins, we utilized the lipid-soluble, cleavable, homobifunctional cross-linking reagent DSP (Pierce). Initial experiments, using fresh rat synaptosome preparations, analyzed the effects of cross-linking on the migration of SV2 in nonreducing SDS-PAGE gels. Following treatment with DSP, approximately 50% of the SV2 shifted from its normal migration at ~ 70-85 kDa to the top of the gel (Fig. 1), suggesting that SV2 is part of a large protein complex. Similar results were obtained by treating a crude synaptic vesicle preparation with the water-soluble cross-linking reagent 3,3′-dithiobis(sulfosuccinimidyl propionate) (data not shown), which suggests that SV2’s interactions are mediated by its cytoplasmic domains. Due to the size of the SV2 complex, we could not determine its molecular weight on polyacrylamide gels. To estimate its mass, we separated cross-linked from uncleaved SV2 by gelatin gradient velocity separation and resolved the fractions by agarose-gel electrophoresis. Using this method, we observed multiple SV2-containing complexes. The three major ones were approximately 250, 500, and >800 kDa (data not shown).

To begin to identify the components of the SV2 complex, we analyzed cross-linked synaptosome preparations for two proteins previously reported to co-immunoprecipitate with SV2, synaptophysin and synaptotagmin (11). Fig. 1 demonstrates that synaptotagmin, like SV2, migrates on SDS-PAGE as a

![SV2-Synaptotagmin Interaction](image)
large molecular weight complex after cross-linking, whereas synaptophysin does not. Nearly all of the synaptotagmin appears to be cross-linked following DSP treatment, compared to approximately 50% of the SV2, suggesting either that SV2 is present in excess to synaptotagmin, or that synaptotagmin is also part of other protein complexes that do not contain SV2. Synaptotagmin also co-migrated with SV2 in agarose-gel separations of cross-linked samples, providing further evidence that the two proteins are in the same complex (not shown). As stated, synaptophysin did not co-migrate with SV2 after cross-linking. We did observe anti-synaptophysin immunoreactive bands of approximately 56 and 76 kDa, which confirmed previous reports of synaptophysin interactions with itself and the 18-kDa synaptic vesicle protein VAMP (15–17). Both synaptotagmin and synaptophysin are major protein constituents of the synaptic vesicle (18, 19). The selectivity of the co-migration of SV2 with synaptotagmin and not synaptophysin suggests that treatment with DSP does not randomly cross-link SV2 to major synaptic vesicle proteins, but rather that cross-linking reflects associations present in the vesicle membrane.

Cross-linking Increases Co-immunoprecipitation of SV2 and Synaptotagmin—To determine whether SV2 and synaptotagmin are in the same protein complex, we asked whether cross-linking affects co-immunoprecipitation of the two proteins. Synaptosomes, treated with vehicle or cross-linking reagent, were solubilized in 1% SDS to disrupt interactions not secured by cross-linking. The resulting extract was diluted 10-fold in 1% Triton X-100 and used in immunoprecipitation experiments employing an antibody directed against the cytoplasmic portion of synaptotagmin I. Western analyses of precipitates (treated with reducing agents to cleave the cross-linking reagent) revealed that cross-linking significantly increases the co-immunoprecipitation of SV2 with synaptotagmin (Fig. 2). As a control, we probed for synaptophysin, which does not co-migrate in SDS-PAGE gels with either SV2 or synaptotagmin after cross-linking. Synaptophysin did not precipitate with synaptotagmin under either condition. The co-immunoprecipitation of a small amount of SV2 with synaptotagmin from uncross-linked samples suggests either that a proportion of the SV2-synaptotagmin complex is resistant to SDS, or, more likely, that the interaction is re-forming in Triton X-100 upon dilution of the SDS. In similar experiments with anti-SV2 antibodies we found that we could not immunoprecipitate the SV2 complex (not shown). These observations suggest that SV2's interactions are mediated by its amino terminus, which contains the epitopes recognized by available anti-SV2 antibodies.

Synaptotagmin Binds Recombinant SV2A Amino Terminus Peptides—SV2 contains two large cytoplasmic domains that could mediate its interactions with other proteins, an amino-terminal region and a loop between the 6th and 7th transmembrane domains (3). Based on the observation that antibodies directed against the amino terminus do not immunoprecipitate the SV2 complex, we predicted that this region of SV2 mediates at least some of its interactions with other proteins. To test if SV2's amino terminus is sufficient for interaction with synaptotagmin and to determine whether the interaction is direct, we assayed the binding of synaptotagmin to SV2-GST fusion protein encoding the amino-terminal cytoplasmic domain of both SV2A and SV2B. The ability of native synaptotagmin to bind SV2 amino-terminal peptides was tested by incubating extracts of crude synaptic vesicle preparations with GST-SV2 peptides attached to glutathione-agarose beads followed by Western analysis of bound material. Synaptotagmin bound the SV2A but not the SV2B peptide (Fig. 3A). This indicates 1) that the interaction is isoform-specific and 2) that the amino terminus of SV2 is sufficient to mediate interaction with synaptotagmin.

While these results suggest that SV2 and synaptotagmin interact, it remained possible that the observed interaction is mediated by another synaptic protein present in synaptic vesicle extracts. To resolve this issue, we asked whether recombinant protein fragments corresponding to the cytoplasmic domain of synaptotagmin I would bind GST-SV2 amino-terminal peptides. Fig. 3B shows that recombinant synaptotagmin I does bind directly to SV2A peptide. As with native synaptotagmin, the recombinant synaptotagmin peptide did not bind SV2B. These results indicate that SV2A interacts directly with synaptotagmin via SV2's amino-terminal cytoplasmic domain.

SV2A Binds the Second Protein Kinase C Homology Domain (C2B) of Synaptotagmin I—Synaptotagmin has been reported to interact with a number of molecules including syntaxin (20, 21), neurexins (22, 23), the clathrin adaptor protein AP2 (24), inositol polyphosphates (25), the soluble protein B-SNAP (26), and with other synaptotagmins (27). With the exception of neurexin, all of these proteins interact with synaptotagmin via one of its two protein kinase C homology (C2) domains. To determine which region of synaptotagmin mediates its interaction with SV2, we analyzed the binding of native SV2 to several synaptotagmin I-GST fusion constructs (diagrammed in Fig. 4A). We observed that native SV2 binds peptides containing the C2B domain (Fig. 4B). To be certain that the C2A domain does not interact with SV2, we employed multiple, slightly different C2A peptides. None of them bound SV2. While it remains possible that SV2 interacts with synaptotagmin via its carboxyl terminus (region 5), which was present in the C2B peptides, the effects of calcium on the interaction (see below) suggest that the calcium-binding C2 domain is involved (region 4).
TritonX-100-solubilized synaptic vesicle protein were incubated with 1 protein bound to glutathione-agarose. Like native synaptotagmin, reacting with an EC50 of approximately 10

...ects that increasing calcium concentrations inhibit binding to the C2B domain of synaptotagmin. Fig. 5 demonstrates that the SV2 complex.

The experiments reported here utilized peptides corresponding to, or antibodies directed against, synaptotagmin I. Therefore, while we can conclude that synaptotagmin I interacts preferentially with SV2A, we do not know whether other isoforms of synaptotagmin also interact with SV2A or preferentially with SV2B. Experiments measuring the binding of native synaptotagmin II to the amino terminus of SV2A and SV2B suggest that this isoform of synaptotagmin binds both isoforms...
The SV2-synaptotagmin interaction may provide calcium-regulated modulation of one or both proteins' actions. There are at least three ways in which this interaction may function. First, synaptotagmin may regulate a transporter or channel function of SV2. This model predicts that association with synaptotagmin either inhibits or promotes transport by SV2, with reversal of this regulation at high calcium concentrations during exocytosis. Interestingly, expression of synaptotagmin's C2B domain transforms an endogenous choline transport activity in *Xenopus* oocytes to one with the characteristics of neuronal choline transporters, indicating that C2B domains can regulate transporters (34). Moreover, the effect of C2B on transport activity in this system was eliminated by high calcium concentrations, a result which parallels the calcium effects we see in the SV2-synaptotagmin interaction. In the synap-}

![Image](image_url)

**FIG. 5.** Increasing calcium concentrations inhibit SV2 binding to the C2B domain of synaptotagmin. A, calcium inhibits the SV2-synaptotagmin interaction. Solubilized synaptosome proteins were incubated with GST or GST-synaptotagmin-3–5 fusion proteins. Lanes labeled GST demonstrate that SV2 does not bind to the GST peptide at any calcium concentration. Lanes labeled C2B demonstrate that increasing calcium concentrations inhibit binding of SV2 to the C2B domain of synaptotagmin. Ponceau staining confirms that equal amounts of fusion proteins were present in the incubations. B, quantification of calcium inhibition establishes an EC\textsubscript{50} of approximately 10 μM. Four replicate experiments were probed with anti-SV2 monoclonal antibody followed by 125I-labeled goat anti-mouse antibody. Immunoreactivity was quantified by phosphorimaging. The pixel intensity of the 0 μM calcium lane was set to 100% for each experiment and the remaining intensities normalized to that value. Points represent the mean value ± S.E. of normalized pixel intensity at each calcium concentration. Values are plotted on a log scale (0 μM calcium is approximated by 1 nM). The EC\textsubscript{50} for the inhibition of the SV2-synaptotagmin interaction by calcium is approximately 10 μM.

of SV2, although with much lower affinity than the synapto-}

tin I-SV2A interaction (not shown). These results suggest that isoform specificity in both binding partners could mediate subtle differences in synaptic functioning.

Our study adds to a growing collection that demonstrates potentially important interactions involving the C2B domain of synaptotagmin. C2B interacts with a number of molecules, including clathrin AP2 (24), high isoositol polyphosphates (25), phospholipids (31), B-SNAP (26), and syntaxin (20, 21). These interactions are calcium-independent. However, C2B does contain the C2-key calcium binding motif (28) and has recently been shown to mediate homo-oligomerization of synaptotagmin at high calcium concentrations (27). In addition, the C2B domain may be important functionally. Microinjection of peptides corresponding to a region of the C2B domain were more efficacious in disrupting neurotransmission in the squid giant synapse than peptides corresponding to the analogous region of the C2A domain (32). Furthermore, mices in which synaptotagmin has been disrupted in the C2B domain lack the fast, low calcium-affinity component of regulated secretion (33), suggesting that the C2B domain may mediate synaptotagmin's role as a low affinity calcium sensor.
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