Neuronal exocytosis occurs via the fusion of docked synaptic vesicles with the presynaptic plasma membrane (1–3). Fusion is triggered by increases in intracellular Ca2+ (4, 5) that act via Ca2+ -sensing proteins to abruptly open a fusion pore through which neurotransmitters escape into the synaptic cleft (6). Subsequent dilation of the fusion pore can result in the complete fusion of the synaptic vesicle membrane with the presynaptic plasma membrane. After complete fusion, vesicles are endocytosed and recycled for future rounds of release (7). Reciprocal dilation of the fusion pore can result in the complete fusion of the synaptic vesicle membrane with the presynaptic plasma membrane. After complete fusion, vesicles are endocytosed and recycled for future rounds of release (8).

Genetic disruption experiments in mice and Drosophila (9–11) have provided evidence that the Ca2+-binding synaptic vesicle protein, synaptotagmin I (12–14), is essential for rapid and efficient Ca2+-triggered release of neurotransmitters. These findings are consistent with the proposed function of synaptotagmin as a Ca2+-sensor for regulated exocytosis (13). In addition, biochemical studies demonstrated that synaptotagmin is a high affinity receptor for the clathrin adaptor protein complex, AP-2 (15). Genetic studies in Caenorhabditis elegans indicate that loss of synaptotagmin also results in defective synaptic vesicle recycling (16). Thus, synaptotagmin is thought to function in both the exo- and endocytotic limbs of the synaptic vesicle cycle.

Synaptotagmin is represented by a large family that currently contains 12 members (17–19). Each member spans the vesicle membrane once and has a short C-terminal intravesicular domain and a large cytoplasmic region that contains two C2 domains, designated C2A and C2B (14). C2A is the membrane proximal domain and mediates the Ca2+-dependent interaction of synaptotagmin with anionic phospholipids (20, 21). The membrane distal C2B domain mediates Ca2+-dependent oligomerization of synaptotagmin, potentially clustering the release machinery into a collar or ring-like structure (22–24). The C2B domain has also been reported to mediate Ca2+-dependent interactions. These include the above described interaction with AP-2, as well as β-SNAP (25), inositol polyphosphates (26), and the II-III cytoplasmic loop or “synprint” region of N- and P/Q-type Ca2+ channels (27, 28). The direct interaction of synaptotagmin with Ca2+ channels may contribute to the speed of exocytosis, which occurs with a lag time of 60–200 μs (29, 30). Finally, both C2 domains are required for high affinity binding to the pre-synaptic proteins syntaxin 1A (22, 31) and SNAP-25,2 although some binding is preserved in the isolated C2A domain (32, 46). These latter interactions are promoted by Ca2+ concentrations (31) similar to those required for neuronal exocytosis (34). Syntaxin and SNAP-25 form a complex with the synaptic vesicle protein synaptobrevin that is capable of catalyzing membrane fusion in vitro (35) and in vivo (36). The mechanism by which synaptotagmin may regulate the fusion complex is not yet known.

To elucidate the mechanism by which synaptotagmin functions in exo- and endocytosis, it is essential to determine how synaptotagmin engages effector molecules. These studies will make it possible to address the function of specific interactions using site-directed inhibitory peptides or by genetic analysis based on loss-of-function mutations. Details concerning the molecular mechanisms by which C2A interacts with effector molecules have recently begun to emerge (37, 38, 53). However, very little is known concerning the molecular basis for the wide range of C2B target-protein interactions. Here, we have begun to address the structural basis for how the C2B domain of synaptotagmin recognizes and binds different effector proteins.

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1 R. L. Gerona, J. A. Kowalchyk, E. C. Larsen, and T. F. J. Martin, submitted for publication.
We demonstrate that distinct isoforms of synaptotagmin form hetero-oligomers and bind the synprint region of N-type Ca\(^{2+}\) channels. Using chimeric and truncated C2 domains, we identified a common region of C2B that mediates oligomerization and AP-2 binding. Furthermore, we report point mutations that have graded effects on the ability of the C2B domain to engage effector proteins. Finally, we demonstrate that synprint can competitively inhibit the oligomerization of synaptotagmin and the binding of AP-2. These findings provide the means to selectively disrupt synaptotagmin-effector interactions for functional studies.

**EXPERIMENTAL PROCEDURES**

**Materials**—cDNA encoding rat synaptotagmin I (14), III (47, 48), and IV (47, 49) was kindly provided by T. C. Sudhof (Dallas, TX), S. Seino (Chiba, Japan), H. Herschman (Los Angeles, CA), and N. Fukuda (Saitama, Japan). cDNA encoding rat syntaxin IA (50) and the pTrcHis-\(\alpha\)B-synprint expression vector (40) were kindly provided by R. H. Scheller (Stanford, CA) and W. A. Catterall (Seattle, WA), respectively.

Mouse monoclonal antibodies directed against \(\alpha\)-adaptin (100.2) and the T7 tag were purchased from Sigma and Novagen, respectively. Mouse monoclonal antibodies directed against synaptotagmin I (604.4 (21)), syntaxin (HPC-1 (51)), and synaptophysin (7.2 (52)) were kindly provided by S. Engers and R. Jahn (Göttingen, Germany).

**Recombinant DNA and Proteins**—Vectors encoding GST\(^{2}\) fusion proteins to express wild type, mutant, chimeric and truncated regions of synaptotagmin I, and the cytoplasmic domains of synaptotagmins III and IV were prepared by polymerase chain reaction and subcloned into pGEX-2T (Amersham Pharmacia Biotech) as described (31). Mutations and chimeras were prepared using the overlapping primer method as described (21) and were confirmed by DNA sequencing. All GST fusion proteins were purified using glutathione-Sepharose (Amersham Pharmacia Biotech) chromatography as described (22). Proteins were quantified by SDS-PAGE and staining with Coomassie Blue using bovine serum albumin as a standard. Full-length rat syntaxin 1A and \(\alpha\)B-synprint were expressed using pTrcHis vectors (In Vitrogen) as described (40).

**Binding Assays**—Rat brain detergent extracts (1 mg/ml) were prepared as described previously (31) in HEPES-buffered saline (HBS); 50 \(\mu\)M HEPES, pH 7.4, 100 \(\mu\)M NaCl with 1% Triton X-100. 1-mI aliquots were incubated with immobilized GST fusion proteins as described in the figure legends. For synprint competition experiments, 4.2 \(\mu\)M purified synprint was included in the rat brain detergent extract binding assays. Bound proteins were detected by immunoblot analysis as described (31, 22) using enhanced chemiluminescence. We observed that purified recombinant proteins containing the C2B domain of synaptotagmin lost native synaptotagmin I binding activity after 3 days. Therefore, all binding experiments were carried out using purified recombinant proteins that were 2 days old. All synprint binding assays were carried out in HBS plus 0.1% Triton X-100, as described in the figure legends. Phospholipid binding assays were carried out in HBS using liposomes composed of 25% brain derived phosphatidylerine and 75% phosphatidylcholine (Avanti Polar Lipids) labeled with \(\gamma\)-3-phosphatidyl [\(\text{N-methyl-}^{3}\text{H}\)]phosphatidylethanolamine,1,2-dipalmityl (Amersham Life Science) as described (20, 37).

**RESULTS**

The C2B domain of synaptotagmin I mediates Ca\(^{2+}\)-trigged homo-oligomerization (22). In the first series of experiments, we determined whether oligomerization is a conserved property among distinct isoforms of synaptotagmin. To address this question, the cytoplasmic domains of synaptotagmins I, III, and IV were immobilized as GST fusion proteins and incubated with rat brain detergent extracts in the presence of Ca\(^{2+}\) or EGTA. The binding of native synaptotagmin I to the immobilized fusion proteins was monitored by immunoblotting with an anti-synaptotagmin I-specific luminal domain monoclonal antibody. As shown in Fig. 1, native synaptotagmin I efficiently bound to recombinant synaptotagmin I, III, and IV in a Ca\(^{2+}\)-dependent manner. These findings demonstrate that Ca\(^{2+}\) not only triggers the self-association of synaptotagmin I but also promotes hetero-oligomerization of distinct synaptotagmin isoforms. These interactions are specific; immunoblotting with an anti-synaptophysin antibody demonstrated that other abundant synaptosomal proteins did not associate with the immobilized synaptotagmins. Consistent with previous results, oligomerization was mediated solely by the C2B domain of synaptotagmin I (22). As an additional control, we compared the anionic lipid binding properties of the immobilized synaptotagmin isoforms (Fig. 1). In agreement with previous reports (39), synaptotagmin I and III but not IV bound liposomes composed of phosphatidylserine/phosphatidylcholine in a Ca\(^{2+}\)-dependent manner. Thus, whereas synaptotagmin IV cannot bind liposomes in a Ca\(^{2+}\)-dependent manner via its C2A domain, this isoform is able to engage in Ca\(^{2+}\)-dependent hetero-oligomerization.

We next tested the ability of recombinant synprint, which comprises the II-III loop of the \(\alpha\)B subunit of N-type Ca\(^{2+}\) channels (40), to bind synaptotagmins I, III, and IV. Purified synprint was incubated with immobilized synaptotagmins I, III, and IV, and binding was detected by immunoblot analysis. Consistent with previous reports, synprint bound to the cytoplasmic domain of synaptotagmin I in a Ca\(^{2+}\)-independant manner (27). Synprint also efficiently bound to synaptotagmin III and IV in a Ca\(^{2+}\)-independant manner, demonstrating that the interaction with Ca\(^{2+}\) channels is a conserved property among distinct synaptotagmin isoforms. We next examined the interaction of synprint with the isolated C2 domains of synaptotagmin I. As reported previously, in the absence of Ca\(^{2+}\), synprint bound to C2B but not to C2A (27). However, addition of 1 mM free Ca\(^{2+}\) resulted in dramatic binding of synprint to C2A and modestly promoted synprint binding to C2B (Fig. 1). This effect was observed in four independent experiments. These findings demonstrate that the interaction of synprint with the synprint region from the \(\alpha\)B subunit of N-type Ca\(^{2+}\) channels, unlike oligomerization (22) or binding to AP-2 (15), is not perfectly preserved within an isolated C2 domain. For this reason, \(\alpha\)B-synprint binding will not be included in the domain mapping studies described below. The C2B domain of synaptotagmin has also been reported to mediate synaptotagmin binding to SV2 (41) and \(\beta\)-SNAP (25). We found that the C2B-SV2 interaction is disrupted by physiological concentrations of Mg\(^{2+}\) (1 mM),\(^2\) and although we observe an interaction between GST-synaptotagmin and His\(_6\)-\(\beta\)-SNAP, we cannot detect binding between the native proteins under our experimental conditions.\(^3\) Therefore, the analysis of the C2B-effector recognition site, described below, is focused on the self-association of synaptotagmin I and the binding of synaptotagmin to AP-2.

The major goal of this study was to delineate the region(s) of the C2B domain of synaptotagmin I (hereafter referred to as synaptotagmin) that interacts with effector proteins. To address this issue, we first opted to map effector binding sites using chimeric C2 domains composed of C2A and C2B. The rationale for this approach was based on two observations. First, C2A does not mediate oligomerization and also fails to bind AP-2. Second, C2 domains are eight-stranded \(\beta\)-sandwich structures in which the strands are connected in a complex manner (Refs. 42–44 and see Fig. 7). Thus, linear truncations would be expected to have drastic effects on the overall confor-

\(^{2}\) The abbreviations used are: GST, glutathione S-transferase; HBS, HEPES-buffered saline; PAGE, polyacrylamide gel electrophoresis.

\(^{3}\) E. R. Chapman and M. Wolowick, unpublished observations.

\(^{4}\) E. R. Chapman and R. C. Desai, unpublished observations.


**Fig. 1.** Comparison of the synaptotagmin I, synprint, and phospholipid binding properties of synaptotagmins I, III, and IV. **Top panel,** 20 μg of GST alone, GST fused to the cytoplasmic domains of synaptotagmins (GST-stg isoforms) I, III, and IV, and GST fused to the isolated C2A and C2B domains of synaptotagmin I (GST-stg I) were immobilized onto 17 μl of glutathione-Sepharose. To assay for native synaptotagmin I binding, beads were incubated for 2 h with 1 ml (1 mg/ml) rat brain detergent extract in the presence of 2 mM EGTA (−) or 1 mM Ca²⁺ (+) in HBS. Beads were washed three times, and bound proteins were eluted by boiling in SDS sample buffer. Samples were resolved by SDS-PAGE, and bound native synaptotagmin was detected by immunoblotting with an anti-synaptotagmin luminal domain antibody (604.4). Immunoreactive bands were visualized using enhanced chemiluminescence. 10 μg of the extract (total) and 15% of the bound material were loaded onto the gel. As a control, samples were also analyzed for the presence of synaptophysin. For synprint binding assays, 30 μg of the GST-synaptotagmin isoforms and isolated C2 domains of synaptotagmin I were immobilized on 30 μl of glutathione-Sepharose and incubated with 2.6 μg of recombinant synprint in 200 μl of HBS with 0.1% Triton X-100 in the presence of either 2 mM EGTA (−) or 1 mM Ca²⁺ (+). Samples were processed as described above, and bound synprint was detected using an anti-T7 tag monoclonal antibody (Novagen) and enhanced chemiluminescence. 3% of the total binding reaction (total) and 12% of the bound material were loaded onto the gel. **Bottom panel,** 15 μg of the cytoplasmic domains of GST-synaptotagmin I (stg-I), III (stg-III), and IV (stg-IV) were immobilized on 10 μl of glutathione-Sepharose. Phospholipid binding assays were carried out as described (20, 37) using [³H]phosphatidyl choline-labeled liposomes composed of 25% phosphatidyl serine and 75% phosphatidyl choline, in either the presence of 2 mM EGTA (−) or 1 mM Ca²⁺ (+). Error bars represent the standard deviation of triplicate measurements.

mation of the domain. The most notable structural difference among C2 domains studied thus far lies in the two different connectivity patterns for a β-strand that results in two distinct topologies. The C2B domain of synaptotagmin is predicted to conform to the same type I topology as the C2A domain (45). Thus, it should be possible to generate correctly folded chimeras.

To determine the minimal region of C2B that confers oligomerization and AP-2 binding we “swapped” regions of C2B and C2A as shown in Fig. 2. To further ensure that the chimeras were properly folded, chimeric junctions were selected that corresponded to perfectly conserved regions within the β-strands. Chimeras were immobilized as GST fusion proteins and incubated with rat brain detergent extracts in the presence of EGTA or Ca²⁺. Binding of native synaptotagmin and AP-2 was assayed by immunoblot analysis. As shown in Fig. 2, Ca²⁺-dependent binding of synaptotagmin and Ca²⁺-independent binding of AP-2 were mediated by the C2B domain of synaptotagmin; C2A failed to bind in both the presence and absence of Ca²⁺. Binding of both synaptotagmin and AP-2 was preserved in a chimera (C2B/A) in which the N-terminal half of C2B was fused to the C-terminal half of C2A. Neither synaptotagmin nor AP-2 bound to the reciprocal chimeric C2 domain (C2A/B) that contained the N-terminal half of C2A and the C-terminal half of C2B. To further localize the region of C2B that confers effector binding, we subdivided the N-terminal half of C2B and generated chimeras in which either the first (C2B/A/A) or second quarter (C2A/B/A) of the chimeric sequence corresponded to C2B, with the balance of the C2 domain composed of C2A sequence. Synaptotagmin and AP-2 bound the chimera that contained the second quarter of C2B (C2A/B/A) but did not bind the chimera that contained the first quarter of C2B (C2B/A/A). We further divided C2A/B/A into C2B/A/B/A and C2A/A/B/A and observed trace levels synaptotagmin and AP-2 binding to the C2A/B/A chimera and no binding to the C2A/B/A/A chimera. In summary, the replacement of residues 165–195 of C2A with homologous residues 296–328 from C2B, resulted in a C2 domain (C2A/B/A) that contained the second quarter of C2B (C2B/A/A) but did not bind the chimera that contained the first quarter of C2B (C2B/A/A). We further divided C2A/B/A into C2B/A/B/A and C2A/A/B/A and observed trace levels synaptotagmin and AP-2 binding to the C2A/B/A chimera and no binding to the C2A/B/A/A chimera. In summary, the replacement of residues 165–195 of C2A with homologous residues 296–328 from C2B, resulted in a C2 domain (C2A/B/A) that contained the second quarter of C2B (C2B/A/A) but did not bind the chimera that contained the first quarter of C2B (C2B/A/A). We further divided C2A/B/A into C2B/A/B/A and C2A/A/B/A and observed trace levels synaptotagmin and AP-2 binding to the C2A/B/A chimera and no binding to the C2A/B/A/A chimera. In summary, the replacement of residues 165–195 of C2A with homologous residues 296–328 from C2B, resulted in a C2 domain (C2A/B/A) that contained the second quarter of C2B (C2B/A/A) but did not bind the chimera that contained the first quarter of C2B (C2B/A/A).
the loss of flanking contact sites and/or steric hindrance due to GST. To better define the minimal requirements for C2B-effector interactions, we carried out a systematic truncation analysis of C2B and analyzed the truncation mutants for native synaptotagmin binding activity. C-terminal truncations, which removed essentially the C-terminal half of C2B, were tolerated; a fragment comprised of residues 248–328 was able to mediate Ca\(^{2+}\)-dependent oligomerization. These data demonstrate the

**FIG. 2.** Chimeric C2 domains reveal the region of the C2B domain of synaptotagmin that confers AP-2 binding and synaptotagmin self-association. Left panel, the C2A and C2B domains of synaptotagmin I, as well as a series of chimeric C2 domains were prepared, purified, and immobilized as GST fusion proteins as described under “Experimental Procedures.” In the diagrammatic representations, C2B is shaded, and C2A is depicted by open rectangles. Dashed lines correspond to the chimeric junctions. Binding of native synaptotagmin I and AP-2 to the immobilized fusion proteins was assayed as described in the legend to Fig. 1. 4 \(\mu\)g of detergent extract (total) and 11\% of the bound material was subjected to SDS-PAGE and immunoblot analysis using an anti-synaptotagmin I luminal domain antibody. To assay for AP-2 binding, 3 \(\mu\)g of the extract (total) and 30\% of the bound material was subjected to immunoblot analysis using anti-\(\alpha\)-adaptin mouse monoclonal antibodies. Right panel, a chimeric C2 domain mediates both C2A- and C2B-specific effector interactions. GST, GST-C2A, GST-C2B, and the GST-C2A/B/A chimera, which bound to AP-2 and mediated Ca\(^{2+}\)-dependent binding of native synaptotagmin I, were assayed for Ca\(^{2+}\)-dependent phospholipid binding as described in the legend to Fig. 1.

**FIG. 3.** Truncation analysis of the synaptotagmin self-association domain. 10–20 \(\mu\)g of C2A, C2B, and truncated versions of C2B were immobilized on 60 \(\mu\)l of glutathione-Sepharose. The sequences of the truncated C2B domains are represented schematically with the domain corresponding to residues 296–328, as described in the legend to Fig. 2, indicated with dashed lines. Binding of native synaptotagmin I was assayed as described in the legend to Fig. 1. 8 \(\mu\)g of the extract (total) and 18\% of the bound material was subjected to SDS-PAGE and immunoblot analysis using the anti-synaptotagmin I luminal domain antibody (604.4) and enhanced chemiluminescence. C-terminal truncation from residues 328 to 326 (\(\Delta\)KKT) or N-terminal truncation from residues 321 to 329 (\(\Delta\)KRLKKKKKT) abolished synaptotagmin self-association. This region is shown in the bottom panel where the critical residues, 326–328, are underlined and in bold. Note that the alignment of C2A and C2B breaks down between \(\beta\)-strands 3 and 4, necessitating the introduction of a gap in the sequence of C2A.
fectability of using a truncation approach to map the C2B-effector binding domain. Analysis of additional truncation mutants demonstrated that further C-terminal truncation to amino acid 326 abolished binding. Thus, amino acids 326–328 are essential for oligomerization. Significant N-terminal truncations were also tolerated because binding was preserved in a fragment encoding 321–421. Further N-terminal truncation to residue 329 resulted in the complete loss of binding. In summary, C-terminal truncation from 328 to 325 or N-terminal truncation from 321 to 329 results in the loss of binding activity. Thus, residues 321–328 comprise an important region for mediating oligomerization. Within this region, amino acids 326–328 appear to be essential. These data are consistent with the chimera studies, described above, in which we delineated 296–328 as the region that confers C2B-specific effector protein interactions. It is notable that residues 326–328 (amino acids KKT) lie in the region of C2B which exhibits a breakdown in its structure of recombinant synaptotagmin.

The domain encoded by residues 293–328 also failed to bind AP-2 (Fig. 4). We therefore used the truncation mutants that corresponded to the borders of the oligomerization domain to determine whether AP-2 bound to a similar site on C2B, as suggested by the chimeric C2 domain analysis described above. Consistent with those results, AP-2 bound to 248–328 but failed to bind the 248–325 truncation mutant. Binding was also observed using 321–421 of C2B. Like oligomerization, AP-2 binding activity was lost by further truncation of C2B to 329–421. These data demonstrate that oligomerization and AP-2 binding are mediated by a common region of C2B.

The analysis described above demonstrated that truncation of the 248–328 fragment to 248–325 resulted in the loss of AP-2- and native synaptotagmin binding to C2B. To further explore the role of lysines 326 and 327 in binding synaptotagmin effectors, these amino acids were replaced individually, or in tandem, with alanine residues. As shown in Fig. 5, substitution of lysine 327 with an alanine reduced oligomerization, AP-2 binding, and synprint binding by approximately 50%. Substitution of both lysines 326 and 327 with alanines essentially abolished all three interactions. CD spectra of the wild type and lysine mutant synaptotagmin were identical. In addition, the double lysine mutant retained partial ability to co-immunoprecipitate with syntaxin. These findings indicate that the mutations did not result in disruption of the overall structure of recombinant synaptotagmin.

Mapping Synaptotagmin C2B Domain–Effector Binding Interfaces

**DISCUSSION**

C2 domains are conserved motifs of approximately 130 amino acid residues that are found in over 60 different proteins (45). These proteins include lipid-modifying enzymes (lipases and kinases), protein kinases, GTPase-activating proteins, regulators of membrane traffic, proteins involved in ubiquitin-mediated protein degradation, and a pore-forming protein secreted by cytolytic T-cells. Many C2 domains bind Ca^{2+} ions and interact with other molecules. To date, little is known...
concerning the molecular basis by which C2 domains recognize, bind, and regulate effectors. Elucidating the molecular mechanism by which synaptotagmin functions in exo- and endocytosis necessitates a detailed analysis of the structure and function of its two C2 domains.

The crystal structures of the C2A domain of synaptotagmin (42), phospholipase C51 (43), and cytoplasmic phospholipase A2 (44) have been reported and are compact eight-stranded β-sandwich structures. Three flexible loops that protrude from one end of the domain contain Ca\(^{2+}\) ligands that coordinate two to three metal ions. Details regarding the interaction of C2A with effectors have recently begun to emerge. A fluorescence study revealed that Ca\(^{2+}\)-binding loop 3 of the C2A domain of synaptotagmin directly penetrates into lipid bilayers, potentially providing movement or force to trigger neuronal exocytosis (37). Subsequent NMR studies demonstrated that residues in loops 2 and 3 form contact sites for monodisperse anionic lipids (53). NMR studies have also provided evidence that residues within each of the three flexible loops of C2A and in particular Ca\(^{2+}\)-binding loops 1 and 3 make contacts with syntaxin (38). However, less is known concerning the basis for synaptotagmin C2B-effector interactions. The only interaction to be mapped in detail is with inositol polyphosphates. Fukuda et al. (47) mapped the inositol 1,3,4,5-tetraphosphate-binding site to residues 315–346 of the C2B domain of synaptotagmin II. Within this region, lysine residues 327, 328, and 332 were critical for binding. As described in more detail below, these residues correspond to lysines 326, 327, and 331 in the sequence of synaptotagmin I.

In this study we sought to determine the structural basis for the interaction of the C2B domain of synaptotagmin with AP-2 and synprint and to map the region of C2B that mediates Ca\(^{2+}\)-dependent self-association. Analysis of chimeric C2 domains revealed that residues 296–328 contain critical elements for C2B-mediated synaptotagmin oligomerization and AP-2 binding. Consistent with these data, independent truncation experiments demonstrated that residues 325–328 were essential for C2B-effector interactions. Furthermore, substitution of lysine 327 with an alanine residue reduced synaptotagmin oligomerization, AP-2-binding, and synprint binding by approximately 50%, whereas substitution of both lysine residues 326 and 327 with alanines abolished all three interactions. As noted above, these residues precisely correspond to lysines 327 and 328 in the inositol 1,3,4,5-tetraphosphate-binding domain of synaptotagmin II. Thus, a common region of C2B is involved in mediating interactions with a number of different molecules. This observation is supported by our findings that synprint is an effective inhibitor of C2B-mediated synaptotagmin interactions. Micromolar concentrations of synprint completely abolished Ca\(^{2+}\)-triggered oligomerization of synaptotagmin as well as the interaction of synaptotagmin with AP-2 (Fig. 6). Interestingly, two previous studies reported that the introduction of synprint into neurons resulted in the inhibition of synaptic transmission (54, 55). This effect was assumed to be due to inhibition of N-type Ca\(^{2+}\) channel-syntaxin interactions. From the data reported here, it is clear that synprint can have multiple inhibitory effects on the molecular machinery that mediates exo- and endocytosis. For example, the inhibitory effects of synprint on synaptic transmission may be due to blockade of Ca\(^{2+}\)-triggered synaptotagmin oligomerization. It will be interesting to determine whether synprint peptides can affect endocytosis via inhibition of the synaptotagmin-AP-2 interaction.

Lysines 326 and 327 lie in a region of C2B that yields a breakdown in its alignment with C2A, necessitating the introduction of a gap of two residues in C2A. This observation suggests that this region diverged during evolution such that each C2 domain evolved to carry out independent and distinct
functions. It should be noted, however, that in some cases both C2 domains of synaptotagmin cooperate to interact with effectors. This principle is illustrated in Fig. 1 in which isolated C2 domains fail to mediate α1B-synprint binding in the same manner as the intact cytoplasmic domain of synaptotagmin. Similar results have been observed for the interaction of synaptotagmin with phosphatidylinositol polyphosphates (26), SNAP-25, and syntaxin (22, 31, 46).

Synaptotagmin is a member of a large gene family (17–19). The ability of distinct isoforms of synaptotagmin to bind to one another (Fig. 1) indicates that a substantial array of heterologomers, each with distinct properties, may assemble in vivo. This property could provide a mechanism to fine tune the regulation of membrane traffic in distinct cell types and compartments. Future studies are required to determine whether individual secretory vesicles possess multiple isoforms of synaptotagmin and whether hetero-oligomers exhibit differences in their abilities to interact with and modulate effectors.

The finding that synaptotagmin IV bound the synprint peptide was somewhat surprising. Synaptotagmins I and III are thought to function as Ca2+-sensors during exocytosis, whereas synaptotagmin IV, due the substitution of an aspartate that normally serves as a Ca2+-ligand, does not bind Ca2+ via its C2A domain (19). Why would a presumably Ca2+-insensitive isoform retain Ca2+-channel binding properties? One possibility is that its C2B domain retains its ability to bind Ca2+ and that this interaction functions in some capacity to couple Ca2+ to the regulation of secretion. This hypothesis is supported by primary sequence analysis of C2B. Five acidic amino acid residues, which serve as Ca2+-ligands in C2A (42), are present in the same positions in C2B (14). However, Ca2+ binding has not yet been demonstrated for the C2B domain of any isoform of synaptotagmin.

An important outcome of this study is the generation of graded loss-of-function mutations. The K327A mutation reduced oligomerization, AP-2 binding, and synprint binding by 50%, whereas the double mutation (K326A,K327A) abolished these interactions. These mutations provide tools for gene replacement experiments to begin to address the function of these interactions in vivo. An additional principle that has emerged is the overlapping or coincident nature of the binding sites for different C2B-binding molecules. The ability of synprint to inhibit C2B-effector interactions suggests that synaptotagmin engages in these interactions in a sequential manner. Furthermore, the inhibitory effect of synprint provides yet another tool to address the physiological function of C2B-effector interactions.

Previous studies on the C2A domain of synaptotagmin demonstrated that residues within loops 2 and 3 form contacts with lipids (37, 53), whereas residues within loops 1 and 3 interact with syntaxin (38). In the present study, we provide evidence that C2B makes contacts with effector proteins via Ca2+-binding loop 1 along with two β-strands that form a concave face of its putative three-dimensional structure (Fig. 7). From these data we can begin to build more detailed molecular models for the mechanism of C2 domain-effector recognition. Future studies will refine this model and, using loss-of-function mutations, will make it possible to address the role of specific effector interactions in exo- and endocytosis in vivo.
Delineation of the Oligomerization, AP-2 Binding, and Synprint Binding Region of the C2B Domain of Synaptotagmin
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