The C2A domain of synaptotagmin I, which binds Ca$^{2+}$ and anionic phospholipids, serves as a Ca$^{2+}$ sensor during excitation-secretion coupling. We have used multidimensional NMR to locate the region of C2A from rat synaptotagmin I that interacts, in the presence of Ca$^{2+}$, with phosphatidylserine. Untagged, recombinant C2A was double-labeled with $^{13}$C and $^{15}$N, and triple-resonance NMR data were collected from C2A samples containing either Ca$^{2+}$-alone or Ca$^{2+}$-plus 6:0 phosphatidylserine. Phospholipid binding led to changes in chemical shifts of backbone atoms in residues Arg253 and Phe259 of loop 3 (a loop that also binds Ca$^{2+}$) and His198, Val200, and Phe208 of loop 2. These residues lie along a straight line on a surface ridge of the C2A domain. The only other residue that exhibited appreciable chemical shift changes upon adding lipid was His254; however, because His254 is located on the other side of the molecule from the phospholipid docking site defined by the other residues, its shifts may result from nonspecific interactions. These results show that the "docking ridge" responsible for Ca$^{2+}$-dependent membrane association is localized on the opposite side of the C2A domain from the transmembrane and C2B domains of synaptotagmin.

Neuronal exocytosis is strictly regulated by Ca$^{2+}$ ions (1). Ca$^{2+}$ triggers the fusion of synaptic vesicles with the presynaptic plasma membrane on the submillisecond time scale; this sets an aggregate upper limit for the $t_{1/2}$ values of protein conformational changes that couple Ca$^{2+}$ binding to exocytotic membrane fusion (2, 3). Although several proteins that function in exocytosis have been identified (4–6), little is known concerning the Ca$^{2+}$-driven conformational changes or protein-lipid interactions that catalyze lipid bilayer fusion. The key to unraveling this mechanism lies in the Ca$^{2+}$ sensor for exocytosis. Recent gene disruption experiments (7–10) have established that the Ca$^{2+}$-binding synaptic vesicle protein, synaptotagmin I (11–13), is essential for rapid and efficient Ca$^{2+}$-triggered release of neurotransmitters. Thus, synaptotagmin has been proposed to function as the major Ca$^{2+}$ sensor of regulated exocytosis.

The 12 known members of the synaptotagmin gene family (14–16) span the vesicle membrane once, have a short amino-terminal intravesicular domain and a large cytoplasmic region that contains two C2 domains, designated "C2A" and "C2B", which interact with a variety of distinct molecules (14). The C2A domain mediates the interaction of synaptotagmin with anionic phospholipids (17, 18), but both C2 domains are required for high affinity binding to plasma membrane proteins syntaxin (19) and SNAP-25.2 These interactions are regulated by Ca$^{2+}$ (20) at calcium ion concentrations similar to those required for neuronal exocytosis (21). Syntaxin and SNAP-25 form a complex with the synaptic vesicle protein synaptobrevin that can catalyze membrane fusion in vitro (22) and in vivo (23).

C2 domains are found in more than 50 distinct proteins, including lipid and serine/threonine kinases, phospholipases, GTPase-activating proteins, proteins involved in ubiquitin-mediated protein degradation and cytosolic pore formation, and a number of proteins involved in membrane traffic (24). Despite the growing number of proteins that possess C2 domains, relatively little is known concerning the structural elements that mediate C2 domain-effector interactions. The crystal structures of three distinct C2 domains have been determined (25–27), and the fold of each is an eight-stranded β-sandwich. In each, three flexible loops (designated loops 1–3) protrude from one end of the domain. Loops 1 and 3 contain the metal-binding ligands that serve to bind a maximum of two or three Ca$^{2+}$ ions.

A number of C2 domains bind membranes in a Ca$^{2+}$-dependent manner (17, 18, 28–35). Because the Ca$^{2+}$-dependent interaction of the C2A domain of synaptotagmin with membranes is likely to serve as an important step in excitation-secretion coupling, we have used multidimensional, multinuclear NMR spectroscopy to investigate the structural basis for this inter-
action. We report that short acyl chain phosphatidylserine interacts exclusively with residues in loops 2 and 3 of C2A. Loops 2 and 3 become fully ordered only after phospholipid is bound, not simply upon binding Ca2+.

EXPERIMENTAL PROCEDURES

Plasmid Construction, Protein Expression, and Purification—cDNA encoding rat synaptotagmin I (12) was kindly provided by T. C. Sudhof (Dallas, TX). The region of the gene coding for the C2A domain (residues 140–267) of synaptotagmin I was amplified by PCR, creating NdeI (5′) and BamHI (3′) sites. This PCR product was digested with NdeI and BamHI and inserted into a pET9a vector (Novagen, Madison, WI), which had been cut previously with the same two enzymes. The resulting plasmid, named pET9a/C2A, was transformed into BL21(DE3) containing pLysS. A single colony picked from a plate was used to inoculate 50 ml of minimal (LB) medium; following overnight growth, this was used to inoculate 1 liter of LB medium. The cells were induced when A600 (absorbance at 600 nm) reached 1.0–1.3 and harvested by centrifugation 3 h later. Protein labeled uniformly with 13C and 15N was produced from 1 liter of minimal medium containing [13C]glucose and [15N]ammonium chloride (Isotec, Miamisburg, OH). The harvested cells were resuspended in 10 mM Tris buffer at pH 8.5 and subjected to a freeze-and-thaw step to break the cell walls followed by a sonication step. The cell lysate was centrifuged at 30,000 × g for 15 min, and the supernatant was retained, filtered, and loaded onto a Mono Q column (Amersham Pharmacia Biotech) on an FPLC system (Amersham Pharmacia Biotech). C2A eluted around 150 mM NaCl, and the fractions containing C2A were pooled, concentrated, and loaded onto a Superdex 75 (Amersham Pharmacia Biotech) column. C2A eluted around 96 ml. The purity was tested by SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining. The typical yield of C2A was around 40 mg/liter of culture.

NMR Sample Preparation—All NMR samples contained 1 mM C2A, 50 mM Tris, pH 7.5, 200 mM NaCl, 2 mM sodium 2,2-dimethyl-2-silapentane-5-sulfonate, and 10% D2O. Semisynthetic 6:0 phosphatidylserine (6PS) was purchased from Avanti Polar Lipids (Alabaster, AL). One sample contained 5 mM Ca2+ and the other contained 5 mM Ca2+ and 5 mM 6PS.

NMR Spectroscopy and Data Processing—All data were collected on a Bruker (Billerica, MA) DMX-500 NMR spectrometer at 303 K. For both samples, three-dimensional HNCA (36, 37) and HNCOCA (38) spectra were collected as 1024*(1H) × 64*(13C) × 48*(15N) and

FIG. 1. Two-dimensional ([H-15N] HSQC spectrum (at 500 MHz [1H]) of the isolated C2A domain of rat synaptotagmin I (residues 140–267) at pH 7.5. The spectrum (thick lines) of the ternary complex, C2A(Ca2+)-[6PS], is overlaid on top of that (thin lines) of the binary complex, C2A(Ca2+). Resonances exhibiting appreciable shift changes are labeled with amino acid type and residue number.
1024*(1H) × 46*(13C) × 48*(15N) matrices, respectively, where the number followed by an asterisk indicates the number of complex data points. Raw data were converted and processed with FELIX (Molecular Simulations, Inc., San Diego, CA) software. All data were digitally filtered to remove negative data points. The convolution difference solvent suppression routine in FELIX was applied, and the resulting data were apodized by a square-cosine window function, Fourier-transformed, and phase-corrected. The size of the data was halved by removing the right half of the spectrum. To data in the 13C dimension, a square-cosine window function was applied; data in this dimension were zero-filled to 256 points, Fourier-transformed, and phased. To data in the 15N dimension, mirror image linear prediction was applied; the resulting data were apodized by a Kaiser window function, zero-filled to 128 points, Fourier-transformed, and phased. The final matrices were 512 (1H) × 256 (13C) × 128 (15N), where the numbers refer to the numbers of real data points. The Sparky software developed at

**Fig. 2.** NMR chemical shift differences in signals from the isolated C2A domain of rat synaptotagmin I, at pH 7.5 in the presence of Ca^{2+}, induced by the addition of 60% phosphatidylserine plotted as a function of residue number: top, amide protons, HN; middle, a carbons, Ca; bottom, amide nitrogens, N.
University of California, San Francisco (http://www.cgl.ucsf.edu/Research/Sparky.html) was used for peak-picking.

RESULTS AND DISCUSSION

Sequence-specific Resonance Assignments—Sequential assignments were determined from the combined interpretation of HNCA (36, 37) and HNCOCA (38) spectra. Resonances from residues Met173, Gly 174, Asp 188, Lys 213, Arg 233, and Phe 234 were not observed in spectra of the binary $C_2A(Ca^{2+})$, complex, probably because of line broadening resulting either from hydrogen exchange with solvent water or from dynamic disorder (25). Signals from these residues were identified, however, in NMR spectra of the $C_2A(Ca^{2+})_2(6PS)$, ternary complex, suggesting that loops 2 and 3 of $C_2A$ are more ordered in the ternary complex than in the binary complex.

6PS Versus PS/PC Liposomes—The $C_2A$ domain of synaptotagmin exhibits preferential interactions with liposomes that contain anionic phospholipids, such as phosphatidylserine (17, 18). 6PS was used here because of its high solubility, which made it possible to saturate the lipid binding sites of $C_2A$ while keeping the free phospholipid concentration below its critical micellar concentration. The relative concentrations used here ($C_2A:Ca^{2+}:6PS = 1:5:5$) were chosen on the basis of results suggesting that the maximum number of metal ions bound is three (26) and that the number of phospholipid molecules is limited (29). Lipid binding was followed by following chemical shifts changes in the two-dimensional $^1H-^{15}N$ HSQC spectrum of $[^{15}N]C_2A$ as a function of added 6PS (Fig. 1).

As a test of the relevance of 6PS as a model for longer chain phosphatidylserine, it was determined that, in the presence of $Ca^{2+}$, 6PS induces the same level of resistance to limited proteolysis as PS/PC liposomes (made with PS derived from brain with acyl chain composition: 40% 18:0, 31% 18:8, 8.5% 22:6) (17). This suggests that, in the presence of $Ca^{2+}$, 6PS induces the same kind of structural rearrangement (or protection from proteolysis) produced by the PS/PC liposomes.

Comparison of the Binary and Ternary Complexes—To maximize the chemical shift comparisons (Fig. 2), two gaps in the data at pH 7.5 (Arg233 and Phe234, whose signals were not seen in the binary complex but sharpen up in the ternary complex) were filled by chemical shifts for the binary complex at pH 5.0 where the peaks are sharper (39). Because the referencing system used by these authors (39) was different from that used here (40), their data set was first brought into register by minimizing the shift differences of signals common to both data sets.

Of the residues (Fig. 2) showing the largest shifts upon addition of 6PS (His198, Val205, Phe206, Arg233, Phe234, His254), all but His254 are on loops 2 and 3. Because His254 is located on the opposite side of the molecule from loops 2 and 3, its shift likely results from a nonspecific interaction with 6PS or possibly a 6PS-mediated protein-protein interaction. Consistent with this interpretation, a fluorescent reporter (tryptophan) placed at residue 153, which lies near His254 in the three-dimensional structure of $C_2A$, was found not to interact with lipid bilayers (41). Evidence for the involvement of $Ca^{2+}$-binding loop 3 in lipid binding is consistent with the fluorescence study of Chapman and Davis (41), which indicated that residue 234 interacts directly with liposomes. The residues on loops 2 and 3 implicated in lipid binding (His198, Val205, Phe206, Arg233,

![Fig. 3. Molscript (44) representations of x-ray structures of $C_2$ domains highlighting residues of the synaptotagmin $C_2A$ domain (or their homologues in the other two structures; see Table I) whose backbone atoms exhibited large $^1H$, $^{13}C$, or $^{15}N$ NMR chemical shift changes upon addition of semisynthetic 6:0 phosphatidylserine in the presence of $Ca^{2+}$. A, x-ray structure of the rat synaptotagmin I $C_2A-Ca^{2+}$ complex (25). B, comparison of three $C_2$ domain structures: top, $C_2A$ domain of rat synaptotagmin I with one bound $Ca^{2+}$ ions (25); middle, $C_2$ domain of human cytosolic phospholipase $A_2$ with two bound $Ca^{2+}$ ions (27); bottom, $C_2$ domain of rat phospholipase $C_2$ with two bound $Ca^{2+}$ ions (26).](image-url)
Phe^{234} are oriented along a straight line on a surface ridge of C_{2A} (Fig. 3A). An important finding of the present work is that this “docking ridge” is located on the opposite side of the C_{2A} domain from the transmembrane and C_{2B} domains of synaptotagmin I.

Results reported here show that the phospholipid binding site(s) of C_{2A} are localized to loops 2 and 3. In contrast, the largest shifts that occurred upon the binding of C_{2A} to a fragment of synaptotagmin I served as a major Ca^{2+} binding sites, and the residue of C_{2A} that exhibited the largest shift perturbation on forming the C_{2A}-synaptotagmin-fragment complex, did not shift significantly upon binding 6PS (Fig. 2). It is localized to a region of loop 3 distinct from the proposed membrane docking site (Fig. 3A). Thus, loop 3 interacts with both lipids and syntaxin, whereas loops 1 and 2 interact preferentially with syntaxin and lipids, respectively. Future studies will determine whether C_{2A} can engage both lipids and syntaxin simultaneously.

Localization of the phospholipid binding site adjacent to the calcium binding sites helps explain the positive cooperativity of Ca^{2+} and phospholipid binding to C_{2A} (17). Through sequence alignments, residues analogous to amino acids 198, 205, 206, 233, 234, and 254 in the C_{2A} domain of rat synaptotagmin I were determined for the other two C_{2A} domains whose structures are known, human cytosolic phospholipase A_{2} (27) and rat phospholipase C_{a1} (26) (Table I). In all three cases, these residues (excluding residue 254) lie along a straight line on the protein surface, are adjacent to known Ca^{2+} binding sites, and involve loops 2 and 3 of the C_{2A} domain (Fig. 3B). The net charge on these residues at physiological pH is consistent with what is known about headgroup preferences for these domains: synaptotagmin (net charge for these residues of +1 or +2) has a preference for negatively charged phospholipids such as PS, whereas cytosolic phospholipase A_{2} (net charge zero for these residues) has a preference for positively charged phospholipids, such as phosphatidylcholine. The lipid preference for the C_{2A} domain of phospholipase C_{a1} is unknown. Given the proximity of the residues that are perturbed by 6PS binding to the calcium binding sites (Fig. 3), it appears probable that the serine headgroup interacts directly with Ca^{2+}, displacing water in open coordination sites. This kind of direct interaction would explain how PS binding increases the affinity of C_{2A} for Ca^{2+}.

Genetic and biochemical studies have provided strong evidence that synaptotagmin I serves as a major Ca^{2+} sensor for neuronal exocytosis (14). A major challenge is to determine how interactions of synaptotagmin with membranes and components of the membrane fusion complex (22, 23) serve to trigger fusion. In this study we have begun to elucidate the molecular mechanism by which the C_{2A} domain of synaptotagmin docks onto membranes. Further structural studies should shed additional light on the conformational changes that couple Ca^{2+} influx to exocytosis.

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