The phospholipid-binding specificities of C2 domains, widely distributed Ca\(^{2+}\)-binding modules, differ greatly despite similar three-dimensional structures. To understand the molecular basis for this specificity, we have examined the synaptotagmin 1 C2A domain, which interacts in a primarily electrostatic, Ca\(^{2+}\)-dependent reaction with negatively charged phospholipids, and the cytosolic phospholipase A\(_2\) (cPLA\(_2\)) C2 domain, which interacts by a primarily hydrophobic Ca\(^{2+}\)-dependent mechanism with neutral phospholipids. We show that grafting the short Ca\(^{2+}\)-binding loops from the tip of the cPLA\(_2\) C2 domain onto the top of the synaptotagmin 1 C2A domain confers onto the synaptotagmin 1 C2A domain the phospholipid binding specificity of the cPLA\(_2\) C2 domain, indicating that the functional specificity of C2 domains is determined by their short top loops.

C2 domains are widely distributed, independently folding domains that are present in many signal transduction and membrane trafficking proteins, such as phospholipases, protein kinase C, and synaptotagmins (reviewed in Refs. 1–4). Most C2 domains bind Ca\(^{2+}\) and interact with phospholipid membranes upon Ca\(^{2+}\) binding. The three-dimensional structures of several C2 domains have been elucidated at atomic resolution (5–11). These structures revealed that C2 domains, despite a low overall sequence identity, are composed of similar \(\beta\)-sandwiches with flexible loops at the top and the bottom. However, the \(\beta\)-strands in the \(\beta\)-sandwiches exhibit two distinct topologies, resulting in type 1 (e.g. the C2A domain of synaptotagmin 1) and type 2 C2 domains (e.g. the C2 domain of cytosolic phospholipase A\(_2\) (cPLA\(_2\))\(^1\) (5, 11). The two topologies are circular permutations of each other, such that the first \(\beta\)-strand in type 1 C2 domains is the eighth \(\beta\)-strand of type 2 C2 domains, and the eighth \(\beta\)-strand of type 1 C2 domains corresponds to the seventh \(\beta\)-strand of type 2 C2 domains as illustrated in the sequence alignment in Fig. 1A. In C2 domains, Ca\(^{2+}\) binds exclusively to the top loops, which coordinate 2–3 Ca\(^{2+}\) ions primarily via multidentate aspartate residues (7, 11–15). The Ca\(^{2+}\)-binding sites are similar among C2 domains with relatively low but variable intrinsic binding affinities (7, 13, 14, 16). The best characterized function of C2 domains consists of their Ca\(^{2+}\)-dependent binding to phospholipid membranes, although not all C2 domains appear to share this function. Phospholipid binding mediates the Ca\(^{2+}\)-stimulated recruitment of C2 domain proteins to membranes upon stimulation (1–4). Phospholipids dramatically increase the overall Ca\(^{2+}\) affinity of C2 domains, most likely because the phospholipid head groups provide additional coordination sites for Ca\(^{2+}\) ions at the tip of the C2 domains (15, 17–19).

At least two different phospholipid-binding modes have been observed in C2 domains, as best described for the C2A domain of synaptotagmin 1 and the C2 domain of cPLA\(_2\) (Table I). In both C2 domains, the top loops bind the Ca\(^{2+}\) ions, but the architecture and stoichiometry of the Ca\(^{2+}\)-binding site differ (Fig. 1A; Refs. 13 and 14). The most important difference between the two C2A domains, however, lies in their binding specificity. The synaptotagmin C2A domain binds to negatively charged phospholipids in a primarily electrostatic interaction (17, 22). In contrast, the cPLA\(_2\) C2 domain interacts with neutral phospholipids in a hydrophobic reaction (Table I; Refs. 16, 22, and 23). Since Ca\(^{2+}\) and phospholipids bind to the top loops of C2 domains, it is plausible to hypothesize that the top loops may be instrumental in determining their respective properties, but this has never been demonstrated. The top loops are relatively short, the cPLA\(_2\) and synaptotagmin C2 domains exhibit a low overall sequence similarity and contain different \(\beta\)-strand topologies (Fig. 1), suggesting that the loops may not be sufficient in specifying the properties of a C2 domain. To address this question, we have now tested if replacing the top loops of the synaptotagmin 1 C2A domain with those of the cPLA\(_2\) C2 domain will “reprogram” the phospholipid-binding mode of the synaptotagmin C2A domain.

**MATERIALS AND METHODS**

**Construction of Expression Vectors and Protein Expression—** pGEX-cPLA2-C2 domain encoding the human cPLA\(_2\) C2 domain (residues 1–141) fused to GST was constructed by PCR amplification of the C2 domain (primer sequences: A = CCGGAAATTTACAAGTTCTTCTTCTTAGTACCTTATACG; B = CCGAACTTCTAGTACAGTTCACACAACTTCACGGAGACATATGGCACATTC) of the PCR product into the EcoRI/HindIII sites of pGEX-KG (24). The synaptotagmin 1 C2A domain expression vector (pGEX-56; residues 140–267) was described previously (20). The synaptotagmin 1/CPLA2 Loop-Swap C2 domain expression vectors (pGEX-56/cytosolic phospholipase A\(_2\); PC, phosphatidylcholine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; FS, phosphatidylserine; GST, glutathione S-transferase; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis. **

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Stefan H. Gerber‡, Josep Rizo§, and Thomas C. Südhof¶

From the ‡Center for Basic Neuroscience, Department of Molecular Genetics, and Howard Hughes Medical Institute and the ¶Departments of Biochemistry and Pharmacology, University of Texas Southwestern Medical Center, Dallas, Texas 75390

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The abbreviations used are: cPLA\(_2\), cytoplasmic phospholipase A\(_2\); PC, phosphatidylcholine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; FS, phosphatidylserine; GST, glutathione S-transferase; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.
Phospholipid Binding Assays—Phospholipids (1.75 mg total; Avanti Polar Lipids, Alabaster AL) were solubilized in chloroform, mixed in the indicated weight ratios with a trace of 3H-labeled PC (to 0.01% of total; Amersham Pharmacia Biotech), and dried under nitrogen. Dried lipids were resuspended in 10 ml of 50 mM HEPES-NaOH, pH 7.4, 0.1 M NaCl by vortexing (1 min), sonicated (5 min) in a waterbath sonicator (modelmodel; Laboratory Supply, Co. Inc., Hicksville, NY; output: 80 kc, 80 watts), and centrifuged (15 min) at 5,000 × g to remove aggregates. Beads containing ~25 μg of recombinant protein (1 g/liter wet glutathione beads) were equilibrated in 0.1 ml of the respective binding buffers (50 mM HEPES-NaOH, pH 6.8, 0.1 M NaCl) for 25 min at room temperature with vigorous shaking in an Eppendorf shaker, briefly centrifuged, and washed three times with 800 μl of the respective binding buffers. Phospholipid binding was quantified by scintillation counting of the beads (LS6000SC; Beckman Instruments, Fullerton, CA). All buffers were made in high resistance MilliQ water using a 1 M CaCl2 stock solution.

RESULTS AND DISCUSSION

We produced GST fusion proteins of the C2A domain of rat synaptotagmin 1, the single C2 domain of human ePLA2, and chimeric C2A domains in which the synaptotagmin Ca2+-binding loops were replaced with those from the ePLA2 C2 domain (“loop swaps”; Fig. 1, A and B). The five C2A domain proteins expressed well in bacteria and were isolated in an electrophoretically pure form on glutathione-agarose (data not shown). Using these proteins, we first tested if the loop-swap C2A domains were still capable of Ca2+-dependent phospholipid binding.

As described previously (17, 18, 20, 21, 25, 26), we found that the cPLA2 C2 domain bound equally well to liposomes composed of 100% PC, 50% PC/50% PE, 30% PS/70% PC, or 50% PI/50% PC, whereas the synaptotagmin 1 C2A domain only bound to liposomes that contained the negatively charged phospholipids PS or PI (Fig. 2 and data not shown). All binding was Ca2+-dependent. The chimeric loop-swap C2A domains that contained either only loops 1 and 2 or loop 3 exhibited no Ca2+-dependent binding. Hence, Ca2+-dependent binding of the loop-swap C2A domain proteins was entirely dependent on the Ca2+-binding loops of the C2A domain.

Functional Specificity of C2 Domains

Purification and characterization of GST fusion proteins immobilized on glutathione-agarose (18). Amounts, purity, and integrity of proteins were standardized by SDS-PAGE and Coomassie Blue staining. GST fusion proteins were purified on glutathione-agarose by standard procedures (24) and used for phospholipid binding measurements with GST fusion proteins immobilized on glutathione-agarose (18). Amounts, purity, and integrity of proteins were standardized by SDS-PAGE and Coomassie Blue staining.

TABLE I

Comparison of the properties of the C2A domain of synaptotagmin 1 and the C2 domain of cytosolic phospholipase A2

| Property                      | C2A domain of synaptotagmin 1 | C2 domain of phospholipase A2 |
|-------------------------------|-------------------------------|------------------------------|
| Type of C2 domain             | Type 1                        | Type 2                       |
| Number of Ca2+ ions bound     | 3                             | 2                            |
| Intrinsic Ca2+ binding affinity | ~50 μM, ~0.5 mM, and ~5 mM for sites 1, 2, and 3 | ~10 and ~60 μM for sites 1 and 2 |
| Apparent Ca2+ affinity with phospholipid membranes | ~5–25 μM (depends on phospholipid composition) | ~3 μM (with 100% PC) |
| Phospholipid binding specificity | Negatively charged phospholipids | Neutral phospholipids |
| Phospholipid binding mode     | Electrostatic; no conformational change | Hydrophobic; with conformational change |

FIG. 1. Structure-based sequence alignment of the C2 domain from human ePLA2 and the C2A domain of rat synaptotagmin 1 (Syt1) and schematic diagram of the C2 domain constructs used in the current study. A, sequences of the ePLA2, and synaptotagmin 1 C2 domains aligned for maximal homology, with the locations of β-strands and top Ca2+-binding loops based on the atomic structures of the domains (5, 6, 10, 11) indicated above and below the sequences. Vertical lines identify the positions at which the top loops from the ePLA2 C2 domain were grafted onto the synaptotagmin 1 C2A domain. Residues shared between the synaptotagmin 1 and ePLA2 C2 domains are shaded, and residues involved in Ca2+ binding in the top loops are shown in white on a black background. Note the divergence of the Ca2+-binding sites between the two C2 domains where the synaptotagmin 1 C2A domain binds three Ca2+ ions via five aspartate and one serine residues, whereas the ePLA2 C2 domain binds two Ca2+ ions via three aspartate, two asparagine, and one threonine residues (13, 14) (see Table I for a description of the distinct properties of these C2 domains). B, schematic overview of the chimeric C2 domain constructs used.

Ca2+-dependent binding of 100% PC liposomes and little binding to liposomes composed of 30% PS/70% PC. Very high Ca2+ concentrations were tested in these experiments (10 mM) to exclude the possibility that these partial loop swaps may simply have a very low Ca2+-affinity. By contrast, the complete loop-swap C2 domain bound to phospholipids as a function of Ca2+ and thus was functional (Fig. 2). Strikingly, although the loop-swap C2A domain contains only the top loops of the ePLA2 C2 domain and is otherwise identical with the synaptotagmin C2A domain, it exhibited similar binding to neutral phospholipids (PC and PE) as the ePLA2 C2 domain (Fig. 2 and data not shown). Quantitations revealed that the loop-swap C2A domain bound as much neutral liposomes as the ePLA2 C2 domain, but was less capable of binding negatively charged liposomes than the ePLA2 C2 domain (data not shown).
We next examined Ca\(^{2+}\)/H\(_{11001}\)-dependent binding of the cPLA\(_2\) C\(_2\) domain, the synaptotagmin C\(_2\)A domain, and the complete loop-swap C\(_2\) domain to PC and PS liposomes in the presence of increasing concentrations of NaCl to test whether phospholipid binding is primarily electrostatic or hydrophobic (Fig. 3). The rationale of this experiment is that high NaCl should stabilize hydrophobic interactions but disrupt electrostatic interactions. In agreement with previous studies (18, 22), hydrophobic binding is primarily electrostatic or hydrophobic (Fig. 3). The rationale of this experiment is that high NaCl should stabilize hydrophobic interactions but disrupt electrostatic interactions. In agreement with previous studies (18, 22), hydrophobic bind-
ing of the cPLA₂ C₂ domain to phospholipids was not impaired by high salt. In contrast, the primarily electrostatic binding of the synaptotagmin C₂A domain to phospholipids was highly salt-sensitive and was abolished by 0.6 M NaCl. In the same experiments, the loop-swap C₂A domain behaved like the cPLA₂ C₂ domain, with phospholipid binding resistant to interference by NaCl at concentrations of up to 1.0 M (Fig. 3).

The functionality of the loop-swap C₂A domain composed of the synaptotagmin C₂A domain with only short Ca²⁺-binding loops from the cPLA₂ C₂ domain is surprising considering the fact that the cPLA₂ and synaptotagmin C₂ domains have divergent properties and distinct β-strand topologies. Studies with different divalent cations showed that in all three C₂ domains, Mg²⁺ was unable to stimulate phospholipid binding at high concentrations, whereas Ba²⁺ and Sr²⁺ at least partly substituted for Ca²⁺, albeit with lower affinity (data not shown). The cPLA₂ C₂ domain and the loop-swap C₂ domain were also similar with respect to divalent cation binding in that Ba²⁺ and Sr²⁺ were slightly more effective in replacing Ca²⁺ in these C₂ domains than in the synaptotagmin C₂ domain (data not shown). To test more accurately if the precise conformation of the β-strand sandwich contributes to the Ca²⁺ binding properties of the overall C₂ domains, we examined their apparent Ca²⁺ affinities in the presence of phospholipid membranes. Ca²⁺/EGTA buffers were used in these experiments to measure the Ca²⁺ dependence of phospholipid binding to the various immobilized C₂ domains. Since Ca²⁺-dependent phospholipid binding by the synaptotagmin C₂ domain is primarily electrostatic, the apparent Ca²⁺ affinity of the synaptotagmin C₂ domain depends on the phospholipid composition of the liposomes used for such measurements (18). Therefore we measured the apparent Ca²⁺ affinity of all three C₂ domains with four types of liposomes containing an increasing abundance of negatively charged phospholipids (Table II).

As shown in an exemplary experiment in Fig. 4, the cPLA₂ C₂ domain exhibited a slightly higher Ca²⁺ affinity than the synaptotagmin C₂A domain, as also shown in earlier studies (16, 18, 20, 22). The complete loop-swap C₂ domain displayed a significantly lower Ca²⁺ affinity (5-15-fold depending on phospholipid composition) than either the cPLA₂ C₂ domain or the synaptotagmin C₂A domain (Fig. 4 and Table II). As predicted, the Ca²⁺ affinity of the synaptotagmin C₂A domain was dependent on phospholipid composition, and two to three times higher in 45% PS/55% PC than in 22.5% PS/77.5% PC. By contrast, the cPLA₂ C₂ domain exhibited similar affinities in the presence of liposomes independent of the surface charge (Table II). Unexpectedly, the apparent Ca²⁺ affinity of the loop-swap C₂ domain was dependent on the phospholipid composition with the opposite relationship as the synaptotagmin C₂A domain. This affinity decreased almost 5-fold with increasing negatively charged liposomes (Table II). Together these data suggest that the precise conformation of the underlying β-sandwich has little influence on the phospholipid binding specificity or on the hydrophobic versus electrostatic binding mechanism mediated by the top Ca²⁺-binding loops, but has a marked effect on the overall Ca²⁺ affinity of the C₂ domain (Table II).

Summary—The C₂A domain of synaptotagmin 1 and the C₂ domain of cPLA₂ are composed of β-sandwiches that contain flexible Ca²⁺-binding loops on top, which were previously hypothesized to contribute to the Ca²⁺-dependent interactions of these domains with phospholipid membranes. However, the two C₂ domains have different β-strand topologies and bind phospholipids with distinct Ca²⁺-dependent mechanisms and

### Table II

**Apparent Ca²⁺-binding affinities of C₂ domains**

| Liposome phospholipids | cPLA₂ EC₅₀ (µM) | n | Loop-swap EC₅₀ (µM) | n | Synaptotagmin 1 EC₅₀ (µM) | n |
|------------------------|-----------------|---|---------------------|---|-------------------------|---|
| 100.0% PC              | 8.6 ± 0.7       | 4 | 48.3 ± 1.3          | 3 | No binding              |   |
| 22.5% PS/77.5% PC      | 4.2 ± 0.1       | 3 | 115.3 ± 27.1        | 3 | 21.0 ± 1.4              | 3 |
| 30.0% PS/70.0% PC      | 6.2 ± 1.7       | 4 | 180.8 ± 26.0        | 4 | 14.7 ± 0.9              | 3 |
| 45.0% PS/55.0% PC      | 5.1 ± 0.2       | 2 | 256.5 ± 28.5        | 2 | 8.9 ± 0.6               | 3 |

Figure 4. Ca²⁺ concentration dependence of phospholipid binding to the C₂ domain of cPLA₂, the complete loop-swap C₂ domain (Loop-Swap), and the C₂ domain of synaptotagmin 1 (Syt1) measured with liposomes composed of 100% PC (top) or 30% PS/70% PC (bottom). Binding reactions were carried out with immobilized GST fusion proteins in Ca²⁺/EGTA buffers to clamp the free Ca²⁺ concentration. Data shown are means ± S.E. from a representative experiment performed in triplicate. Mean values obtained from multiple independent experiments with liposomes composed of 100% PC, 22.5% PS/77.5% PC, 30% PS/70% PC, and 45% PS/55% PC are summarized in Table II.
specificities, suggesting that the top loops may not be sufficient for the specific properties of the various C₂ domains. To test this directly, we have now grafted the top loops of the cPLA₂ C₂ domain onto the tip of the synaptotagmin C₂A domain. We find that grafting all of the Ca²⁺-binding loops of the cPLA₂ C₂ domain enables the synaptotagmin C₂A domain to bind to neutral phospholipids in a salt-insensitive reaction similar to the cPLA₂ C₂ domain, whereas grafting only one or two of the three loops is insufficient. Furthermore, the loop-swap C₂ domain exhibited a lower Ca²⁺ affinity, indicative of a contribution of the backbone of the C₂ domain to the precise positioning of the Ca²⁺-binding loops. These data demonstrate that the functional features and phospholipid binding modes of the cPLA₂ and synaptotagmin C₂ domains are largely determined by their top loops, a surprising result considering the shortness of these sequences in the various C₂ domains.

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The Top Loops of the C₂ Domains from Synaptotagmin and Phospholipase A₂
Control Functional Specificity
Stefan H. Gerber, Josep Rizo and Thomas C. Südhof

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