Specificity of Ca^{2+}-Dependent Protein Interactions Mediated by the C_2A Domains of Synaptotagmins

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ABSTRACT: Synaptotagmins represent a family of neuronal proteins thought to function in membrane traffic. The best characterized synaptotagmin, synaptotagmin I, is essential for fast Ca^{2+}-dependent synaptic vesicle exocytosis, indicating a role in the Ca^{2+} triggering of membrane fusion. Synaptotagmins contain two C_2 domains, the C_2A and C_2B domains, which bind Ca^{2+} and may mediate their functions by binding to specific targets. For synaptotagmin I, several putative targets have been identified, including the SNARE proteins syntaxin and SNAP-25. However, it is unclear which of the many binding proteins are physiologically relevant. Furthermore, more than 10 highly homologous synaptotagmins are expressed in brain, but it is unknown if they execute similar binding reactions. To address these questions, we have performed a systematic, unbiased study of proteins which bind to the C_2A domains of synaptotagmins I−VII. Although the various C_2A domains exhibit similar binding activities for phospholipids and syntaxin, we found that they differ greatly in their protein binding patterns. Surprisingly, none of the previously characterized binding proteins for synaptotagmin I are among the major interacting proteins identified. Instead, several proteins that were not known to interact with synaptotagmin I were bound tightly and stoichiometrically, most prominently the NSF homologue VCP, which is thought to be involved in membrane fusion, and an unknown protein of 40 kDa. Point mutations in the Ca^{2+} binding loops of the C_2A domain revealed that the interactions of these proteins with synaptotagmin I were highly specific. Furthermore, a synaptotagmin I/VCP complex could be immunoprecipitated from brain homogenates in a Ca^{2+}-dependent manner, and GST−VCP fusion proteins efficiently captured synaptotagmin I from brain. However, when we investigated the tissue distribution of VCP, we found that, different from synaptic proteins, VCP was not enriched in brain and exhibited no developmental increase paralleling synaptogenesis. Moreover, binding of VCP, which is an ATPase, to synaptotagmin I was inhibited by both ATP and ADP, indicating that the native, nucleotide-occupied state of VCP does not bind to synaptotagmin. Together our findings suggest that the C_2A-domains of different synaptotagmins, despite their homology, exhibit a high degree of specificity in their protein interactions. This is direct evidence for diverse roles of the various synaptotagmins in brain, consistent with their differential subcellular localizations. Furthermore, our results indicate that traditional approaches, such as affinity chromatography and immunoprecipitations, are useful tools to evaluate the overall spectrum of binding activity for a protein but are not sufficient to estimate physiological relevance.

Synaptotagmins I and II are abundant synaptic vesicle proteins composed of a short intravesicular N-terminal sequence, a single transmembrane region, and two C_2 domains which are separated from the transmembrane region by a connecting sequence (*1*−*3*). In all vertebrates and invertebrates investigated, closely related homologues of synaptotagmin I are known, suggesting a high degree of evolutionary conservation (*4*−*7*). Genetic studies in mice, *Drosophila*, and *C. elegans* revealed that synaptotagmin I is essential for normal synaptic transmission (*5, 8*−*10*). Mice carrying a mutant synaptotagmin I gene die immediately after birth. Cultured hippocampal neurons from the mutant mice exhibit a selective defect in Ca^{2+}-triggered neurotransmitter release while Ca^{2+}-independent release is normal (*8*). These results showed that in mice, synaptotagmin I is not essential for synaptic vesicle exocytosis as such but is required for normal Ca^{2+} triggering of exocytosis.

The first and second C_2 domain of synaptotagmins (the C_2A and C_2B domains, respectively) are independently folding Ca^{2+}-binding domains (*11*−*13*) that exhibit characteristic sequence differences in all synaptotagmins (*1, 2, 4*). Crystal and NMR structures for the C_2A domain of synaptotagmin I are available (*14, 15*). In contrast, for the C_2B domain only the NMR structure of the similar C_2B domain from rabphilin, a related protein, has been determined (*16*). The three-dimensional structures show that the C_2-domains of synaptotagmins are composed of similar β-sandwiches formed by eight β-strands with loops emerging from the top

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[1] Abbreviations used: ECL, enhanced chemiluminescence; GST, glutathione S-transferase; NSF, *N*-ethyl-maleimide sensitive factor; SDS−PAGE, SDS-polyacrylamide gel electrophoresis; VCP, vasoline-containing peptide.

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and bottom. Ca^{2+} binds exclusively to the top loops in the C_2A and the C_2B domains, without causing a major conformational change (*16*−*18*). Three Ca^{2+} ions bind to the top of the C_2A domain, and two Ca^{2+} ions to top of the C_2B domain. The conclusions derived from the studies of the synaptotagmin and rabphilin C_2 domains appear to be generally applicable to other C_2 domains which also bind multiple Ca^{2+} ions at the top as revealed by X-ray crystallography, although the intrinsic Ca^{2+} affinity of various C_2 domains differs (*19*−*21*).

The fact that the two C_2 domains of synaptotagmin I bind multiple Ca^{2+} ions and that synaptotagmin I is essential for Ca^{2+}-dependent exocytosis suggests that Ca^{2+} binding to the C_2 domains of synaptotagmin I triggers exocytosis. Thus, one strategy to elucidate the mechanism of action of synaptotagmin I in exocytosis has been to identify molecules that bind to it as a function of Ca^{2+}. Several such binding partners have been found. The C_2A domain of synaptotagmin I binds to phospholipids and the t-SNARE syntaxin, while the C_2B domain binds to the vesicle protein SV2A and to itself in a Ca^{2+}-dependent manner (*11*−*13*, *22*−*25*). In addition, Ca^{2+}-independent interactions of synaptotagmins I and II with β-SNAP (but not α-SNAP), SNAP-25, AP-2, all subtypes of Ca^{2+}channels, and phosphoinositides have been reported (*26*−*34*). Although it is tempting to speculate that some of these interactions participate in triggering exocytosis, their physiological relevance has not yet been demonstrated. In this respect, an interesting phenotype was obtained when squid neurons were injectioned with antibodies specific for either the C_2A domain or the C_2B domain of synaptotagmin (*35*, *36*). The C_2A domain antibodies inhibited exocytosis selectively without an effect on vesicle numbers, while the C_2B domain antibodies resulted in a depletion of synaptic vesicles from the nerve terminals. These observations suggested an important role particularly for the C_2A domain in triggering exocytosis.

Synaptotagmins I and II are expressed in a differential pattern in the vertebrate brain; synaptotagmin I is primarily present in rostral brain regions and synaptotagmin II in caudal brain regions (*2*, *37*). It seems likely that synaptotagmin II performs a function analogous to that of synaptotagmin I in the caudal brain areas that do not express synaptotagmin I (*2*). In addition to synaptotagmins I and II, at least 10 other synaptotagmins are synthesized in vertebrate brains, although at much lower levels than synaptotagmins I and II (*23*, *37*−*44*). All of these "other" synaptotagmins exhibit the same domain organization with a single transmembrane region and two cytoplasmic C_2 domains; some of these other synaptotagmins were recently shown to be conserved in invertebrates (*45*). In most synaptotagmins, the Ca^{2+} binding sites that were characterized in the C_2A domain of synaptotagmin I are retained, suggesting that most synaptotagmins bind Ca^{2+}. Furthermore, most synaptotagmins also bind to phospholipids and syntaxin in a Ca^{2+}-dependent manner, although with slightly different properties (*23*, *37*, *46*). The exceptions are synaptotagmins IV, VIII, XI, and XII (*23*, *37*, *39*, *43*, *44*) which contain amino acid substitutions in the C_2A domains that abolish Ca^{2+} binding. Most of the synaptotagmins have been studied only at the DNA level. A protein product has been demonstrated only for synaptotagmins III and VI in addition to the original synaptotagmins I and II. Surprisingly, although synaptotagmins III and VI are expressed primarily in brain, they are not enriched on synaptic vesicles (*47*). This is particularly unexpected because synaptotagmin III is highly concentrated in synapses; its precise localization in the synapses is unknown. Thus, at least some of the nonclassical synaptotagmins perform functions outside of synaptic vesicles, a conclusion consistent with the fact that synaptotagmin III (which is coexpressed with synaptotagmin I) cannot compensate for the synaptotagmin I mutation in the knockout mice (*8*).

The fact that the various synaptotagmins are coexpressed in synapses but not colocalized on synaptic vesicles suggests that they may have distinct functions. However, the C_2 domains of synaptotagmins I, II, III, VI, and VII are extremely similar. The C_2A domains of these synaptotagmins exhibit similar in vitro binding actitivities for phospholipids and syntaxins, although different Ca^{2+} affinities. This raises the question if syntaxins are the true targets of C_2A domains of synaptotagmins, which would thus perform similar functions at distinct subcellular locations. An alternative hypothesis would be that the currently identified binding partners of C_2A-domains are not the major interacting proteins, and that synaptotagmins in fact perform very different functions by binding to diverse targets. In the current study we have systematically investigated this question. We analyzed the binding activities of C_2A domains of different synaptotagmins to determine if these domains exhibit specificities toward interacting partners, and to identify the best binding partners for these domains. C_2A domains were chosen because the squid experiments, as well as more recent microinjection experiments in PC12 cells, indicated that these domains are central for synaptotagmin function (*35*, *48*). Unexpectedly, we find that the most tightly binding proteins are not the previously identified interaction partners of synaptotagmins but molecules heretofore not known to bind. The most intriguing of these is the fusion protein VCP which exhibits all of the characteristics of what would generally be considered a specific binding protein.

MATERIALS AND METHODS

Vector Constructions and Protein Expression. The recombinant GST−SytIC_2A fusion protein (residues 140−267) and its mutants (D230N, K190Q, R199Q, K236Q) were described previously (*49*, *50*). Other GST−synaptotagmin fusion proteins were synthesized from the following expression plasmids in the vector pGEX-KG (*51*) encoding the following residues of SytII−SytVII (*23*): pGEX1071/1081 (GST−SytIIC_2A), residues 141−268; pGEX1078/1079 (GST−Syt$IIIC_2A$), residues 292−425; pGEX1082/1083 (GST−SytIVC_2A), residues 148−282; pGEX1184/1185 (GST−SytVC_2A), residues 6−136; pGEX1075/1076 (GST−SytVIC_2A), residues 226−358; and pGEX1072/1073 (GST−Syt$VII\ C_2A$), residues 134−261. pGEXVCP-6 encoding the full-length VCP cDNA was by amplifying the VCP coding sequence by PCR as a 2.4 kb *Nco*I−*Sal*I fragment using rat brain cDNA as a template and oligonucleotides corresponding to the N- and C-termini of VCP as primers (*52*). The fragment was cloned into the *Nco*I−*Sal*I sites of pGEX-KG. pGEXVCP-7 encoding the N-terminal region of VCP (residues 1−213) was constructed by cloning 0.65 kb *Bam*HI−*Nhe*I fragment from pGEXVCP-6 into the *Bam*HI−*Xba*I sites of pGEX-KG. pGEXVCP-8 encoding the C-terminal region of VCP (residues 682−806) was constructed

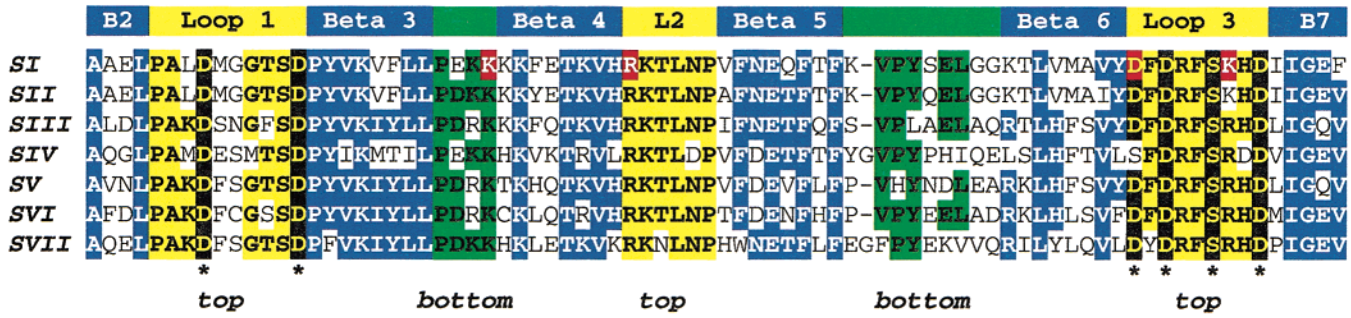

FIGURE 1: Sequences of the Ca^{2+} binding loops and flanking regions of the C_2A domains of synaptotagmins I−VII. Sequences are identified on the left (SI = synaptotagmin I; SII = synaptotagmin II; etc.) and aligned for maximal homology. Residues that are identical in more than 50% of the sequences are highlighted with a color code reflecting the secondary structure of the corresponding sequence: blue = β-strands, yellow = top Ca^{2+}-binding loops; green = bottom loops. The single serine and five aspartate residues whose side chains ligate the three bound Ca^{2+} ions in the C_2A domain of synaptotagmin I are shown on a black background. The four residues in synaptotagmin I that were substituted in the analyses of mutant C_2A domains of synaptotagmin I (see Figure 3 below) are shown on a red background.

by cloning the 0.68 kb *Mfe*I−*Hin*dIII fragment of pGEX-VCP-6 into the *Eco*RI−*Hin*dIII sites of pGEX-KG. All cloning was carried out using standard molecular biology techniques (*53*), and all constructs were verified by DNA sequencing.

Affinity Chromatography on Immobilized GST-Fusion Proteins. Four frozen rat brains were homogenized in 22 mL of 20 mM HEPES−NaOH pH 7.4, 1 mM EGTA, 0.1 g/L PMSF, 10 mg/L leupeptin, 10 mg/L aprotinin, and 1 mg/L pepstatin A (*54*). 22 mL of 20 mM HEPES−NaOH, 0.2 M NaCl, 2% NP-40, 1 mM EGTA, 0.1 g/L PMSF, 10 mg/L leupeptin, 10 mg/L aprotinin, and 1 mg/L pepstatin A were added, and the homogenate was extracted for 1 h at 4 °C. Insoluble material was removed by centrifugation (30 min at 100 000 × g), and $MgCl_2$ and $CaCl_2$ were added to the supernatant to final concentrations of 2.5 mM and 2.0 mM, respectively. The total brain extract was precleared by incubation for 2 h at 4 °C with 0.25 mL of glutathione agarose followed by centrifugation (800 × g for 2 min). 40 mL of the recovered brain extract was incubated overnight at 4 °C with glutathione agarose (0.5 mL) with ~2 mg GST−SytC_2A that was preequilibrated with buffer A (10 mM HEPES−NaOH, pH 7.4, 0.1 M NaCl, 1% NP-40, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$). After overnight incubation, the agarose beads were packed into a polypropylene column with a paper disk (Quick-Sep, Isolab). The column was washed with 20 mL of buffer A, and eluted as described (*54*) with 2 mL volumes of buffer A containing increasing concentrations of NaCl (0.2 M, 0.5 M) followed by 2 mL of 0.5 M NaCl buffer A containing 5 mM EGTA instead of 1 mM $CaCl_2$. Eluted proteins were precipitated with trichloroacetic acid and resusupended in 0.12 mL SDS−PAGE sample buffer. Aliquots were analyzed by SDS−PAGE and Coomassie blue staining (20 μL) or immunoblotting (2 μL) using standard procedures (*55*, *56*).

Protein Identification by Amino Acid Sequencing. To identify proteins that are affinity purified on the C_2A domain of synaptotagmin I, proteins in the eluate were purified by SDS−PAGE, the bands were cut out from the gel, and the proteins were digested with trypsin and tryptic fragments were purified by HPLC and analyzed by Edman degradation as described (*54*). Resulting peptide sequences were used for searching various sequence databanks using the BLAST program suite of the NCBI.

Immunoprecipitation. Total brain extract prepared in a manner similar to the GST-pulldown experiments was applied to the protein G-sepharose to which monoclonal antibody against SytI (Cl 41.1 or a mixture of Cl 604.3, 604.4, 604.5, 606.6) (*57*) was cross-linked with DMP (PIERCE). After washing the column extensively with the loading buffer containing 1 mM $CaCl_2$, the column was eluted with 2 mL of the buffer containing 5 mM EGTA. The EGTA elution, as well as 2 mL of the final wash by the loading buffer, was concentrated to 160 μL by TCA precipiation, and 40 or 10 μL of the material was analyzed by SDS−PAGE followed by Coomassie blue staining or immunoblotting, respectively.

Miscellaneous Procedures. Polyclonal antibodies to VCP (K330) were raised against GST fusion proteins encoding full-length VCP (GST−VCP). Homogenates from different rat tissues and from rat brains at various developmental timepoints were obtained using standard procedures.

RESULTS AND DISCUSSION

Purification of Brain Proteins on Immobilized C_2A Domains of Synaptotagmins. C_2 domains are compact β-sandwiches that bind multiple Ca^{2+} ions via sequence loops emerging at the top of the domain (*14*−*21*). The C_2A domains of synaptotagmins are closely related and exhibit only relatively minor sequence variations, leading to the expectation that they should bind to similar proteins. The sequence similarity is illustrated in Figure 1 which exhibits an alignment of the sequences of the Ca^{2+} binding loops of the C_2A domains of synaptotagmins I−VII. Although only Ca^{2+} binding to the C_2A domain of synaptotagmin I has been analyzed in detail (*17*, *18*), the residues that are involved in Ca^{2+} binding (highlighted in black in Figure 1) and flanking sequences (highlighted in yellow in Figure 1) are conserved in most of the C_2A domains. Of the C_2A domains shown, only synaptotagmin IV contains an amino acid substitution that interferes with Ca^{2+} binding (*43*). The conservation of the Ca^{2+} binding loops would suggest that most C_2A domains are functionally similar even if the various synaptotagmins are differentially localized (*47*). To test this hypothesis, we systematically studied the binding activities of the C_2A domains of the seven synaptotagmins shown in Figure 1. For this purpose, we prepared GST-fusion proteins from these domains, immobilized them on glutathione agarose, and used them as affinity matrices for Ca^{2+}-dependent chromatography

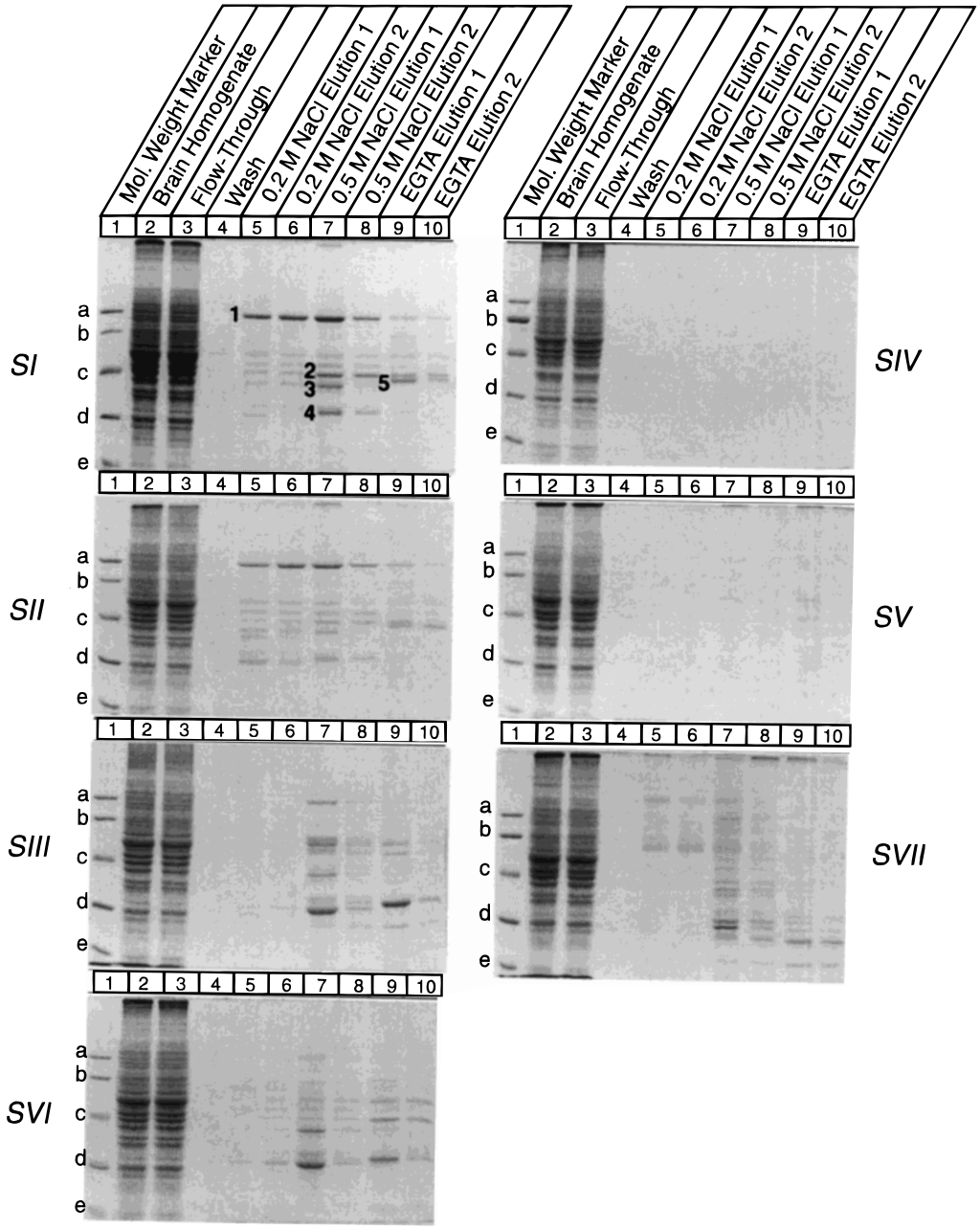

FIGURE 2: Affinity chromatography of rat brain proteins on immobilized GST-fusion proteins of the C_2A domains of synaptotagmins I−VII. Total rat brain homogenate solubilized in NP-40 (lane 1) was applied to columns containing immobilized GST-fusion proteins of the C_2A domains of synaptotagmins I to VII in a buffer containing Ca^{2+} and 0.1 M NaCl. After collecting the flow-through (lane 3), columns were washed extensively with the loading buffer (lane 4) and sequentially eluted with 0.2 M NaCl, 1 mM Ca^{2+} (lanes 5, 6), 0.5 M NaCl, 1 mM Ca^{2+} (lanes 7,8), and 0.5 M NaCl, 5 mM EGTA to chelate Ca^{2+} (lanes 9, 10). Equivalent amounts of all fractions were analyzed by SDS−PAGE and Coomassie blue staining; next to each gel panel, the synaptotagmins whose GST−C_2A domain fusion proteins were used for the affinity chromatography are identified (SI = synaptotagmin I, SII = synaptotagmin II, etc.). In the synaptotagmin I gel, the five major binding proteins are marked by letters 1−5; amino acid sequences obtained from these proteins and their identification are listed in Table 1. Letters on the left point to molecular weight markers run in lane 1 (*a* = 97 kDa, *b* = 66 kDa, *c* = 45 kDa, *d* = 31 kDa, *e* = 21 kDa).

of rat brain proteins (Figure 2). Proteins were applied to the columns in the presence of Ca^{2+}, and the columns were then eluted with Ca^{2+}-containing buffers with increasing NaCl concentrations, followed by a final elution in an EGTA buffer to chelate Ca^{2+}. Proteins in the various fractions were analyzed by SDS−PAGE, immunoblotting, and Coomassie staining.

Inspection of the resulting protein patterns reveals that the various C_2A domains affinity-purify different sets of proteins (Figure 2). As shown in a comparison of the protein eluants from different C_2A domains on the same gel in Figure 3, it is not so that simply the same proteins are purified to variable degrees on different C_2A domains. Instead, the major proteins differ between the classes of C_2A domains. For example, a 97 kDa protein (labeled "1" in Figure 2) is prominently bound to the C_2A domains of synaptotagmins I and II but not to those of the other synaptotagmins, which instead contain a slightly larger protein (Figure 3). This indicates that the different C_2A domains exhibit distinct and surprising binding specificities.

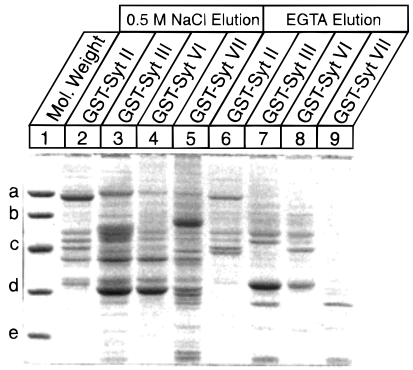

FIGURE 3: Side by side comparisons of rat brain proteins that bind to C_2A domains of synaptotagmin II, III, VI, VII. Fractions of eluted proteins with 0.5 M NaCl (lanes 2−5) or 5 mM EGTA (lanes 6−9) from the columns containing GST-fusion proteins of C_2A domains of synaptotagmin II, III, VI, VII were compared with side by side. Letters on the left identify the molecular weight markers run in lane 1 (a = 97 kDa, b = 66 kDa, c = 45 kDa, d = 31 kDa, e = 21 kDa).

Most proteins that bind to a given C_2A domain are eluted by increasing the NaCl concentration in the presence of Ca^{2+}; however, these proteins nevertheless bind in a Ca^{2+}-dependent manner since no binding was observed in the absence of Ca^{2+} (data not shown). We observed a single protein that bound to synaptotagmin I in a manner resistant to 0.5 M NaCl (labeled "5" in Figure 2); this protein could only be eluted by Ca^{2+}removal with EGTA. The C_2A domains of synaptotagmins IV and V did not bind any proteins under our experimental conditions. At least in case of synaptotagmin IV, this is not surprising since this synaptotagmin contains an amino acid substitution in the Ca^{2+} binding site and probably does not bind Ca^{2+} (Figure 1; ref. *43*). The interactions of different synaptotagmins with proteins are not random because more closely related C_2A domains bind the same sets of proteins. For example, synaptotagmins I and II are most closely related and bind the same proteins; similarly, synaptotagmins III and VI are also most closely related to each other and also bind the same set of proteins, which in turn are different from that bound to either synaptotagmins I/II or VII (Figure 2). This specificity is quite surprising considering the fact that the sequence differences between the various C_2A domains are rather small (Figure 1). Together these results indicate that although synaptotagmins exhibit a high degree of homology in their C_2A domains, the C_2A domains are involved in distinct Ca^{2+}-dependent interactions.

Identification of Binding Proteins. To identify the proteins that were affinity-purified on the immobilized C_2A domains, we obtained peptide sequences from selected bands. In these experiments, we focused on synaptotagmin I because it is the only synaptotagmin with a known function, and microinjection experiments suggested a role for the isolated C_2A-domain in release (*35*, *48*). We sequenced all proteins identified by Coomassie staining (numbered in Figure 2); the resulting sequences and their identifications are described in Table 1. Most of the sequences obtained corresponded to known proteins. The most prominent binding protein for the C_2A domains of synaptotagmins I and II, the 97 kDa band, was identified as VCP (#1 in Figure 1). Three proteins that eluted at high salt in the presence of Ca^{2+} (#2, 3, and 4 in Figure 1) were identified as actin, mitochondrial p32, PHAP2/SET, and PHAP1, respectively. Finally, a 40 kDa protein which only eluted upon removal of Ca^{2+} by EGTA did not correspond to any known protein in the databanks. All of these proteins also bound to synaptotagmin II as shown in Figure 2. It is surprising that none of the known binding proteins of synaptotagmins, including syntaxin and Ca^{2+} channels, were affinity-purified in a manner detectable by Coomassie staining. The failure to purify these proteins is not due to the fact that these proteins are not abundant, because at least syntaxin is a very abundant brain protein. Furthermore, syntaxin can be affinity-purified on immobilized GST−munc18 (data not shown), suggesting that the lack of purification of syntaxin on the immobilized C_2A domain is not due to a sequestration of syntaxin in an inaccessible complex.

Which of these protein interactions are likely to be right? The binding of actin is difficult to evaluate since actin binds to a large number of proteins in vitro. PHAP1 and PHAP2/SET contain very acidic C-terminal regions which could mediate an artifactual interaction with synaptotagmin; in addition, these proteins are thought to be largely nuclear (*58*, *59*). On the other hand, these proteins are phosphatase 2A inhibitors, a function that may be important in membrane fusion since, at least in yeast, a phosphatase is essential for vacuole fusion (*60*). p32 is unlikely to be a physiological binding protein since it is localized to mitochondria (*61*). The most tightly bound protein was the 40 kDa protein (#5 in Figure 2) whose interest cannot be evaluated because its peptide sequences did not match any known protein, suggesting that it is a novel protein. The fact that this pioneer protein was the only protein resistant to 0.5 M NaCl elution is indicative of a particularly stable interaction. However, the most interesting binding protein is the 97 kDa protein that was the most abundant band on the gels. This protein was identified as VCP, a protein with a known function in membrane fusion, albeit not in synaptic fusion (*62*−*64*).

Specificity of the Binding of Brain Proteins to the C_2A Domain of Synaptotagmin I. To test if the interaction of the various proteins with the C_2A domain of synaptotagmin I is mediated by Ca^{2+} binding to the domain, we investigated if point mutations in the Ca^{2+} binding loops affect these interactions. In these experiments, we used point mutations in the C_2A domain of synaptotagmin I that were previously characterized structurally and shown to either abolish Ca^{2+} binding, or have no effect, or even enhance Ca^{2+} binding (*49*, *50*). When we used a mutant synaptotagmin I C_2A domain in which one of the five residues involved in coordinating the three Ca^{2+} ions was substituted (D230N), none of the proteins that bound to wild-type synaptotagmin I were captured (Figure 4). This result indicates that all of these proteins were bound via an interaction with the Ca^{2+} binding loops at the top of the C_2 domain. Two point mutations in positively charged residues that surround the Ca^{2+} binding site, R199 or K233, also abolished most of the binding activity of the C_2A domain; only the 40 kDa protein was still significantly bound (Figure 4). This result agrees well with the fact that all of the interactions of the C_2A domain were salt sensitive, i.e., electrostatic, except for the binding of the 40 kDa protein (Figure 2). The R199Q and K233Q substitutions do not inhibit Ca^{2+} binding but instead enhance it (*49*). Conversely, when we mutated an arginine residue that is localized to the bottom of the C_2A

Table 1: Sequences of Proteins Purified by Affinity Chromatography on the Immobilized C_2A Domain of Synaptotagmin I[a]

protein	sequence	identification	
1	GxDLxTAILK	rat VCP	residues 9−18
	LAGESESNLRK	(acc# P46462)	residues 278−288
	TLLAK	residues 525−529	
	GGNIGDGGGAADRVINQI	residues 588−604	
2	DQYVGDEAQSK	rat actin	residues 53−63
	LDLAGR	(acc# CAA24539)	residues 180−185
	IIAPPER	residues 330−337	
3	QPFFQK	human PHAPII	residues 65−70
	VEVTEFEDIK	(acc# CAA52982)	residues 109−119
	EFHLNESGDPSSK	residues 142−154	
4	LAEELPSLTHLN	human PHAPI	residues 83−94
	LLxQLNYLDxYxR	(acc# CAA52981)	residues 138−150
	AFVEFLTDEIKEER	rat p32	residues 78−91
	ITVTFNISNNSI	(acc# Q35796)	residues 126−136
5	FLLK	unknown	
	MLVQR		
	YVEGA		
	LQTNYSK		
	QMSVTEEDLEEFQLALK		
	ETSPSSNNSSEELSxALQL		

[a] Proteins that were affinity purified on immobilized C_2A domain of synaptotagmin I (see Figure 1) were digested by trypsin. Tryptic peptides were purified by HPLC and subjected to amino acid sequencing using Edman degradation as described (*54*). Sequences are shown in single letter amino acid code. Identifications were made by BLAST searches of GenBank, with the GenBank accession numbers shown in brackets.

domain (K190Q), no effect on binding was observed. Together these data testify to a high degree of specificity in these interactions. They reveal that all of the interactions observed involve the top of the C_2 domain that includes the Ca^{2+} binding site.

VCP Specifically Binds to Synaptotagmin I/II. The purification of VCP on immobilized synaptotagmin I is intriguing because VCP, a homologue of NSF, is known to function in membrane fusion, although not at the synapse (*62*−*64*). Therefore we decided to explore the characteristics of its binding. For this purpose, we generated an antibody against VCP and used it to immunoblot the fractions obtained by affinity chromatography on the various C_2A domains (Figure 5). The results show that for the C_2A domains of synaptotagmins I and II, VCP is partly removed from the flow-through and highly enriched in the eluate. VCP binding is much stronger than that of any previously characterized potential targets of synaptotagmin I and is Ca^{2+} dependent, since no binding was observed in the absence of Ca^{2+} and binding was abolished in mutants that lack one of the three Ca^{2+} binding sites (Figure 2 and data not shown). VCP binding to synaptotagmins I and II was absolutely specific for synaptotagmins I and II: even the sensitive immunoblotting technique did not detect any VCP in the fractions from the C_2A domains of the other synaptotagmins (Figure 5). This result confirms the conclusion that the C_2A domains from the various synaptotagmins mediate highly specific protein interactions.

To explore the interaction of VCP with synaptotagmin I further, we studied its Ca^{2+} concentration dependence (Figure 6). Very high concentrations of Ca^{2+} were required for binding, similar to the concentrations needed for syntaxin binding (*23*). These concentrations correspond to those required for filling the third Ca^{2+} binding site in the C_2A domain which has a very low affinity in the absence of phospholipids. If the C_2A domain of synaptotagmins I and II truly interact with VCP, it should be possible to turn the interaction around and affinity-purify full-length synaptotagmin on immobilized VCP-fusion proteins. To test this, we employed three VCP−GST fusion proteins containing either full-length VCP or the N- and C-terminal halves of VCP (Figure 7). Using affinity chromatography experiments, we confirmed a specific, Ca^{2+}-dependent interaction of VCP with full-length synaptotagmin I. Furthermore, we found that the N-terminal fragment of VCP but not its C-terminal fragment efficiently captured synaptotagmin I, suggesting that the binding is to the N-terminus (Figure 7).

Immunoprecipitation of a Synaptotagmin I/VCP Complex from Brain. To investigate if a complex of VCP and synaptotagmin I is present in brain, we immunoprecipitated synaptotagmin I from solubilized brain homogenates and analyzed the resulting fractions by Coomassie blue staining and immunoblotting. We observed efficient coimmunoprecipitation of VCP with synaptotagmin in a manner that was absolutely Ca^{2+} dependent (Figure 8). Syntaxin, as reported previously, was also coimmunoprecipitated but to a lesser extent, and only partly in a Ca^{2+}-dependent manner. VCP was the only major band detectable on Coomassie blue stained gels, indicating that in brain homogenates, VCP is the major Ca^{2+}-dependent interaction partner for synaptotagmin I.

The Distribution and Developmental Expression Profile of VCP Differs from That of Typical Synaptic Proteins. By all classical criteria, the data shown above suggest that VCP is an excellent candidate for a synaptotagmin I target protein: VCP is nearly stoichiometrically bound to GST−synaptotagmin I, and conversely, GST−VCP captures synaptotagmin I from brain in a Ca^{2+}-depenendent manner. Both of these interactions are sequence specific; even closely related C_2A domains do not exhibit them. Furthermore, a Ca^{2+}-dependent VCP−synaptotagmin I complex can be immunoprecipitated from brain. However, if VCP was a physiological partner for synaptotagmin I, it should be codistributed with synaptotagmin I and exhibit similar tissue distributions and developmental time courses. Thus we investigated if VCP has the characteristics of a synaptic protein as would be expected if this interaction was physiological.

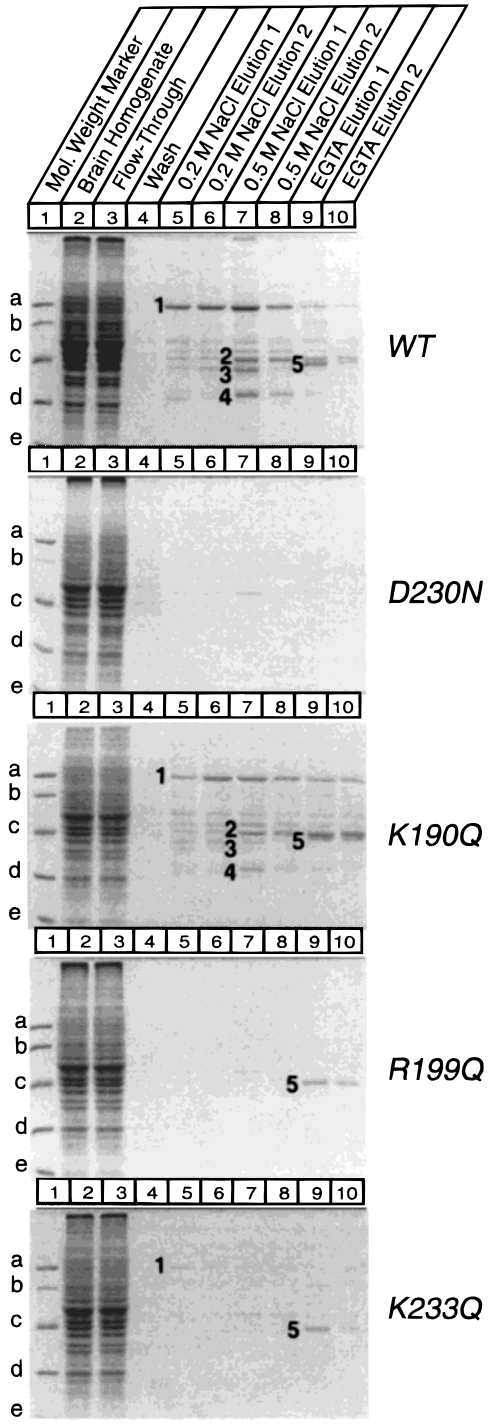

FIGURE 4: Analysis of protein binding to mutant C_2A domains from synaptotagmin I. GST-fusion proteins of the wild-type C_2A domain from synaptotagmin I (WT) or of the C_2A domain with amino acid substitutions in the top Ca^{2+} binding loops (D230N, R199Q, and K233Q) or in the bottom Ca^{2+} independent loops (K190Q) were immobilized on glutathione agarose beads in a column. Columns were used for affinity chromatography with rat brain proteins as described in Figure 2.

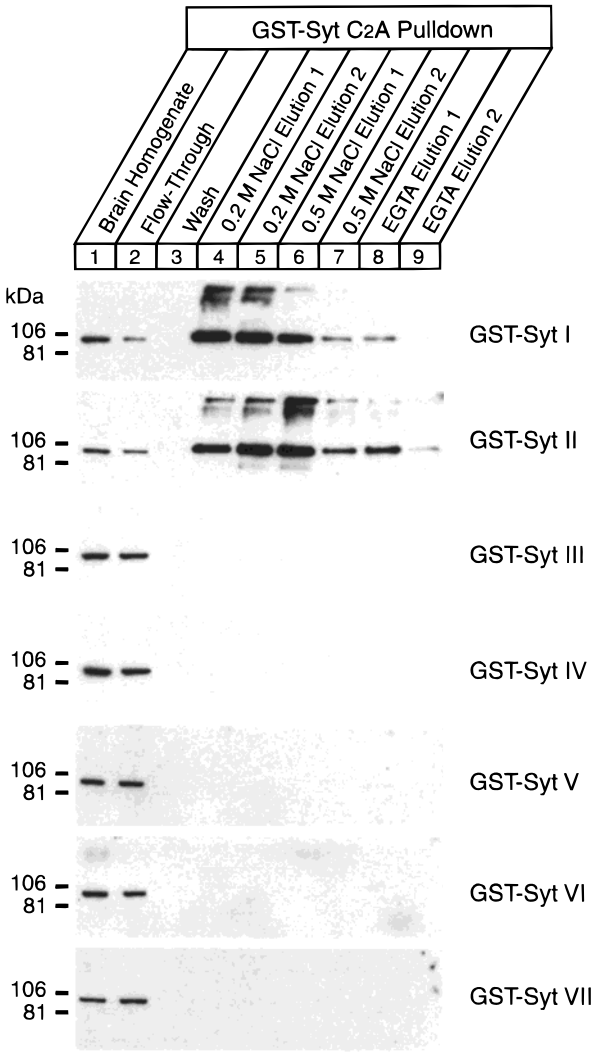

FIGURE 5: Immunblotting analysis of VCP binding to the C_2A domain of synaptotagmin I/II. Fractions obtained during affinity chromatography with the immobilized GST-fusion proteins of C_2A domains of synaptotagmin I−VII, Figure 2, were analyzed by immunoblotting using ECL detection with the antibody to VCP.

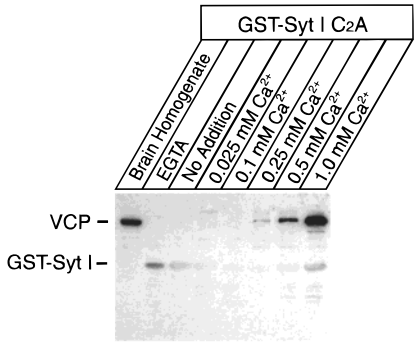

FIGURE 6: Ca^{2+} dependence of VCP binding to the C_2A domain of synaptotagmin I. The immobilized GST-fusion protein of the synaptotagmin I C_2A domain was used for pulldowns of rat brain proteins in buffers containing 2.5 mM Mg^{2+} and the indicated Ca^{2+} concentrations. Beads were washed in the incubation buffers, and bound proteins were analyzed by immunoblotting for VCP.

In brain, synapses develop primarily after birth to become one of the most abundant subcellular structures. As a result, proteins with a function in synaptic membrane traffic often are expressed in brain at much higher levels than in other tissues, even if they are ubiquitously synthesized in all cells. An example for this phenomenon is the distribution of NSF which is an essential chaperone for membrane traffic in all cells but nevertheless present in brain at levels that are an order of magnitude higher than its levels in other tissues (*65*, *66*). When we probed the tissue distribution of VCP, however, and compared it to that of NSF, we found a dramatically different pattern (Figure 9). This suggests that

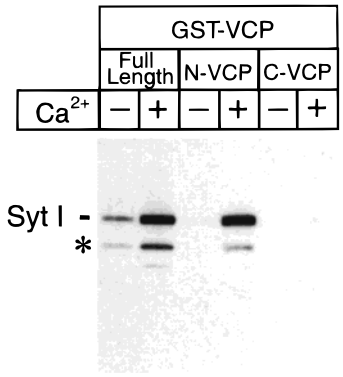

FIGURE 7: Binding of native rat brain synaptotagmin I to GST-fusion proteins of VCP. Recombinant GST-fusion proteins of full-length VCP or the N- and C-terminal regions of VCP (N−VCP = residues 1−213; C−VCP = residues 682−806) were used for GST-pulldown experiments with rat brain homogenates in the absence and presence of Ca^{2+}. Beads were washed in the incubation buffers, and bound proteins were analyzed by immunoblotting for synaptotagmin I. The asterisk marks the position of the major proteolytic breakdown product of synaptotagmin I.

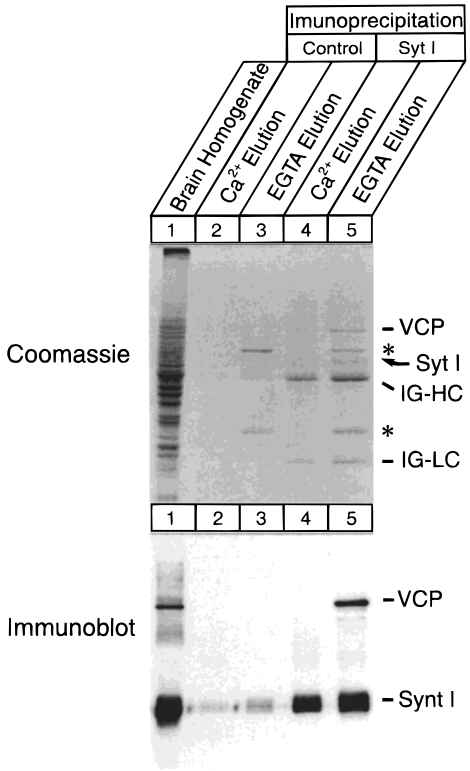

FIGURE 8: Immunoprecipitation of synaptotagmin I. Brain extracts were incubated with protein G-Sepharose alone (control) or protein G-sepharose with cross-linked monoclonal antibody (Cl41.1) against synaptotagmin I (Syt I). After extensive washing, bound proteins were eluted with the incubation buffer containing Ca^{2+} (Ca^{2+} elution; lanes 2 and 4) followed by incubation buffer containing EGTA (EGTA elution; lanes 3 and 5). Samples were analyzed by Coomassie blue staining (upper panel) and by immunoblotting for syntaxin I and VCP (lower panel). The positions of various proteins (VCP, synaptotagmin I [Syt I], heavy and light chains of IgG [IG-HC and IG-LC, respectively], and syntaxin I [Synt I]) are indicated on the right. Note that a minor amount of IgG and syntaptotagmin I leaks off the column. Asterisks mark the two proteins that are similarly purified on protein G-Sepharose as a function of Ca^{2+} under control and test conditions.

VCP performs no preferential brain function as would be expected for a synaptic protein.

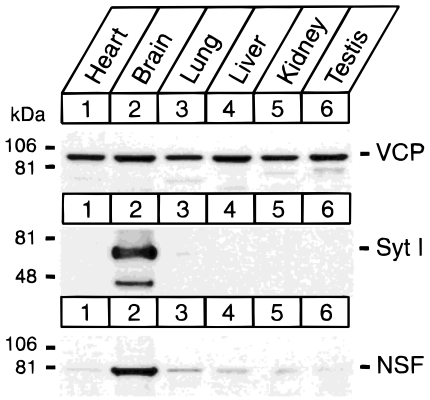

FIGURE 9: Tissue distribution of VCP expression. Equal amounts (50 μg) of homogenates from the indicated rat tissues were analyzed by immunoblotting with antibodies to VCP (top panel), synaptotagmin I (Syt I, middle panel), and NSF (bottom panel). Numbers on the left indicate positions of molecular weight markers.

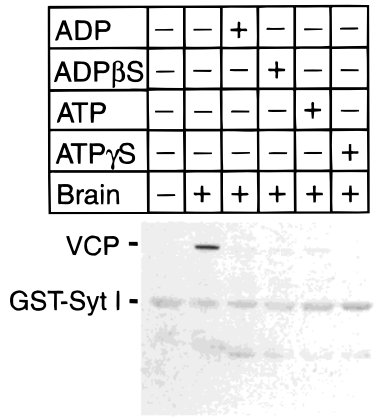

FIGURE 10: Effects of adenosine nucleotides on the binding of VCP to the C_2A domain of synaptotagmin. The GST-fusion protein of the C_2A domain from synaptotagmin I was used for pulldowns of rat brain proteins in control buffer or in the presence of 0.5 mM of the indicated nucleotides (ADP, ADPβS, ATP, and ATPγS). The proteins were washed extensively in the incubation buffers, and bound proteins were analyzed by immunoblotting for VCP. Since the VCP antibodies (K330) were raised against GST−VCP, they were preabsorbed extensively with GST to reduce the crossreactivitiy with the GST−synaptotagmin fusion protein (GST−Syt I).

Synapses develop late, largely postnatally. As a result, most synaptic proteins become abundant only after birth. Therefore, an indirect test for a protein with a synaptic function is that its abundance increases dramatically postnatally. Again, when we tested VCP in this regard, we found that in contrast to true synaptic proteins, there was no postnatal increase in expression (data not shown). Even a relatively ubiquitously acting protein like rab5 exhibited a more pronounced "synaptic" pattern. This result further supports the notion that VCP may not have a specialized synaptic function.

Binding of VCP to Synaptotagmin Is Inhibited by Nucleotides. VCP is an ATPase that is presumably involved in fusion as a kind of chaperone (*62, 63*). Therefore we tested the effect of various nucleotides on the interaction of VCP with synaptotagmin (Figure 10). Surprisingly, with any nucleotide tested, hydrolyzable or nonhydrolyzable, inhibition of synaptotagmin binding was observed. Synaptotagmin did not bind to VCP whenever VCP was complexed to a nucleotide. This result suggests that only unfolded VCP

lacking nucleotides binds to synaptotagmin I. Since it is unlikely that in a cell VCP is ever without nucleotides, the interaction of VCP appears to be artifactual as it only operates with unfolded VCP.

SUMMARY

The current study reports two findings that may be generally important for our understanding of the function of synaptotagmin I in triggering neurotransmitter release. First, we show that closely related C_2A domains from different synaptotagmins exhibit an exquisite binding specificity for distinct proteins. This finding resolves a paradox, namely the observation that synaptotagmins I and III interact with similar targets (phospholipids and syntaxin) even though they have distinct subcellular localizations and functions (*23*, *47*). The results suggest that the synaptotagmins of synaptic vesicles and the nonvesicular synaptotagmins perform their distinct functions by interacting with different proteins. At least four synaptotagmins are expressed in invertebrates, indicating that the diversification of synaptotagmin functions is evolutionarily conserved (*45*). Furthermore, our observations show that the different Ca^{2+}-dependent protein interactions observed for the C_2A domains are mediated by their top loops, revealing a high degree of specificity that must be encoded in the relatively small sequence variations of these loops.

Our second major finding is that even proteins which bind stoichiometrically, specifically, and Ca^{2+}-dependently to a C_2A domain are not necessarily physiologically relevant. It may seem a somewhat frivolous exercise to document in detail an interaction of synaptotagmin I with VCP, two interesting proteins involved in membrane fusion, only to conclude in the end that the interaction is likely unphysiological. However, we feel that this is a very instructive example of the difficulties of such binding studies. Few interactions reported for synaptic proteins are as compelling as the binding of the C_2A domain of synaptotagmin I to VCP: First, it is stoichiometric; VCP is the major protein purified on the column. Second, it can be observed with both orientations, i.e., with GST−synaptotagmin and with GST−VCP. Third, it is specific since closely related isoforms of synaptotagmin do not execute this interaction. Fourth, it is absolutely Ca^{2+} dependent and requires the same Ca^{2+} concentration as syntaxin binding to synaptotagmin I. Finally, a stoichiometric complex of VCP with synaptotagmin I can be immunoprecipitated from brain. Based on this evidence, a physiological interaction of VCP with synaptotagmin seemed likely. Nevertheless, we would like to propose that this is not so: VCP is a chaperone which contains two ATP-binding subunits; it is unlikely to be ever nucleotide-free in a cellular enrivonment. The fact that any nucleotide inhibits binding suggests that the interaction of VCP with synaptotagmin I involves the unfolded state of VCP which is not physiological. Additional evidence against a physiological interaction of VCP with synaptotagmin I was provided by the fact that VCP is not enriched in brain, and that its levels to not increase developmentally in parallel with other synaptic markers. One corollary of these findings is that functional implications of interactions observed in vitro, including immunoprecipitations, should be assessed carefully until these interactions are found to make biological sense.

With a large number of proteins cloned, and an increasing number of genes knocked out, for many proteins now the major question is not what they do, but how they do it. One of the frequently used techniques to address this question is to find interacting proteins, with the underlying idea that membrane traffic is carried out by protein−protein interactions and that the identification of the binding partners of a protein will give insight into its mechanism of action. As a result, assays for protein−protein interactions have become important, with the most frequently employed techniques consisting in GST pulldowns, immunoprecipitations, and yeast two-hybrid studies. However, in the case of synaptotagmin, as in the case of several other proteins, these approaches are primarily useful as a screening method that allows formulation of testable hypotheses, but does not yield finished results in their own right.

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