The Evolutionary Pressure to Inactivate

A SUBCLASS OF SYNAPTOTAGMINS WITH AN AMINO ACID SUBSTITUTION THAT ABOLISHES Ca\textsuperscript{2+} BINDING

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Synaptotagmin I is a Ca\textsuperscript{2+}-binding protein of synaptic vesicles that serves as a Ca\textsuperscript{2+} sensor for neurotransmitter release and was the first member found of a large family of trafficking proteins. We have now identified a novel synaptotagmin, synaptotagmin XI, that is highly expressed in brain and at lower levels in other tissues. Like other synaptotagmins, synaptotagmin XI has a single transmembrane region and two cytoplasmic C\textsubscript{2}-domains but is most closely related to synaptotagmin IV with which it forms a new subclass of synaptotagmins. The first C\textsubscript{2}-domain of synaptotagmin I (the C\textsubscript{2A}-domain) binds phospholipids as a function of Ca\textsuperscript{2+} and contains a Ca\textsuperscript{2+}-binding site, the C\textsubscript{2}-motif, that binds at least two Ca\textsuperscript{2+} ions via five aspartate residues and is conserved in most C\textsubscript{2}-domains (Shao, X., Davletov, B., Sutton, B., Südhof, T. C., Rizo, J. R. (1996) Science 273, 248–253). In the C\textsubscript{2A}-domains of synaptotagmins IV and XI, however, one of the five Ca\textsuperscript{2+}-binding aspartates in the C\textsubscript{2}-motif is substituted for a serine, suggesting that these C\textsubscript{2A}-domains do not bind Ca\textsuperscript{2+}. To test this, we produced recombinant C\textsubscript{2A}-domains from synaptotagmins IV and XI with either wild type serine or mutant aspartate in the C\textsubscript{2}-motif. Circular dichroism showed that Ca\textsuperscript{2+} stabilizes both mutant but not wild type C\textsubscript{2A}-domains against temperature-induced denaturation, indicating that the mutations restore Ca\textsuperscript{2+}-binding to the wild type C\textsubscript{2A}-domains. Furthermore, wild type C\textsubscript{2A}-domains of synaptotagmins IV and XI exhibited no Ca\textsuperscript{2+}-dependent phospholipid binding, whereas mutant C\textsubscript{2A}-domains bound phospholipids as a function of Ca\textsuperscript{2+} similarly to wild type synaptotagmin I. These experiments suggest that a class of synaptotagmins was selected during evolution in which the Ca\textsuperscript{2+}-binding site of the C\textsubscript{2A}-domain was inactivated by a single point mutation. Thus, synaptotagmins must have Ca\textsuperscript{2+}-independent functions as well as Ca\textsuperscript{2+}-dependent functions that are selectively maintained in distinct members of this gene family.

Synaptotagmins represent a large protein family with at least ten genes that probably function in membrane traffic (1–9). All synaptotagmins are characterized by a single N-terminal transmembrane region and two cytoplasmic C\textsubscript{2}-domains followed by a short conserved C terminus. Synaptotagmins I and II probably serve as Ca\textsuperscript{2+} sensors in fast Ca\textsuperscript{2+}-dependent exocytosis (10, 11). Synaptotagmin III may have a distinct function at the synapse, possibly in mediating the slow component of Ca\textsuperscript{2+}-dependent release (12), while the other synaptotagmins are thought to function in related neuronal and nonneuronal membrane trafficking reactions (6). However, the total number of synaptotagmins and their localizations and functions are unknown. The current study was initiated to determine if there are additional synaptotagmins in mammals that could be grouped into subclasses with distinct properties.

Most synaptotagmins are Ca\textsuperscript{2+}-binding proteins (1). In synaptotagmin I, the first C\textsubscript{2}-domain (C\textsubscript{2A}-domain) binds to phospholipids and syntaxin as a function of Ca\textsuperscript{2+} (6, 13). The second C\textsubscript{2}-domain mediates the Ca\textsuperscript{2+}-dependent binding of synaptotagmin to itself, leading to Ca\textsuperscript{2+}-dependent homomultimers (14, 15). A crystal structure of the C\textsubscript{2A}-domain of synaptotagmin I revealed that it represents a compact domain composed of eight \( \beta \)-strands forming two \( \beta \)-sheets (16). Three sequence loops emerge from the top, and four from the bottom of the domain. Ca\textsuperscript{2+} binding to the C\textsubscript{2A}-domain stabilizes it (17) but does not induce a major conformational change (18, 19). Detailed studies of Ca\textsuperscript{2+} binding to the C\textsubscript{2A}-domain by NMR spectroscopy demonstrated that it binds at least two Ca\textsuperscript{2+} ions at a site formed by the top three loops (19). Ca\textsuperscript{2+} is coordinated by five aspartate residues, three of which coordinate both Ca\textsuperscript{2+} ions. Thus the C\textsubscript{2A}-domain contains an unusual Ca\textsuperscript{2+}-binding site, designated the C\textsubscript{2}-motif, that is formed by aspartate residues on discontinuous sequence loops.

More than 50 C\textsubscript{2}-domain sequences are present in the data banks, suggesting that it is a widespread domain (20). The C\textsubscript{2}-motif is conserved in many of these C\textsubscript{2}-domains that are thus likely to bind Ca\textsuperscript{2+} similarly to the C\textsubscript{2A}-domain of synaptotagmin I. In agreement with the binding of at least two Ca\textsuperscript{2+} ions to the C\textsubscript{2A}-domain of synaptotagmin I, Ca\textsuperscript{2+} cooperatively activates phospholipid binding to native synaptotagmin I (12, 21) and to recombinant C\textsubscript{2A}-domain (13). Phospholipid binding is promiscuous and only requires negatively charged phospholipids (12). In addition to phospholipids, C\textsubscript{2A}-domains bind syntaxin as a function of Ca\textsuperscript{2+} but with distinct Ca\textsuperscript{2+} affinities that suggest different functions in membrane traffic (6).

Although most C\textsubscript{2A}-domains from synaptotagmins contain the C\textsubscript{2}-motif and bind phospholipids as a function of Ca\textsuperscript{2+}, those of synaptotagmins IV and VIII do not (1, 6, 11). Synaptotagmin VIII contains many changes in the sequences of the Ca\textsuperscript{2+}-binding loops, indicating that the C\textsubscript{2}-motif is not formed in this synaptotagmin. By contrast, the C\textsubscript{2A}-domain of synaptotagmin IV is very similar to that of the other synaptotagmins and contains all residues of the C\textsubscript{2}-motif except that one of the five Ca\textsuperscript{2+}-coordinating aspartates is substituted for a serine. In
this study, we now describe the identification of a novel synaptotagmin, synaptotagmin XI, that is highly homologous to synaptotagmin IV and also contains the aspartate to serine substitution in the C2-motif. Since the changed aspartate coordinates both Ca\(^{2+}\) ions in the C2-motif, the substitution is predicted to abolish Ca\(^{2+}\)-binding, and indeed no Ca\(^{2+}\)-dependent phospholipid binding was observed with standard liposomes for the C2A-domain of synaptotagmin IV (11).

In addition to participating in Ca\(^{2+}\)-dependent interactions, C2-domains from synaptotagmins also exhibit Ca\(^{2+}\)-independent activities. For example, the second C2-domain of all synaptotagmins tested binds AP2, a clathrin adaptor protein complex predicted to abolish Ca\(^{2+}\) currents (6, 11). Such a demonstration could be obtained, however, if reversal of an inactivating amino acid substitution restored Ca\(^{2+}\)-dependent properties. Indeed, the notion that the C2A-domain of synaptotagmin IV is a Ca\(^{2+}\)-independent domain was challenged by a report of Ca\(^{2+}\)-dependent binding of the C2A-domain of synaptotagmin IV to phospholipids consisting of 100% PS (23). It is thus important to establish if synaptotagmins IV and XI are Ca\(^{2+}\)-independent because this would support a general function for C2-domains in Ca\(^{2+}\)-independent reactions. Therefore, a further goal of the current study was to determine if the C2A-domains of synaptotagmins IV and XI are selectively inactivated in evolution as Ca\(^{2+}\)-binding modules by a single substitution, or if these C2-domains contain additional changes that differentiate them from other synaptotagmins. Our data reveal that synaptotagmins IV and XI contain a single point mutation that selectively abolishes Ca\(^{2+}\) binding but leaves other properties of the C2-domain intact.

**EXPERIMENTAL PROCEDURES**

Cloning of synaptotagmin XI and sequence analysis—Searches of GenBank with a consensus sequence from the C-terminal domain of synaptotagmins and double C2-domain proteins (1) uncovered a human EST sequence (accession number D38552) that represents a novel member of this family. PCR primers were designed based on the human sequence (sequences: GCGGATTCTGGTAATCTAGATTGACGACCTTAAAGCTGAGT- GAA/C, TGT and GGCGCTGACTGATA TCAGCTGACAGTGGCGC- A/C, TTTC/C, T/A, GGGC, letters in brackets indicate redundant posi-

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1 The abbreviations used are: PS, phosphatidylserine; EST, expressed sequence tag; GST, glutathione S-transferase; PC, phosphatidylcholine; PCR, polymerase chain reaction.
region with multiple neighboring cysteines, has two C2-domains, and has a short C-terminal sequence homologous to the C-terminal domains of other double C2-domain proteins (Fig. 1). In continuation of the naming of other members of this protein family, we named this protein synaptotagmin XI.

Expression of Synaptotagmin XI—To learn which rat tissues express synaptotagmin XI, we performed an RNA blot analysis. High levels of synaptotagmin XI mRNA were observed in brain (Fig. 2). In addition, low levels of the mRNAs could be detected in almost all tissues tested, suggesting that synaptotagmin XI, similar to synaptotagmins IV, VI, and VII (6), is not brain-specific but ubiquitously expressed in low abundance.

Definition of a Subclass of Synaptotagmins—With the new synaptotagmin described here, synaptotagmins now constitute a family of 11 proteins (2–9). Although all synaptotagmins are composed of similar domains, their N-terminal regions (intravesicular sequence, transmembrane region, and linker between the transmembrane region and the C2A-domain) exhibit little similarity to each other except for pairwise similarities between synaptotagmins I and II and between III and VI (1, 6).

By contrast, the C-terminal domains (the two C2-domains and the short C-terminal domain) are highly homologous between all synaptotagmins. Sequence analysis of synaptotagmin XI demonstrates that it is most closely related to synaptotagmin IV (53% overall identity; Fig. 1). Synaptotagmins IV and XI are homologous to each other in their N-terminal 73 amino acids which exhibit no sequence similarity to other synaptotagmins, defining them as a separate subgroup. In addition, the C2-domains of synaptotagmins IV and XI are more closely related to each other than to those of other synaptotagmins. The sequence differences between different synaptotagmins are not random variations since they are evolutionarily conserved (98% identity between rat and mouse synaptotagmin IV) (11).

Similarly, the C-terminal 104 amino acids of human synaptotagmin XI encoded by EST D38522 exhibits only a single amino acid change compared with the rat sequence.

The C2-domain of synaptotagmin I binds two Ca2+ ions through five aspartate residues located in two loops (19). Three of the aspartates are bifunctional and ligate both Ca2+ ions. Alignment of different C2A-domains shows that most contain either aspartates or glutamates at the five positions that are involved in Ca2+ binding (Fig. 3). The C2A-domains of synaptotagmins IV and XI, however, are different. The general spacing is the same, but, in one position (corresponding to aspartate 230 in synaptotagmin I), a serine is substituted for the aspartate (Fig. 3). The serine is proposed to act as a sequencing or cloning artifact since it is present in both the C2A-domains of synaptotagmins IV and XI, however, are different. The general spacing is the same, but, in one position (corresponding to aspartate 230 in synaptotagmin I), a serine is substituted for the aspartate (Fig. 3). The serine is proposed to act as a sequencing or cloning artifact since it is present in both
synaptotagmin IV and XI and since the serine is evolutionarily conserved in synaptotagmin IV (11).

Aspartate 230 is a critical residue in synaptotagmin I since it coordinates both Ca$^{2+}$ ions and is at the center of the C$_2$-motif (19). Modeling of the analogous Ca$^{2+}$-binding site in synaptotagmins IV and XI suggests that in the presence of serine instead of aspartate, Ca$^{2+}$ binding is unlikely (Fig. 4A). This model also suggests that Ca$^{2+}$ binding could potentially be restored if the serine is mutated to aspartate (Fig. 4B). To test these hypotheses, we constructed expression vectors that direct the synthesis of GST-fusion proteins with the C$_2$A-domains from synaptotagmins IV and XI either in the wild-type forms containing serine or in mutant forms containing aspartate instead of serine at the appropriate position.

Figure 4. Model of the Ca$^{2+}$-binding site in the C$_2$-motif of the C$_2$A-domain from synaptotagmin XI as wild type sequence containing serine (A) and as mutant with the serine to aspartate substitution (B). The diagram is based on the bipartite Ca$^{2+}$-binding motif modeled for the C$_2$A-domain of synaptotagmin I (19); residues are identified by numbers (Fig. 1). In the wild type form (A), serine 247 is predicted to be unable to act as a bidentate ligand for the two Ca$^{2+}$ ions. In the serine to aspartate substitution mutant (B), aspartate 247 can contribute to bind the two Ca$^{2+}$ ions as in the C$_2$A-domain of synaptotagmin I. An analogous model is proposed for synaptotagmin IV, with the corresponding changes in residue numbers. Dashed lines illustrate the coordination of the two Ca$^{2+}$ ions. The protein backbone linking aspartate residues in the same loop is represented by solid curves.

Figure 5. Effect of Ca$^{2+}$ on the temperature-dependent denaturation of the wild type and mutant C$_2$A-domains from synaptotagmins IV and XI. Purified recombinant wild type C$_2$A-domains from synaptotagmins IV and XI (A and C, respectively) and mutant C$_2$A-domains from the same synaptotagmins (B and D, respectively) were incubated with 0.5 mM EGTA in the absence of Ca$^{2+}$ (open circles) or in the presence of 5.5 mM Ca$^{2+}$ (closed circles). Unfolding of the domains as a function of temperature was monitored by CD absorbance at conformation-dependent wavelength (217 nm). The mutants contain a single amino acid substitution exchanging the serine in the C$_2$-motif for aspartate (see Figs. 3 and 4).
no Ca\(^{2+}\)-dependent binding of phospholipids consisting of 29% PS and 71% PC was observed to the wild type C\(_2\)A-domains from synaptotagmins IV and XI. In contrast, the C\(_2\)A-domain of synaptotagmin I exhibited robust Ca\(^{2+}\)-specific phospholipid binding. No Ca\(^{2+}\)-independent phospholipid binding was observed as the background level of phospholipids bound corresponds to that obtained with GST alone. The mutant C\(_2\)A-domains, however, exhibited marked Ca\(^{2+}\)-dependent phospholipid binding that was not observed with Mg\(^{2+}\) (Fig. 6).

These data suggest that the C\(_2\)A-domains of the synaptotagmin IV/XI subclass contain a selective, evolutionarily conserved single amino acid substitution that inactivates Ca\(^{2+}\) binding and Ca\(^{2+}\)-dependent phospholipid binding.

Dependence of Ca\(^{2+}\)-dependent Phospholipid Binding on Liposome Composition—Recently, it was reported that the C\(_2\)A-domain of synaptotagmin IV can bind 100% PS in a Ca\(^{2+}\)-dependent manner although it does not bind to liposomes composed of mixtures of PS and PC (23). Given the structure of the Ca\(^{2+}\)-binding site in C\(_2\)A-domains (Fig. 4), this is a surprising finding since the C\(_2\)A-domain is not expected to bind Ca\(^{2+}\) with the aspartate to serine substitution. However, PS itself binds Ca\(^{2+}\) at the concentrations used, and it is possible that clusters of negative charges by PS may also allow Ca\(^{2+}\) binding to the protein with unanticipated properties. Furthermore, stable bilayers are difficult to obtain with 100% PS, which tends to aggregate. Since the assay used by Fukuda et al. (23) utilized soluble GST-fusion proteins that were co-precipitated with lipids, the possibility arises that Ca\(^{2+}\)-dependent PS aggregation could artifactually trap the C\(_2\)A-domain.

To test if 100% PS induces a novel Ca\(^{2+}\)-binding site in wild type synaptotagmin IV, we investigated the Ca\(^{2+}\)-dependent interactions of the wild type and mutant C\(_2\)A-domains from synaptotagmin IV with 100% PS in an assay that does not depend on lipid co-precipitation to avoid artifacts. No binding of 100% PS to the wild type C\(_2\)A-domain was observed (Fig. 6, D and E). Even with the mutant C\(_2\)A-domain, which exhibits robust Ca\(^{2+}\)-dependent binding to liposomes composed of 29% PS, 71% PC, Ca\(^{2+}\) only had an insignificant effect on binding of 100% PS. Since, in the C\(_2\)A-domain of synaptotagmin I, only negatively charged phospholipids bind as a function of Ca\(^{2+}\), the absence of Ca\(^{2+}\)-dependent binding of 100% PS to the mutant C\(_2\)A-domain of synaptotagmin IV is paradoxical. This result can best be explained by the assumption that a stable lipid bilayer may be required. To test this hypothesis, we made liposomes from 80% PS, 20% PC, which are more likely to contain stable bilayers. Now, Ca\(^{2+}\)-dependent phospholipid binding to the mutant C\(_2\)A-domain was observed although it was not as strong as that observed with our standard liposomes. The wild type C\(_2\)A-domain, however, was still unable to bind. Together, these data provide further evidence for the conclusion that the wild type C\(_2\)A-domain of synaptotagmin IV is not a Ca\(^{2+}\)-binding domain and suggest that, even under conditions of PS enrichment, no Ca\(^{2+}\)-dependent properties can be demonstrated.

DISCUSSION

Synaptotagmins form a large family of genes with putative functions in membrane traffic (1). We now report the structure and properties of the 11th member of this family. Since it seems likely that additional synaptotagmins remain to be discovered, synaptotagmins now form one of the largest protein families in membrane traffic second only to rab proteins.

The structure of synaptotagmin XI reveals that it defines a new subclass of synaptotagmins together with synaptotagmin IV. Synaptotagmins IV and XI are highly expressed in brain, and at lower levels in other tissues (Fig. 2; see Ref. 6). This indicates a function that is concentrated in brain but also
A Subclass of Ca\textsuperscript{2+}-dependent Synaptotagmins

Operative in nonneural tissues, synaptotagmins I and II probably serve as Ca\textsuperscript{2+} sensors in neurotransmitter release (10, 11), suggesting that the other synaptotagmins also represent membrane trafficking proteins at the plasma membrane, possibly in regulating different forms of exocytosis. In addition, synaptotagmins may function in endocytosis since all synaptotagmins tested are high affinity binding proteins for the clathrin adapter protein complex, AP2 (6, 11, 22).

The subgroup of synaptotagmins composed of synaptotagmins IV and XI is characterized by an homologous N-terminal region and by identical deviations from the C\textsubscript{2}-domain consensus sequence shared by most synaptotagmins (Figs. 1 and 3). The most remarkable change in the C\textsubscript{2}-domains of synaptotagmins IV and XI is the substitution of one of the aspartates of the C\textsubscript{2}-binding site for a serine. This raises the question if a C\textsubscript{2A}-domain with this substitution is capable of Ca\textsuperscript{2+} binding. We show by two assays, the Ca\textsuperscript{2+}-binding assay and Ca\textsuperscript{2+} denaturation curve and Ca\textsuperscript{2+} concentration dependent shift in the heat denaturation curve and Ca\textsuperscript{2+}-dependent bottom (1). This would imply that in the synaptotagmin IV/XI subgroup, evolutionary pressure led to a selective retention of only the Ca\textsuperscript{2+}-independent properties. Unfortunately no Ca\textsuperscript{2+}-independent activities of a C\textsubscript{2A}-domain have been identified yet. Once these have been discovered, however, it will be important to test them in different synaptotagmins to determine if they are selectively retained in the Ca\textsuperscript{2+}-independent forms.

In a very interesting study, synaptotagmin IV was identified as an immediate early gene (26). This suggests that a switch from Ca\textsuperscript{2+}-dependent to Ca\textsuperscript{2+}-independent synaptotagmins may occur during strong stimulation of neurons. Such a switch could be particularly useful during pathological hypoxecitation that is accompanied by unimpeded Ca\textsuperscript{2+} influx. A switch to a Ca\textsuperscript{2+}-unresponsive synaptotagmin under those conditions would eliminate a Ca\textsuperscript{2+} target and maybe inhibit excessive neurotransmitter release. Future studies will have to test this hypothesis and also investigate if synaptotagmin XI is also an immediate early gene.

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