Calcium-dependent Interaction of the Cytoplasmic Region of Synaptotagmin with Membranes

AUTONOMOUS FUNCTION OF A SINGLE C2-HOMOLOGOUS DOMAIN*

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The synaptic vesicle protein synaptotagmin has been implicated in the docking and subsequent calcium-regulated exocytosis of synaptic vesicles. We demonstrate that synaptotagmin is a major constituent of synaptic vesicle membranes, comprising 7–8% of the total vesicle protein. A proteolytic fragment of synaptotagmin, containing two repeats homologous to the C2-domain of protein kinase C, bound to a variety of natural membranes in a calcium-dependent manner (EC$\text{so}^\text{30} \sim 30 \mu\text{M calcium}). Binding was insensitive to proteolysis of the acceptor membranes suggesting an interaction with the lipid constituents. This interaction was confirmed using a recombinant fusion protein, containing both C2-like domains of synaptotagmin, that bound to artificial liposomes in a calcium-dependent manner. Phospholipid binding properties were preserved in a 114-amino acid domain corresponding to the first C2-like repeat of the protein and represents the shortest functional cassette yet reported. Furthermore, deletion of a highly conserved 9-amino acid motif, within this region, was sufficient to abolish the calcium-dependent phospholipid binding properties of this domain. This mutation may provide a means to selectively disrupt individual C2-domains in order to assess their relative contributions to function.

Synaptotagmin represents a small family (Perin et al., 1990; Geppert et al., 1991; Wendland et al., 1991) of transmembrane proteins present in synaptic vesicles (SV)† and large secretory granules of neurons and endocrine cells (Walch-Solimena et al., 1993) and references therein). Synaptotagmin binds calcium and phospholipids in a mutually dependent manner, making the molecule an attractive candidate as the calcium receptor for regulated exocytosis (Brose et al., 1992). In addition, synaptotagmin has been implicated in vesicle docking since it interacts, in a calcium-independent manner, with the plasma membrane proteins neurexin and syntaxin (Bennett et al., 1992; Petrenko et al., 1991; Hata et al., 1993). A calcium-dependent interaction has also been reported between synaptotagmin and putative receptors for protein kinase C (Mochly-Rosenthal et al., 1992).

The precise function of synaptotagmin in the secretory machinery remains to be established. The physiological function of the protein has been addressed in a number of recent studies. For instance, calcium-dependent secretion from large dense-core vesicles occurs in PC12 cell lines lacking synaptotagmin (Shoji-Kasai et al., 1992). Mutations in the synaptotagmin genes of Caenorhabditis elegans (Nonet et al., 1993) and Drosophila (DiAntonio et al., 1993, Littleton et al., 1993) result in severely impaired nervous system function, consistent with defective synaptic transmission. These studies indicate that synaptotagmin is not an absolute requirement for exocytosis to occur but suggest that the protein plays a crucial modulatory role in regulated secretion. This role has been examined by Littleton et al. (1993) who reported that Drosophila larva, with parcial lack-of-function synaptotagmin mutations, exhibited an increase in miniature excitatory potentials and a decrease in calcium-induced neurotransmitter release.

The most striking structural feature of synaptotagmin is the presence of two repeats, in the cytoplasmic domain, which are homologous to the C2-regulatory domain of protein kinase C (Perin et al., 1990), see also Fig. 2. C2-domains are conserved regions shared by calcium-dependent but not calcium-independent isoforms of protein kinase C, suggesting that these regions confer calcium dependence (reviewed by Nishizuka, 1988). Homologous domains have subsequently been identified in other proteins which interact with lipids including cytosolic phospholipase A2 (Clark et al., 1991), phospholipase C-g (Stahl et al., 1988), GTPase-activating protein (Vogel et al., 1988), and rabphilin (Shirakata et al., 1993). A consensus sequence within this conserved domain has been reported by Clark et al. (1991) and is given in Fig. 2. Recent studies indicate that the C2-like domains of synaptotagmin (referred to as C2-domains) may be involved in the regulation of calcium-dependent exocytosis. For instance, microinjection of a recombinant fragment containing the first C2-domain of synaptotagmin inhibited calcium-dependent exocytosis of large dense core vesicles in PC12 cells (Ellerink et al., 1993). Peptides corresponding to sequences within the C2-domains also inhibited neurotransmitter release when injected into squid giant presynaptic nerve terminals (Bonnert et al., 1993).

While such findings suggest that the C2-domains are important for the function of synaptotagmin, surprisingly little is known regarding their biochemical properties. For instance, a deletion encompassing most of the C2-domain of protein kinase C failed to abolish calcium and phospholipid-dependent activity (Kaibuchi et al., 1989). In addition, limited proteolytic cleavage of synaptotagmin (see Fig. 2) appeared to abolish calcium-dependent lipid interactions (Brose et al., 1992) despite the fact that the C2-domains remained intact. These findings question whether C2-domains are solely responsible for the calcium/phospholipid-binding properties of their parent molecules.

In the present study, we have examined the interaction of synaptotagmin with membranes, with particular emphasis on the function of its C2-domains. Reconstitution of the cytosolic

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† To whom correspondence should be addressed. Tel.: 203-737-4454; Fax: 203-787-5334.
‡ The abbreviations used are: SV, synaptic vesicle; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; PI, phosphatidylinositol.
domain of the protein with native membrane preparations revealed a calcium-dependent interaction. Using recombinant fragments of synaptotagmin and artificial liposomes, we observed that the cytoplasmic domain of synaptotagmin selectively binds to negatively charged phospholipids in a calcium-dependent manner. In addition, these properties were preserved in a recombinant fusion protein which contained only the first C2 repeat of synaptotagmin. Furthermore, we identify a 9-aminoc acid motif crucial for the binding properties of this domain.

**EXPERIMENTAL PROCEDURES**

**Purification of Proteins—**Synaptotagmin was purified by affinity chromatography using the anti-synaptotagmin monoclonal antibody 41.1 as described by Brose et al. (1992). Protein concentrations for purified synaptotagmin and the membrane preparations described below were determined using the Pierce BCA (bicinchoninic acid) reagent.

**Reconstitution of the Cytoplasmic Domain of Synaptotagmin with Membranes—**A tryptic fragment comprising most of the cytoplasmic domain of synaptotagmin was prepared by limited proteolysis of highly purified SV membranes. The fragment was purified according to Huttner et al. (1985) and digested with trypsin in a tris:vesicle membrane protease ratio of 0.05:1 (v/v). Membrane protease was added after 15 min at room temperature. Digestion of the C2A domain was terminated by the addition of a 50-fold excess (w/v) of soybean trypsin inhibitor, 2 mM phenylmethylsulfonyl fluoride, 10 μM aprotinin, 10 μg/ml pepstatin A, and 10 μg/ml leupeptin. To examine the interaction of the fragment with purified SV membranes, 25 μg of the trypsin-treated SV membranes was mixed with other membrane preparations described below and assayed for binding, again, by co-sedimentation. In these experiments, the calcium concentrations were titrated, the free calcium concentrations were buffered with EGTA and calculated using immunoblot analysis in which the calcium concentrations were titrated, the free calcium concentrations were buffered with EGTA and calculated using immunoblot analysis. In these experiments, protein concentrations for purified SV membranes were determined using the Pierce BCA (bicinchoninic acid) reagent.

**Preparation of Recombinant Fusion Proteins—**cDNA encoding rat synaptotagmin I was kindly provided by T. C. Sudhof (Dallas, TX; Perin et al., 1990). All polymerase chain reactions (PCR) were carried out using Pfu polymerase (Strategene Corp.). The C2ABs-GST and C2A-GST recombinant fusion proteins were expressed in Escherichia coli (Pharmacia) for comparison with GST standards. Primary antibodies were added as above and incubated for 1 h at room temperature. Blots were washed 5 times, incubated for 1 h with rabbit anti-mouse IgG antibodies (Cappel) diluted 1:1000, washed 5 times, and incubated with 0.1 μg/ml 125I-protein A (DuPont-New England Nuclear) for 1 h, washed 5 times, dried, and autoradiographed.

**Immunoblot Analysis—**All SDS-PAGE was carried out using the Bio-Rad Mini Protein Gel II apparatus. Mouse monoclonal antibody 41.1, raised against a recombinant cytoplasmic domain of synaptotagmin has been described previously (Brose et al., 1992). A new set of monoclonal antibodies was generated by standard procedures (Köhler and Milstein, 1975; John et al., 1985). Using a 12-aminoc acid synthetic peptide derived from the amino terminus of rat synaptotagmin I (CMVSASH-PLEA) as the antigen, we obtained six independent hybridoma lines (Cl 604.1–6).

SDS-polyacrylamide gels were transferred to nitrocellulose, incubated for 1 h in blot (20 mM Tris, pH 7.5, 150 mM NaCl, 5% powdered milk, and 0.1% Tween 20). Mouse monoclonal antibodies 41.1 and 604.4 (anti-41) were added at a 1:20,000 dilution in blot buffer overnight at 4 °C. Blots were washed 3 times with blot buffer and incubated for 1.5 h at room temperature with a goat anti-mouse IgG antibody conjugated to alkaline phosphatase or horseradish peroxidase (Cappel) diluted 1:2000 in blot. Alkaline phosphatase blots were washed 3 times with 100 mM Tris, pH 9.5, 100 mM NaCl, and 5 mM MgCl₂. Immunoreactive bands were visualized by addition of nitroblue tetrazolium (1 mg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (0.17 mg/ml). Horseradish peroxidase blots were washed 3 times in blot, 3 times in phosphate-buffered saline and the immunoreactive bands visualized with 0.5 mg/ml 5-chloro-1-naphthol and 0.05% H₂O₂ in phosphate-buffered saline.

Blots probed with 125I-protein A were blocked overnight in blot. Primary antibodies were added as above and incubated for 1 h at room temperature. Blots were washed 5 times, incubated for 1 h with rabbit anti-mouse IgG antibodies (Cappel) diluted 1:1000, washed 5 times, and incubated with 0.1 μg/ml 125I-protein A (DuPont-New England Nuclear) for 1 h, washed 5 times, dried, and autoradiographed.
10^3 (EGTA) and 25 x 10^3 (calcium) dpm of PS/PC liposomes; 8.3 x 10^3 (EGTA) and 7.4 x 10^3 (calcium) dpm of PC liposomes; 15 x 10^3 (EGTA) and 23 x 10^3 (calcium) dpm of PI/PC liposomes; 14 x 10^3 (EGTA) and 13 x 10^3 (calcium) dpm of PE/PC liposomes. Using PS/PC liposomes, C2A-GST bound a total of 14 x 10^3 (EGTA) and 18 x 10^3 (calcium) dpm, C2A-GST bound 7.5 x 10^3 (EGTA) and 7.0 x 10^3 (calcium). GST alone immobilized to glutathione-Sepharose bound 1.6-2.5 x 10^3 dpm of labeled liposomes regardless of their composition or the presence of EGTA or calcium. The high degree of calcium independent binding of liposomes comprised of PE and PI/PC is likely due to nonspecific ionic and hydrophobic interactions with the immobilized synaptotagmin fusion protein. To eliminate these variables we measured the calcium-dependent component of the binding directly by elution with EGTA. Assays were carried out as described above. Following the washes, phospholipids which bound in a calcium-dependent manner were eluted by incubating the samples for 15 min in 200 μl of 20 mM Tris, pH 7.2, 50 mM NaCl, and 10 mM EGTA at 4 °C. The eluate was collected by centrifugation. Radioactivity in the entire eluate was measured by liquid scintillation counting. These data are plotted in Fig. 6.

Densitometry—Densitometry was carried out using a Visage 2000 scanner (Bio Image Products, Milligen/Biorsearch Division of Millipore).

RESULTS
Synaptotagmin Is a Major Constituent of Synaptic Vesicle Membranes—In the first set of experiments, we measured the amount of synaptotagmin in a highly purified preparation of SVs in order to determine its relative abundance with respect to other SV proteins. For this purpose, synaptotagmin, affinity purified as described (Brose et al., 1992), was used to generate a standard curve in a quantitative immunoblotting procedure (Fig. 1). From these data, synaptotagmin comprises 7–8% (w/w) of total vesicular protein. This is comparable to the abundance of synaptojan (Jahn et al., 1987) and synapsin (Goelz et al., 1981), resulting in a stoichiometric ratio of synaptotagmin: synaptojan of 0.74:1.

Reconstitution of Synaptotagmin/Membrane Interactions—To study synaptotagmin/membrane interactions, we have prepared a tryptic fragment as well as recombinant proteins encompassing cytoplasmic domains of the molecule. It has been reported earlier that synaptotagmin contains a hypersensitive proteolytic cleavage site between residues 111 and 112 (Perin et al., 1991; Tugal et al., 1991); Fig. 2). Purified SVs were incubated with low amounts of trypsin. After the end of the incubation, membranes were sedimented in the presence of calcium chelators. The supernatant and the pellet were analyzed.

Fig. 1. Quantification of synaptotagmin in synaptic vesicle membranes. Synaptotagmin and highly purified SV membranes were subjected to SDS-PAGE and immunoblotted using the anti-synaptotagmin monoclonal antibody 41.1. Bands were visualized by autoradiography using 125I-protein A as described under "Experimental Procedures." Bands from the autoradiograms were quantified by densitometry and plotted as a function of total vesicular protein. This is comparable to the abundance of synaptotagmin (ng) and 100 μg of SV protein.

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Synaptotagmin
N
TMR
C

Tryptic fragments
//

C2ABs
C2A

Consensus sequence:
M
SDPVVK
L
SDPVVK
L
LNPWNE
F
F
P

(A)

Synaptotagmin:
MGST
SDPVVK
L

P

(b)

Protein kinase C α:
MDPGQLSDPVVK
KL

P

(B)

Fig. 2. Schematic alignment of the synaptotagmin constructs and proteolytic fragments used in this study. A schematic representation of synaptotagmin is shown at the top: the transmembrane region (TMR) and the two C2 repeats (boxed) are indicated. The NH2 terminus faces the lumen of the vesicle. Below, the fragments resulting from limited tryptic proteolysis are depicted. Cleavage occurs between residues 111 and 112 (Tugal et al., 1991; Perin et al., 1991). C2ABs and C2A represent the regions of synaptotagmin fused to GST and used in the epitope mapping and phospholipid binding studies. C2ABs corresponds to residues 97–421 and C2A, the first C2 domain of synaptotagmin, corresponds to residues 135–248 (Perin et al., 1990). Shown below is the C2-domain consensus sequence for calcium-dependent lipid-binding proteins (Clark et al., 1991). For comparison, the corresponding sequences from PKCa (Coussens et al., 1986) and the first C2 repeat of synaptotagmin are given (Perin et al., 1990). Bold letters indicate residues which are conserved relative to the consensus sequence. A, denotes the region deleted from C2A resulting in C2AΔ (see "Experimental Procedures"). B, denotes the NH2-terminal border of the 110-residue region deleted from protein kinase Cα described by Kaibuchi et al. (1989).
Fig. 3. Characterization of tryptic and recombinant fragments of synaptotagmin using site-specific monoclonal antibodies. A, purified SV membranes were subjected to limited digestion with trypsin. Membranes were sedimented by centrifugation in 20 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EGTa, and equal fractions of the supernatant (sn) and pellet (p) subjected to SDS-PAGE and immunoblotted using anti-synaptotagmin mouse monoclonal antibodies. Immune complexes were visualized using an alkaline phosphatase-conjugated secondary antibody. Antibody 41.1 recognized the soluble cytosolic fragment of synaptotagmin of approximately M, = 39,000. Antibody 604.4, directed against a synthetic ecto-domain peptide derived from synaptotagmin, bound to the membrane associated fragment of approximately M, = 29,000. B, characterization of recombinant synaptotagmin-GST fusion proteins. The recombinant synaptotagmins were prepared and purified as described under "Experimental Procedures" and their structure is also described in the legend to Fig. 2. Five hundred ng of each purified recombinant protein was subjected to SDS-PAGE and visualized by Coomassie Blue staining (left panel). In the right panel, 50 ng of the recombinant proteins were immunoblotted using antibody 41.1 and visualized using a horseradish peroxidase-conjugated secondary antibody. Monoclonal antibody 41.1 reacted with the recombinant cytoplasmic domain of synaptotagmin, C2ABs-GST, and with the first C2-domain, C2A-GST. However, C2A-GST failed to react with 41.1.

Lyzed by immunoblotting using mouse monoclonal antibody 41.1 (directed against the cytoplasmic domain) and a newly generated monoclonal antibody directed against the NH4-terminal 12 amino acids of synaptotagmin I (clone 604.4). As shown in Fig. 3A, trypsin incubation yielded a soluble fragment of approximately M, = 39,000, recognized by antibody 41.1, and a membrane-associated fragment of approximately M, = 29,000, recognized by antibody 604.4 (Fig. 3A).

To further characterize the epitope recognized by the antibody 41.1, we prepared a set of synaptotagmin-glutathione S-transferase (GST) fusion proteins. The sequence of these fusion proteins is summarized in Fig. 2. The construct C2ABs-GST encodes residues 97–421 and thus contains both C2-domains. The COOH terminus of synaptotagmin, C2A-GST contains the first C2-domain (residues 135–248) as defined previously (Perin et al., 1990). C2AΔGST is identical to C2A-GST except that we have deleted a highly conserved motif (SDPYVKVFL, residues 177–185). As shown in Fig. 3B, 41.1 recognized C2ABs-GST and C2A-GST but did not react with C2AΔ-GST. These data demonstrate that residues 135–248 (C2A) contain the 41.1 epitope and that the sequence 177–185 contains residues critical for 41.1 recognition of either the primary or a higher order structure of synaptotagmin.

In the next series of experiments, we investigated the interactions of the 39-kDa cytoplasmic fragment of synaptotagmin with native membranes in a co-sedimentation assay. For comparison, we utilized SVs as well as synaptosomal and fibroblast membranes as the acceptor membranes. In the absence of calcium, only a small degree of binding (approximately 20%) to each of the target membranes was observed (Fig. 4, left). In the presence of calcium, the synaptotagmin fragment was quantitatively associated with the particulate fraction. This interaction appears to be specific for calcium with respect to magnesium since magnesium was included in all our buffers and failed to mimic the effects of calcium. To determine whether this binding reflected an interaction with proteins or lipids in the acceptor membrane preparations, these membranes were subjected to extensive proteolytic digestion followed by boiling prior to the co-sedimentation assay. As in the untreated membranes, calcium resulted in quantitative binding of the synaptotagmin fragment. These findings suggest that the fragment bound to the phospholipid components of the membrane. The residual membrane binding observed in the absence of calcium was abolished by protease treatment and boiling indicating that this component was mediated by protein/protein interactions.

Since the calcium dependence for the association of synaptotagmin with liposomes is dependent on the phospholipid composition (Brose et al., 1992), the calcium concentration required for binding to native membranes has not been established. We therefore used SV membranes as the tryptic fragment acceptor and examined the calcium dependence for binding. We estimate that the effective concentration for 50% binding (EC50) was approximately 30 μM free calcium (Fig. 5A). Chelation of calcium prior to sedimentation completely reversed binding of the fragment to membranes (Fig. 5B), confirming our previous report (Brose et al., 1992). Addition of calcium in the absence of membranes did not result in sedimentation of the fragment demonstrating that sedimentation in the presence of membranes is not due to nonspecific aggregation of the fragment (Fig. 5C).

Calcium-dependent Phospholipid Binding to Recombinant Fragments of Synaptotagmin—The results described above suggest that a soluble cytoplasmic fragment of synaptotagmin, containing both C2-domains, interacts with membrane phospholipids in a calcium-dependent manner. To further analyze this interaction, we utilized the synaptotagmin-GST fusion proteins described above. To measure binding, all fusion proteins were immobilized on glutathione-Sepharose and mixed with radiolabeled artificial liposomes of defined compositions. Lipids which bound in a calcium-dependent manner were eluted by the addition of EGTA and quantified by liquid scintillation counting.

C2ABs-GST, the recombinant fusion protein which includes
both C_{2}-domains, bound to liposomes comprised of phosphatidylcholine/phosphatidylserine and phosphatidylcholine/phosphatidylinositol in a calcium-dependent manner. Calcium-dependent binding was not observed with liposomes composed of phosphatidylcholine alone or phosphatidylcholine/phosphatidylethanolamine or when GST alone was immobilized to the beads (Fig. 6A).

We further investigated the structural requirements for calcium-dependent phospholipid binding by utilizing the fusion protein containing only the first C_{2}-domain of synaptotagmin. As shown in Fig. 6B, this 114-residue domain conferred calcium-dependent PS binding to GST, and thus represents the shortest defined functional calcium-dependent phospholipid binding motif. Within this domain, deletion of the highly conserved amino acid sequence (SDPVKVKFL) (see Fig. 2) abolished calcium-dependent phospholipid binding (Fig. 6B). These findings suggest that the calcium-dependent phospholipid-binding domain lies in the NH_{2}-terminal region of C_{2}-domains and that the SDPVKVKFL sequence is essential for these properties.

**DISCUSSION**

In the present study, we have demonstrated that the cytoplasmic domain of synaptotagmin interacts with phospholipid membranes in a calcium-dependent manner. Recombinant protein containing most of the cytoplasmic domain also bound to phospholipids in a calcium-dependent manner indicating that this property is encoded in the primary sequence of the protein and is not dependent on post-translational modifications. This interaction was preserved in a recombinant GST fusion protein which only contained the first C_{2}-domain of synaptotagmin. This 114-residue region represents the shortest functional calcium-dependent phospholipid-binding domain reported to date. Interestingly, the deletion of a highly conserved stretch of 9 amino acids within this region (consensus sequence SDPVKFL), abolished calcium-dependent phospholipid binding activity.

The role of C_{2}-domains in the regulation of PKC activity has also been addressed by deletion analysis. A 110-amino acid deletion which encompassed most of the C_{2}-domain of PKCa, including the majority to the consensus sequence (Fig. 2; Clark et al., 1991) failed to abolish the calcium and phospholipid stimulated activity of the enzyme (Kaibuchi et al., 1989). Interestingly, the SDPVKFL motif was the only portion of the consensus sequence preserved in this deletion mutant (Kaibuchi et al., 1989; Fig. 2). These data, together with our findings, indicate that this 9-amino acid motif is crucial for the function of C_{2}-domains. It is notable that this same motif is required for recognition by the monoclonal antibody 41.1. This antibody
may provide an additional tool to perturb the function of synaptotagmin in microinjection experiments as described by Elferink et al. (1993).

The data presented here contradict our previous observations which suggested that proteolytic release of the cytoplasmic domain of synaptotagmin abolished binding activity (Brose et al., 1992). This discrepancy may be due to limitations in the assay system used to monitor binding in our previous study (fluorescence resonance energy transfer as compared to co-sedimentation used here). We assume that tryptic cleavage of synaptotagmin may have resulted in further degradation of the functional 39-kDa fragment such that the amount of energy donor fell below the limits of detection of the energy transfer assay system.

Our data show that synaptotagmin binds efficiently to membranes of neuronal and non-neuronal origin in a calcium-dependent manner, regardless of whether these membranes were pretreated with proteases. Two conclusions can be drawn from these findings: first, synaptotagmin binds to native membranes as well as to phospholipid vesicles and second, the binding is due to an interaction with membrane lipids with and does not require protein components. The calcium requirements for the membrane association were high (EC50 approximately 30 µM) relative to other calcium-binding proteins. This is in agreement with the findings of Brose et al. (1992) using the intact protein and artificial liposomes which contained 25% acidic phospholipids. Such a low affinity for calcium was predicted for the exocytic calcium receptor based on electrophysiological studies (Smith and Augustine, 1988; Linas et al., 1992).

The data presented in this study clarify the structural requirements for the calcium-dependent phospholipid binding properties of synaptotagmin. To date, synaptotagmin is the only calcium-binding membrane protein which has been definitively demonstrated to be present in SVs. Its abundance (7–8% of total vesicle protein) is consistent with a critical regulatory function in secretion as suggested by previous studies (Bommert et al., 1993; DiAntonio et al., 1993; Elferink et al., 1993; Littleton et al., 1993; Nonet et al., 1993). The 9-residue deletion which abolishes calcium-dependent phospholipid binding provides a means to selectively disrupt individual C2-domains. Such an approach may be utilized to determine the physiological function of these domains in, for instance, rescue experiments of synaptotagmin-deficient organisms.

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