Distinct Ca\(^{2+}\)-dependent Properties of the First and Second C\(_2\)-domains of Synaptotagmin I*

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Synaptotagmin I (SytI) is a synaptic vesicle protein that binds Ca\(^{2+}\)-and is essential for fast, Ca\(^{2+}\)-dependent neurotransmitter release in the hippocampus, suggesting that it serves as a Ca\(^{2+}\)-sensor for exocytosis. Although SytI has two cytoplasmic C\(_2\)-domains, only the first C\(_2\)-domain was shown to exhibit Ca\(^{2+}\)-regulation; it binds phospholipids and syntaxin in a Ca\(^{2+}\)-dependent manner. By contrast, the second C\(_2\)-domain is inactive in these assays and only binds putative interacting molecules in a Ca\(^{2+}\)-independent manner. We have now discovered in a yeast two-hybrid screen for SytI-interacting molecules that the C\(_2\)-domains of SytI interact with themselves. Using immobilized recombinant C\(_2\)-domains from SytI and SytII, we found that only the second but not the first C\(_2\)-domains of these synaptotagmins are capable of affinity-purifying native rat brain SytI and that this binding is Ca\(^{2+}\)-dependent, suggesting that only the second C\(_2\)-domain is capable of a Ca\(^{2+}\)-triggered self-association. A relatively high Ca\(^{2+}\) concentration (>100 \(\mu\)M) is required for binding in the presence of Mg\(^{2+}\); Sr\(^{2+}\) and Ba\(^{2+}\) but not Mg\(^{2+}\) can substitute for Ca\(^{2+}\). Our data suggest that the second C\(_2\)-domain of SytI is also a Ca\(^{2+}\)-regulated domain similar to the first C\(_2\)-domain but with distinct binding activities.

Synaptotagmin I (SytI)\(^1\) is a member of a family of neuronal proteins that is characterized by a short N-terminal intravesicular sequence, a single transmembrane region, and a large cytoplasmic sequence containing two C\(_2\)-domains (reviewed in Südhof (1995)). Nine synaptotagmin isoforms have been described in mammals (Perin et al., 1990; Geppert et al., 1991; Wendland et al., 1991; Mizuta et al., 1994; Hilibush and Morgan, 1994; Li et al., 1995a; Craxton and Goedert, 1995; Hudson and Birnbaum, 1995), four of which are also found in non-neural tissues (Li et al., 1995a; Hudson and Birnbaum, 1995). At least three synaptotagmins (SytI, SytII, and SytIII) are synaptic vesicle proteins, of which SytIII is present throughout the brain whereas SytIII and SytII show restricted complementary expression patterns with partially overlapping distributions (Ullrich et al., 1994). Mice in which the SytI gene has been mutated exhibit a lethal phenotype in which there is a selective loss of fast Ca\(^{2+}\)-dependent neurotransmitter release in hippocampal synapses (Geppert et al., 1994). Spontaneous neurotransmitter release and neurotransmitter release evoked by Ca\(^{2+}\)-independent mechanisms are normal, suggesting an essential role for SytI only in the Ca\(^{2+}\)-dependent last step of membrane fusion. Together with the Ca\(^{2+}\)-binding properties of SytI (Li et al., 1995a, 1995b), these data suggest that SytI may serve as an exocytotic Ca\(^{2+}\)-sensor.

Based on the presence of C\(_2\)-domains in SytI and the observation that the C\(_2\)-domain confers Ca\(^{2+}\)-regulation onto protein kinase C, it was speculated early on that SytI may be a Ca\(^{2+}\)-binding protein (Perin et al., 1990). Indeed, experiments with purified SytI demonstrated that it binds Ca\(^{2+}\) and phospholipids (Brose et al., 1992) and that it undergoes a conformational change as a function of Ca\(^{2+}\) (Davletov and Südhof, 1994). Studies on recombinant C\(_2\)-domains showed that the first C\(_2\)-domain of SytI and of most but not all other synaptotagmins binds phospholipids as a function of Ca\(^{2+}\) (Davletov and Südhof, 1993; Chapman and Jahn, 1994; Ullrich et al., 1994; Li et al., 1995a). In addition, Ca\(^{2+}\) triggers binding of syntaxin to the first C\(_2\)-domain of synaptotagmins with a Ca\(^{2+}\)-dependence that is distinct from that of phospholipid binding, implying the presence of two Ca\(^{2+}\)-binding sites in a single C\(_2\)-domain (Li et al., 1995a, 1995b). Surprisingly, the second C\(_2\)-domain of all synaptotagmins is inactive in these assays despite a high degree of sequence homology. Furthermore, the Ca\(^{2+}\)-dependent binding properties of the native cytoplasmic domain of purified brain SytI containing both C\(_2\)-domains has the same properties as the recombinant single first C\(_2\)-domain (Li et al., 1995a, 1995b). Together these data suggest that the known Ca\(^{2+}\)-binding properties of SytI can be entirely accounted for by its first C\(_2\)-domain alone and raised the possibility that the second C\(_2\)-domain may not represent a Ca\(^{2+}\)-binding domain. This possibility was supported by the Ca\(^{2+}\)-independent interactions of the second C\(_2\)-domains of synaptotagmins with clathrin AP2 (Zhang et al., 1994; Li et al., 1995a) and with polyanions such as polyinositol phosphates (Fukuda et al., 1994).

We now report the results of a yeast two-hybrid interaction screen for proteins binding to the C\(_2\)-domains of SytI. Unexpectedly, SytI itself was identified as an interacting partner. In vitro binding assays demonstrated a Ca\(^{2+}\)-dependent self-interaction of SytI that is specific for its second C\(_2\)-domain. These data suggest that in addition to the first C\(_2\)-domain, the second C\(_2\)-domain of SytI is a Ca\(^{2+}\)-regulated domain. However, the two C\(_2\)-domains have distinct Ca\(^{2+}\)-regulated properties, suggesting a functional diversification of C\(_2\)-domains in synaptotagmins.

**EXPERIMENTAL PROCEDURES**

Yeast Two-hybrid (Y2H) Screens and Interaction Assays—A bait vector (pBTM116p65-8) encoding the cytoplasmic domains of SytI starting with the first C\(_2\)-domain was constructed by cloning the 0.9-kilobase pair Sal-Sai fragment from pGEX65-8 (Davletov et al., 1993) into the same sites of pBTM116 (Vignal et al., 1993). This results in a vector
expressing a LexA-fusion protein with SytI starting at residue 120. A cDNA library was constructed in the Ndot site of Y2H prey vector pVP16 (Vojtek et al., 1993) from poly(A)+-enriched rat brain RNA using the Life Technologies, Inc. Choice system. Y2H screens were performed essentially as described (Fields and Song, 1989; Vojtek et al., 1993; Hata and Südhof, 1995) by sequentially transfecting yeast strain L40 with the prey and the cDNA library on high-copy vectors and using the lithium acetate method (Schiestl and Gietz, 1989). Transformants were plated on selection plates lacking histidine, uracil, tryptophan, lysine, and leucine but containing 2.5 mM 3-amino triazole. Positive clones were picked after 4–6 days of incubation at 30°C, and the β-galactosidase activity of the clones was assayed on a nitrocellulose filter. Extractromosomal DNA from clones that grew in the absence of histidine and were β-galactosidase positive was isolated using the glass bead method (Ward, 1990). Prey plasmids were rescued in Escherichia coli HB101 cells by electroporation and selection on M9 plates containing 50 mg/ml proline and 0.1 g/ml ampicillin. 14 million yeast transformants with the cDNA library were screened, and 42 clones positive upon retransformation were isolated and sequenced using the dideoxy chain termination method.

Construction of Expression Vectors—The recombinant GST-synaptotagmin fusion proteins used were synthesized from the following expression plasmids in the vector pGEX-KG (Guan and Dixon, 1993) encoding the following residues of SytI and SytII (Perin et al., 1990; Geppert et al., 1991; pGEX6-4 (GSTSytIC2-A), residues 140–267; pGEX6-9 (GSTSytIC2-B), residues 266–421; pGEX6-8 (GSTSytIC2-A/B), residues 120–421; pGEX1071/1152 (GSTSytIC2-A/B), residues 141–268; pGEX1153/1159 and pGEX1153/1159Y312N (GSTSytIC2-B wild type and mutant, respectively), residues 266–399; pGEX1071/1152Glu141Asp and pGEX1071/1152Asp141Glu (GSTSytIC2-A/B wild type and mutant, respectively), residues 141–422. Recombinant proteins were purified on glutathione-agarose and used immobilized on glutathione-agarose without elution. Amounts of proteins used were standardized based on Coomassie Blue-stained SDS gels.

SytI Binding to Recombinant Proteins—One frozen rat brain (Pel-freeze) was homogenized in 11 ml of 4 mM HEPES-NaOH pH 7.4 containing 0.1 g/liter phenylmethylsulfonyl fluoride. The homogenate was extracted for 4 h at 4°C after addition of 11 ml of 4 mM HEPES-NaOH pH 7.4, 0.1 g/liter phenylmethylsulfonyl fluoride, 0.2 mM NaCl, 2% Nonidet P-40, 0.2 mM EDTA. Insoluble material was removed from the extract by centrifugation (30 min at 100,000 × g), and MgCl2 was added to the supernatant to 3.5 mM final concentration. 1-ml aliquots of the supernatant were incubated overnight at 4°C with glutathione-agarose beads, and binding of endogenous brain SytI to the immobilized fusion proteins was measured as a function of Ca2+ concentration. Fusion proteins containing both C2-domains or containing only the second C2-domain of either SytI or SytII efficiently affinity-purified SytI from total brain (Fig. 1). Strong binding was observed only in the presence of Ca2+, whereas in the absence of Ca2+ weak binding was present. By contrast, the first C2-domains of SytI and of SytII were unable to bind. A point mutation in the second C2-domain of SytII that corresponds to a mutation in Drosophila synaptotagmin, which impairs synaptotagmin function (DiAntonio and Schwarz, 1994), had no effect on Ca2+-dependent binding. Together these results suggest that the C2-domains of synaptotagmins self-associate as a function of Ca2+ via their second but not their first C2-domains. Since the low binding observed in the absence of Ca2+ appears to be sufficient for an interaction observed in the Y2H assay, it is possible to identify Ca2+-dependent binding proteins for SytI using the Ca2+-independent Y2H screen.

Divalent Cation Specificity of Synaptotagmin Self-Interaction—Previous studies on the first C2-domains of synaptotagmin demonstrated that Sr2+ and Ba2+ but not Mg2+ can substitute for Ca2+ in activating phospholipid binding, although with a much lower affinity (Davlletov and Südhof, 1993; Li et al., 1995b). We therefore tested the effects of different divalent cations on the binding of SytI to the second C2-domain of SytI (Fig. 2). Mg2+ was unable to trigger binding and in fact inhibited binding compared with that observed in the absence of divalent cations (Fig. 2). By contrast, both Sr2+ and Ba2+ were capable of activating binding, with Ba2+ having the lowest effect. Parallel incubations with GST alone demonstrated that the binding observed was dependent on the SytI-fusion protein and not due to divalent cation-dependent aggregation of SytI in the homogenate (lower panel in Fig. 2).
Ca\(^{2+}\) Regulation of Second Synaptotagmin C\(_2\) Domain

**DISCUSSION**

SytI is a Ca\(^{2+}\)-binding protein of synaptic vesicles that is essential for fast Ca\(^{2+}\)-dependent neurotransmitter release from hippocampal neurons (Brose et al., 1992; Geppert et al., 1994), suggesting that it serves as the exocytic Ca\(^{2+}\) sensor. Previous studies demonstrated that the first C\(_2\)-domain of SytI serves as a Ca\(^{2+}\)-dependent phospholipidand syntaxin-binding domain (Davletov and Südhof, 1993, 1994; Li et al., 1995a, 1995b). The second C\(_2\)-domain is inactive in these assays but binds AP2 and polyanions in a Ca\(^{2+}\)-independent manner (Zhang et al., 1994, Fukuda et al., 1994). The phospholipid and syntaxin binding properties of a cytoplasmic fragment from SytI containing both C\(_2\)-domains are identical to that of the single recombinant first C\(_2\)-domain (Li et al., 1995a, 1995b). Together these results suggested that SytI may perform its Ca\(^{2+}\) sensor function primarily via its first C\(_2\)-domain whereas the second C\(_2\)-domain may have a distinct function. We now demonstrate that the second C\(_2\)-domain also has a Ca\(^{2+}\)-dependent activity suggestive of a Ca\(^{2+}\)-binding domain. It mediates the Ca\(^{2+}\)-dependent aggregation of SytI and SytII with a Ca\(^{2+}\) concentration dependence that mirrors the Ca\(^{2+}\) dependence of neurotransmitter release if the experiments are performed in the presence of physiological concentrations of Mg\(^{2+}\). These data suggest a model of SytI whereby both C\(_2\)-domains of SytI can serve as Ca\(^{2+}\)-binding modules with distinct functions (Fig. 4).

Even in the absence of Ca\(^{2+}\), SytI is not a monomer but a multimer (Perin et al., 1991). The basic unit of this multimer is an SDS-resistant dimer that can be detected by SDS-PAGE, and this multimerization is mediated by sequences N-terminal to the two C\(_2\)-domains (Brose et al., 1992) (Fig. 4). Our current demonstration of a Ca\(^{2+}\)-dependent binding of SytI to itself via its second C\(_2\)-domain raises the possibility that during nerve terminal depolarization and Ca\(^{2+}\) influx, SytI multimers may be cross-linked by Ca\(^{2+}\) into large superstructures. The function of such a superstructure in fusion is unknown, but it is conceivable that it would aid in forming a pore that must occur during membrane fusion and probably involves assembly of a proteinaceous ring.

A considerable number of interactions has been described for synaptotagmins, not all of which may be physiologically important. Considering the point of action of SytI, it seems likely that Ca\(^{2+}\)-regulated activities are more relevant than constitutive binding activities. Another criterion that supports the potential physiological relevance of an interaction is the colocalization of the binding partners. Based on these two criteria, the interactions of SytI with phospholipids, syntaxin, and itself appear to...
be the most likely to be relevant. The recombinant second C2-domain of Syt I also binds syntaxin (see Fig. 3) and phospholipids (Damer and Creutz, 1994) in a Ca2+ independent manner. However, when the complete double C2-domain fragment from native Syt I prepared by partial proteolytic cleavage is analyzed, phospholipid binding and syntaxin binding are completely dependent on Ca2+, and little Ca2+-independent binding is observed (Li et al., 1995a, 1995b). Two other binding activities were demonstrated for synaptotagmin I that are Ca2+-independent and conceptually intriguing: binding of neurexins and AP2. Although no in vivo data exist to support the physiological significance of neurexin binding, AP2 binding may be physiologically significant since AP2 transiently associates with synaptic vesicles (Pfeffer and Kelly, 1985; Maycox et al., 1992) and synaptic vesicle recycling is severely impaired in synaptotagmin mutants in Caenorhabditis elegans (Jorgensen et al., 1995).

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