Distinct Self-oligomerization Activities of Synaptotagmin Family: Unique Calcium-dependent Oligomerization Properties of Synaptotagmin VII*

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SUMMARY

Synaptotagmins constitute a large protein family, characterized by one transmembrane region and two C2 domains, and can be classified into several subclasses based on phylogenetic relationships and biochemical activities (Fukuda, M., Kanno, E., and Mikoshiba, K. (1999) *J. Biol. Chem.*, **274**, 31421-31427). Synaptotagmin I (Syt I), a possible Ca$^{2+}$ sensor for neurotransmitter release, showed both Ca$^{2+}$-dependent (via the C2 domain) and -independent (via the N-terminal domain) self-oligomerization, which are thought to be important for synaptic vesicle exocytosis. However, little is known about the relationship between these two interactions and the Ca$^{2+}$-dependent oligomerization properties of other synaptotagmin isoforms. In this study, we first examined the Ca$^{2+}$-dependent self-oligomerization properties of synaptotagmin family by co-expression of T7- and FLAG-tagged Syts (full length or cytoplasmic domain) in COS-7 cells. We found that Syt VII is a unique class of synaptotagmins that only showed robust Ca$^{2+}$-dependent self-oligomerization at the cytoplasmic domain with EC$_{50}$ values of about 150 µM Ca$^{2+}$. In addition, Syt VII preferentially interacted with the previously described subclass of Syts (V, VI, and X) in a Ca$^{2+}$-dependent manner. Co-expression of full length and cytoplasmic portion of Syts VII (or II) indicate that Syt VII cytoplasmic domain oligomerizes in a Ca$^{2+}$-dependent manner without being tethered at the N-terminal domain, whereas Ca$^{2+}$-dependent self-oligomerization at the cytoplasmic domain of other isoforms (e.g. Syt II) occurs only when the two molecules are tethered at the N-terminal domain.
INTRODUCTION

Neurotransmitter release is achieved by the fusion of docked synaptic vesicles with the presynaptic plasma membrane in response to a rapid increase in intracellular Ca\(^{2+}\) concentrations (up to about 200 µM) due to the opening of voltage-gated Ca\(^{2+}\) channels (1, 2). Ca\(^{2+}\)-binding proteins on the synaptic vesicles are required to sense this rapid increase in Ca\(^{2+}\) ions. Recent genetic and biochemical studies have indicated that synaptotagmin I (Syt I), a Ca\(^{2+}\), phospholipid, and inositol polyphosphate binding protein present on synaptic vesicles, is the best candidate for the Ca\(^{2+}\)-sensor of neurotransmitter release in the central nervous system (reviewed in Refs. 3-6).

The synaptotagmins now constitute a large protein family with twelve isoforms described in rat or mouse (Ref. 7 and references therein), and are thought to be involved in vesicular trafficking. Each member shares a short amino terminus, a single transmembrane region, and two C2 domains (known as the C2A and C2B domains) homologous to the C2 regulatory region of protein kinase C (reviewed in Refs. 3-6). Syt I, the best characterized isoform of synaptotagmin, is thought to function in an oligomerized state on the basis of genetic analyses of Drosophila synaptotagmin mutants (reviewed in Ref. 8) and in vitro biochemical studies (9-13). Syt I (or II) has two self-oligomerization properties: A SDS-resistant Ca\(^{2+}\)-independent multimerization probably mediated by a region just downstream of the transmembrane region (7, 14, 15) and a Ca\(^{2+}\)-dependent oligomerization mediated by the C2B domain (9-13). However, the functional relationship between these two interactions remains unknown. For instance, it remains undetermined whether the C2B domains oligomerize independent of the Ca\(^{2+}\)-independent oligomerization at the amino (N)-terminal domain (Fig. 1A). In addition, studies of the Ca\(^{2+}\)-dependent oligomerization of the C2B domain have mainly used recombinant proteins from Escherichia coli (9-13), which can be very sticky compared to native proteins prepared from mouse brain, and tend to aggregate in low Ca\(^{2+}\) ion concentrations. Studies of Ca\(^{2+}\)-independent oligomerization using recombinant proteins from E. coli may also be unreliable because native synaptotagmins undergo post-
translational modifications such as palmitoylation (16, 17) or disulfide bond formation (7). Thus, it would be better to examine the oligomerization properties of the synaptotagmin family in mammalian cultured cells by expressing two different epitope (FLAG and T7)-tagged synaptotagmins (7). In this method, we previously showed that Syts III, V, VI and X form homo- and hetero-oligomer through the N-terminal cysteine motif by disulfide bonding (at amino acids 10, 21, and 33 of mouse synaptotagmin III) irrespective of the presence of Ca\(^{2+}\) (7), and that Syt VI\(\Delta\)TM, a major alternatively spliced variant of Syt VI that lacks this cysteine motif, cannot associate with Syt VI \textit{in vitro} or \textit{in vivo} (18). In contrast, other isoforms did not show such cysteine-based homo-oligomerization (7).

In the present study, we examined the Ca\(^{2+}\)-dependent and -independent oligomerization properties of synaptotagmin isoforms (both full length and cytoplasmic portion) using two different epitope-tagged synaptotagmins, and found that Syt VII is the only isoform that shows robust Ca\(^{2+}\)-dependent oligomerization properties. On the basis of these results, we discuss the functional relationship between Ca\(^{2+}\)-dependent and -independent oligomerization of synaptotagmins.
EXPERIMENTAL PROCEDURES

Materials

AmpliTaq DNA polymerase and restriction enzymes were obtained from PE Biosystems, and Toyobo Biochemicals (Tokyo, Japan), respectively. Polyclonal and monoclonal antibodies (M2) against FLAG peptide were obtained from Zymed Laboratories, Inc. and Sigma, respectively. Horseradish peroxidase (HRP)-conjugated anti-T7 tag antibody was from Novagen. All other chemicals were commercial products of reagent grade. Solutions were prepared in deionized water.

Construction of T7- and FLAG-tagged Synaptotagmin I-XI Cytoplasmic Domains

Construction of the full cytoplasmic domains of mouse synaptotagmins I-XI were carried out by polymerase chain reaction using the following oligonucleotides with internal restriction enzyme sites (underlined below; *Bam*HI or *Bcl*I): Syt I, 5’-CGGATCCAGAAAAATGTTTGTTCAAA-3’ (Syt I-cyto primer, sense; amino acid residues 80-85); Syt II, 5’-GGATCCAGAAGTGCTGCTGCAAGAA-3’ (sense; amino acid residues 88-94); Syt III, 5’-CGGATCTGAAAGTTGCTGGGT-3’ (sense; amino acid residues 77-82); Syt IV, 5’-CTGATCACAGAGAAAGATCAGCCAAATC-3’ (sense; amino acid residues 39-45); Syt V, 5’-GGGATCCCTGGGAAAACGCTGCTGGGTTCC-3’ (sense; amino acid residues 75-81); Syt VI, 5’-GGGATCAGCCCTGGGAAAACGCTGCTGGGTTCC-3’ (sense; amino acid residues 86-92); Syt VII, 5’-GGGATCCCTGGGAAAACGCTGCTGGGTTCC-3’ (sense; amino acid residues 43-49); Syt VIII, 5’-GGGATCCCTGGGAAAACGCTGCTGGGTTCC-3’ (sense; amino acid residues 69-75); Syt IX, 5’-GGGATCCCTGGGAAAACGCTGCTGGGTTCC-3’ (sense; amino acid residues 52-57); Syt X, 5’-GGGATCCCTGGGAAAACGCTGCTGGGTTCC-3’ (sense; amino acid residues 77-83), and Syt XI, 5’-GGGATCCCTGGGAAAACGCTGCTGGGTTCC-3’ (sense; amino acid residues 41-47). In the case of FLAG-tagged Syt I-cyto, for example, a cDNA encoding the Syt I cytoplasmic domain was amplified by Syt I-cyto and SP6.
primers using pGEM-T-FLAG-Syt I (7) as a template. Purified PCR products were
digested with BamHI and SalI and then substituted for the BamHI-SalI insert of pGEM-
T-FLAG-Syt I, and verified by DNA sequencing as described above. The resulting
pGEM-T-FLAG-Syt I-cyto plasmid was digested with NotI, and then the NotI insert was
subcloned into the NotI site of pEF-BOS (pEF-FLAG-Syt I-cyto) (19, 20). The pEF-
FLAG-Syts II-XI-cyto clones were similarly constructed. pEF-T7(or FLAG)-Syts I-XI
were prepared as described previously (7). Plasmid DNA was prepared using Wizard-
mini preps (Promega) or QIAGEN Maxi prep kits.

Data Processing

Statistical analysis and curve fitting were done using the GraphPad PRISM
computer program (Version 2.0).

Miscellaneous Procedures

Co-transfection of pEF-T7-Syts and pEF-FLAG-Syts into COS-7 cells (5 × 10^5
cells, the day before transfection/10 cm dish) was carried out by the DEAE-dextran
method as described previously (7). Proteins were solubilized with buffer containing 1%
Triton X-100, 250 mM NaCl, 50 mM HEPES-KOH, pH 7.2, 0.1 mM
phenylmethylsulphonyl fluoride, 10 µM leupeptin and 10 µM pepstatin A at 4 °C for 1 h.
Immunoprecipitation of T7-Syts by anti-T7 tag antibody-conjugated agarose, SDS-
polyacrylamide gel electrophoresis (PAGE), and immunoblotting analyses were also
performed as described previously (7). Immunoreactive bands on X-ray film (X-
OMATAR, Kodak) were captured by Gel Print 2000i/VGA (Bio Image) and analyzed by
Basic Quantifier Software (version 1.0) (Bio Image).
RESULTS

Ca\(^{2+}\)-dependent and -independent Self-oligomerization of Synaptotagmin Isoforms

In a previous study, we showed that Syts III, V, VI, and X formed hetero-oligomers through disulfide bonding irrespective of the presence of Ca\(^{2+}\) (7). To examine the Ca\(^{2+}\)-dependent self-oligomerization of other synaptotagmin isoforms (Syts I, II, IV, VII, VIII, IX and XI), T7- and FLAG-tagged full length Syts were co-expressed in COS-7 cells and the association of these two proteins was evaluated by immunoprecipitation in the presence or absence of 250 µM Ca\(^{2+}\), corresponding to the concentrations required for neurotransmitter release (1, 2). Briefly, proteins were solubilized with 1% Triton X-100, and T7-Syts were immunoprecipitated by anti-T7 tag antibody-conjugated agarose. Co-immunoprecipitated FLAG-Syts were first detected by anti-FLAG antibody (Fig. 1B, upper left panel), and then the same blots were stripped and reprobed with HRP-conjugated anti-T7 tag antibody to ensure the equivalent loading of T7-Syts (Fig. 1B, lower left panel). Co-immunoprecipitated FLAG-Syts were then quantified and normalized by the amount of immunoisolated T7-Syts (Fig. 1C). Based on the differences in homo-oligomerization activities, we classify these isoforms into three distinct groups. The first group included Syts I, II, VIII, and XI, which showed Ca\(^{2+}\)-independent homo-oligomerization activity, but Syt XI oligomerization activity was apparently weaker than that of the others (Syts I, II, and VIII). Among this group, only Syt II oligomerization was slightly activated by Ca\(^{2+}\) (<1.5-fold increase; Fig. 1C). The second group contained Syts IV and IX. Syt IV did not form homo-oligomers in our binding conditions, and faint Syt IX homo-oligomerization was only observed after prolonged exposure to X-ray film. Consistent with this, we previously showed that these isoforms did not form significant SDS-resistant homo-oligomers on SDS-PAGE (7). The third group contained only Syt VII, the homo-oligomerization of which was largely Ca\(^{2+}\)-dependent, though small fractions of Syt VII oligomerize in the absence of Ca\(^{2+}\). The minor higher band of Syt VII is probably produced by partial post-translational modifications such as O-glycosylation or palmitoylation at the N-terminal domain due to
transient overexpression in COS-7 cells. Because of such possible modifications at the N-terminal domain, the minor higher band of Syt VII seems to be more efficiently immunoprecipitated than the lower band, but both bands behaved the same in the presence of Ca$^{2+}$.

Since the homo-oligomerization of Syts, except for Ca$^{2+}$-dependent oligomerization of Syt VII, were difficult to detect when T7- and FLAG-tagged Syts were separately expressed in COS-7 cells and mixed after solubilization (Fig. 1B, upper right panel), the homo-oligomerization presented in Fig. 1B probably reflects in vivo interactions. It is noteworthy that Syt VII Ca$^{2+}$-independent oligomerization could not be observed when T7- and FLAG-Syt VII were separately expressed in COS-7 cells, whereas in the presence of Ca$^{2+}$ Syt VII oligomerized as well as with co-expression. The oligomerization experiments were also performed in the presence of 1 mM Mg$^{2+}$, but Mg$^{2+}$ could not activate synaptotagmin oligomerization (data not shown).

**Ca$^{2+}$-dependent Oligomerization Properties of Cytoplasmic Domains from Synaptotagmins I-XI**

In the next set of experiments, we examined the Ca$^{2+}$-dependent oligomerization properties of synaptotagmin cytoplasmic domains as demonstrated by the interaction of recombinant Syt I or II expressed in bacteria with detergent-solubilized native proteins (Syt I) from brain (9-13). Consistent with the results shown in Fig. 1, only the cytoplasmic domain of Syt VII showed robust Ca$^{2+}$-dependent oligomerization (Fig. 2). Syts II and V cytoplasmic domains also showed apparent Ca$^{2+}$-dependent oligomerization (2.4- and 1.9-fold increase in the presence of Ca$^{2+}$, respectively), but most of the fractions of Syts II and V oligomerization were Ca$^{2+}$-independent. Surprisingly, Ca$^{2+}$-dependent Syts I, III, VI, IX and X homo-oligomerizations were quite weak, and could only be detected when large amounts of anti-T7 tag antibody immunoprecipitant was loaded on SDS-PAGE (data not shown). In contrast, homo-oligomerization of Syts IV, VIII, and XI cytoplasmic domains was not observed in our binding conditions. This result is unlikely to be due to the lower expression levels of the cytoplasmic domains of
Syts than those of full length proteins, because comparable or greater amounts of T7-(or FLAG)-Syts I-XI cytoplasmic domains were expressed in COS-7 cells (data not shown).

Ca$^{2+}$-dependent Hetero-oligomerization of Synaptotagmin VII with Synaptotagmins V, VI, and X

To further examine whether Syt VII also hetero-oligomerizes with other isoforms in a Ca$^{2+}$-dependent manner, FLAG-Syt VII-cyto and various T7-Syt-cytos proteins were co-expressed in COS-7 cells and their association was tested by immunoprecipitation in the presence or absence of Ca$^{2+}$ as described above. Interestingly, the Syt VII cytoplasmic domain preferentially interacted with Syts V, VI, and X, but not Syt III, all of which belong to the same subclass of synaptotagmins (7, 21), in a Ca$^{2+}$-dependent manner (Fig. 3 and data not shown). Syts V, VI, and X were found to hetero-oligomerize as well with Syt VII as Ca$^{2+}$-dependent Syt VII homo-oligomerization. Similar results were obtained even when the full length Syts III, V, VI, VII, and X were used (data not shown).

Functional Relationship between Ca$^{2+}$-dependent and -independent Oligomerization Properties of Synaptotagmins

Since the homo-oligomerization of full length and the cytoplasmic domain of Syt VII were strongly activated by Ca$^{2+}$ (Fig. 1B and Fig. 2), Ca$^{2+}$-dependent oligomerization of the Syt VII cytoplasmic domain would occur between two molecules that do not pre-assemble at the N-terminal domain (see Fig. 1A, right panel). This was evident when three proteins (T7-Syt VII, FLAG-Syt VII, and FLAG-Syt VII-cyto; molar ratio, 1:1:1) were co-expressed in COS-7 cells (Fig. 4B, lane 2) and their associations were analyzed by immunoprecipitation. In the absence of Ca$^{2+}$, only FLAG-Syt VII weakly interacted with T7-Syt VII (Fig. 4A, lane 3), whereas in the presence of Ca$^{2+}$, equivalent amounts of Syt VII cytoplasmic domain (FLAG-Syt VII-cyto) and Flag-Syt VII were co-immunoprecipitated with T7-Syt VII (Fig. 4A, lane 4, arrowheads), indicating that the Syt VII cytoplasmic domain can oligomerize with full length proteins completely in a
Ca\textsuperscript{2+}-dependent manner without being tethered at the N-terminal domain (see Fig. 4C, right panel).

In contrast, when T7-Syt II, FLAG-Syt II, and FLAG-Syt II-cyto were co-expressed in COS-7 cells (molar ratio, 1:1:1), the Syt II cytoplasmic domain (FLAG-Syt II-cyto) could not interact with full length proteins (T7-Syt II) even in the presence of Ca\textsuperscript{2+} (Fig. 4A, lanes 1 and 2), even though the Syt II cytoplasmic domain itself showed Ca\textsuperscript{2+}-dependent oligomerization (Fig. 2). However, FLAG-Syt II could oligomerize well with T7-Syt II irrespective of the presence of Ca\textsuperscript{2+}, as demonstrated in Fig. 1A and 1B. Therefore, the Ca\textsuperscript{2+}-independent oligomerization mediated by around the transmembrane region may restrict the Ca\textsuperscript{2+}-dependent oligomerization mediated by the cytoplasmic domain; i.e. Ca\textsuperscript{2+}-dependent oligomerization of synaptotagmins (Syts I, II, III, V, VI, and X) occurs only when the two molecules are tethered at the N-terminal domain (see Fig. 4C, left panel).

Ca\textsuperscript{2+}-dependency of Self-oligomerization of Cytoplasmic Domains of Syt VII

In the final set of experiments, we examined the Ca\textsuperscript{2+}-dependence of homo-oligomerization of the Syt VII cytoplasmic domain. T7- and FLAG-Syt VII-cyto were co-expressed in COS-7 cells and recombinant proteins were immunoprecipitated by anti-T7-tag antibody in the presence of various concentrations of Ca\textsuperscript{2+}. As shown in Fig. 5A (upper panel), co-immunoprecipitated FLAG-Syt VII-cyto was increased as a function of Ca\textsuperscript{2+} concentration. The EC\textsubscript{50} value (Ca\textsuperscript{2+} concentration at half maximal binding) of Syt VII self-oligomerizations was calculated to be 155 µM Ca\textsuperscript{2+} (Fig. 5B), which closely corresponds to the Ca\textsuperscript{2+} concentrations required for neurotransmitter release (1, 2), and is consistent with the previously described EC\textsubscript{50} values for Syts I and II (9). Interestingly, hetero-oligomerization of Syt VII with Syt V, VI, or X cytoplasmic domain also occurred in a Ca\textsuperscript{2+} concentration range similar to that required for Syt VII homo-oligomerization (EC\textsubscript{50} of about 200-400 µM Ca\textsuperscript{2+}; Fig. 5C and data not shown), suggesting that these associations are physiologically relevant.
DISCUSSION

In this study, we have demonstrated that synaptotagmin isoforms show distinct homo-oligomerization activities by using a dual epitope-tag (T7 and FLAG) expression system. Based on the results shown in Fig. 1B, together with the previously described cysteine-based oligomerization of Syts III, V, VI, and X (7), we classified the synaptotagmin family into four distinct groups. The first group contained Syts III, V, VI, and X, which were strongly associated with each other via the conserved N-terminal cysteine motif by disulfide bonding (7), and accordingly their associations were β-mercaptoethanol-sensitive and Ca\textsuperscript{2+}-independent. At the cytoplasmic domain, however, only Syt V cytoplasmic domain showed apparent Ca\textsuperscript{2+}-dependent homo-oligomerization (Fig. 2). This grouping is consistent with a previous phylogenetic analysis (7) and their reported weak or no inositol 1,3,4,5-tetrakisphosphate binding ability (21).

The second group (Syts I, II, VIII, and XI) also showed Ca\textsuperscript{2+}-independent homo-oligomerization, but these interactions were insensitive to β-mercaptoethanol (7). Syt XI homo-oligomerization activity was significantly lower than those of Syts I, II, and VIII. Despite the high sequence similarity between Syts I and II (7), and the similar biochemical properties of their C2 domains tested so far (9, 20-25), the Syt I and II cytoplasmic domains showed distinct self-oligomerization activities (Fig. 2): the Syt II cytoplasmic domain oligomerized well in the absence of Ca\textsuperscript{2+}, and this association was enhanced by Ca\textsuperscript{2+}, whereas the Syt I cytoplasmic domain showed only very weak Ca\textsuperscript{2+}-dependent oligomerization. Although the oligomerization activity is thought to be protein concentration-dependent, the expression levels of recombinant Syt I in COS-7 cells seems to be sufficient and at physiologically relevant levels, because the levels of expression of Syts I-XI in COS-7 cells are greater than the endogenous levels of expression in brain\textsuperscript{2}. Moreover, previous in vitro binding experiments only showed the existence of Ca\textsuperscript{2+}-dependent oligomerization, but did not address the strength (e.g. \(K_d\) value) of the Syt I oligomerization (9-13). Further work will be required to elucidate whether the Ca\textsuperscript{2+}-dependent oligomerizing capacity of Syt I occurs at physiologically relevant levels. Syt I
Ca\textsuperscript{2+}-independent oligomerization is thought to be mediated by the predicted amphipathic α-helix region just downstream of the transmembrane region (14). However, since Syts II and VIII did not contain such amphipathic α-helix regions and only the Syt II cytoplasmic domain showed Ca\textsuperscript{2+}-independent oligomerization (Fig. 2), different mechanisms are involved in homo-oligomerization of Syts II and VIII. Syts IV and IX belong to the third group, which was characterized by almost no homo-oligomerization activity in our binding conditions.

Syt VII, the only member of fourth group, showed unique Ca\textsuperscript{2+}-dependent oligomerization properties. The Syt VII cytoplasmic domain oligomerizes in a Ca\textsuperscript{2+}-dependent manner without being tethered at the N-terminal domain (Fig. 1A, right panel), whereas the Ca\textsuperscript{2+}-dependent homo-oligomerization of the first and second group Syts at the cytoplasmic domain occurs only when the two molecules are tethered at the N-terminal domain (Fig. 1A, left panel). In addition, we found that Syt VII can completely hetero-oligomerize with Syts V, VI, and X, but not Syt III, in a Ca\textsuperscript{2+}-dependent manner. Since Syts III, V, VI and X can be assembled by disulfide bonding at the N-terminal domain (7), Syt VII binds to the cytoplasmic domain of Syts V, VI, and X in response to Ca\textsuperscript{2+}, which would modulate the function of the Syts III, V, VI and X complexes. Since Syts III, VI, and VII are expressed in all brain regions and Syts V and X are brain-specific isoforms (25-28), at least four isoforms are co-expressed in the same brain regions. Thus, it is possible to form such Ca\textsuperscript{2+}-dependent hetero-oligomers. Currently, however, except for the function of Syt III in insulin secretion from pancreatic β-cells (29), the exact role of Syts III, V, VI, and X in brain remains obscure.

Recently, Chapman et al. reported (12) the C2B effector domain of Syt I (Lys at positions 326 and 327 (K326, and K327)), which is crucial for self-oligomerization (9-13), and for binding to inositol 1,3,4,5-tetrakisphosphate (22, 30), AP-2 (25, 31, 32) and the synprint region of the α1B subunit of N-type Ca\textsuperscript{2+} channels (33). Although these two Lys residues in the C2B domain are well conserved in almost all the isoforms (7, 25), Ca\textsuperscript{2+}-dependent homo-oligomerization activities were apparently different between each isoform (Fig. 2), suggesting that the surrounding sequences would affect and
determine homo-oligomerization capacity. In fact, the Syt VII C2B domain has several unique amino acid residues that are not conserved in other isoforms (e.g. I284, N328, S373 \textit{e.t.c.}). Further detailed mutational analysis is needed to elucidate the structural basis of Ca$^{2+}$-dependent oligomerization of the cytoplasmic domain of Syt VII in the future. In contrast, when the C2B domains of Syts III, V, VI, and X were compared, we could not identify specific amino acid residues unique to Syt III, which are implicated in loss of Ca$^{2+}$-dependent oligomerization with Syt VII.

In summary, we have investigated the Ca$^{2+}$-dependent and -independent homo-oligomerization of synaptotagmin family proteins and show that the Ca$^{2+}$-independent oligomerization at the N-terminal domain is a prerequisite for the Ca$^{2+}$-dependent oligomerization at the cytoplasmic domain of synaptotagmin family proteins (except for Syt VII). Therefore, studies on the Ca$^{2+}$-dependent hetero-oligomerization at the cytoplasmic domain will have to be evaluated by using both full length and cytoplasmic domains. Ca$^{2+}$-dependent and -independent hetero-oligomerization of the synaptotagmin family will be clarified by the T7- and FLAG-tagged Syts co-expression system in the near future.
The abbreviations used are: HRP, horseradish peroxidase; Mr, molecular weight; N, amino; PAGE, polyacrylamide gel electrophoresis; Syt(s), synaptotagmin(s).

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REFERENCES

1. Llinás, R., Steinberg, I. Z., and Silver, R. B. (1992) Science 256, 677-679
2. Heidelberger, R., Heinemann, C., Neher, E., and Matthews, G. (1994) Nature 371, 513-515
3. Südhof, T. C., and Rizo, J. (1996) Neuron 17, 379-388
4. Fukuda, M., and Mikoshiba, K. (1997) BioEssays 19, 593-603
5. Linial, M. J. Neurochem. (1997) 69, 1781-1792
6. Schiavo, G., Osborne, S. L, and Sgouros, J. G. (1998) Biochem. Biophys. Res. Commun. 248, 1-8
7. Fukuda, M., Kanno, E., and Mikoshiba, K. (1999) J. Biol. Chem., 274, 31421-31427
8. Littleton, J. T., and Bellen, H. J. (1995) Trends Neurosci. 18, 177-183
9. Sugita, S., Hata, Y., and Südhof, T. C. (1996) J. Biol. Chem. 271, 1262-1265
10. Chapman, E. R., An, S., Edwardson, J. M., and Jahn, R. (1996) J. Biol. Chem. 271, 5844-5849
11. Damer, C. K., and Creutz, C. E. (1996) J. Neurochem. 67, 1661-1668
12. Chapman, E. R., Desai, R. C., Davis, A. F., and Tornehl, C. K. (1998) J. Biol. Chem. 273, 32966-32972
13. Osborne, S. L., Herreros, J., Bastiaens, P. I. H., and Schiavo, G. (1999) J. Biol. Chem. 274, 59-66
14. Perin, M. S., Brose, N., Jahn, R., and Südhof, T. C. (1991) J. Biol. Chem. 266, 623-629
15. Niinobe, M., Yamaguchi, Y., Fukuda, M. and Mikoshiba, K. (1994) Biochem. Biophys. Res. Commun. 205, 1036-1042
16. Veit, M., Söllner, T. H., and Rothman, J. E. (1996) FEBS Lett. 385, 119-123
17. Chapman, E. R., Blasi, J., An, S., Brose, N., Johnston, P. A., Südhof, T. C., and Jahn, R. (1996) Biochem. Biophys. Res. Commun. 225, 326-332
18. Fukuda, M., and Mikoshiba, K. (1999) J. Biol. Chem., 274, 31428-31434
19. Mizushima, S., and Nagata, S. (1990) *Nucleic Acids Res.* **18**, 5332

20. Fukuda, M., Aruga, J., Niinobe, M., Aimoto, S., and Mikoshiba, K. (1994) *J. Biol. Chem.* **269**, 29206-29211

21. Ibata, K., Fukuda, M., and Mikoshiba, K. (1998) *J. Biol. Chem.* **273**, 12267-12273

22. Fukuda, M., Kojima, T., Aruga, J., Niinobe, M., and Mikoshiba, K. (1995) *J. Biol. Chem.* **270**, 26523-26527

23. Fukuda, M., Kojima, T., and Mikoshiba, K. (1996) *J. Biol. Chem.* **271**, 8430-8434

24. Fukuda, M., Kojima, T., and Mikoshiba, K. (1997) *Biochem. J.* **323**, 421-425

25. Li, C., Ullrich, B., Zhang, J. Z., Anderson, R. G. W., Brose, N., and Südhof, T. C. (1995) *Nature* **375**, 594-599

26. Ullrich, B., and Südhof, T. C. (1995) *Neuropharmacology* **34**, 1371-1377

27. Babity, J. M., Armstrong, J. N., Plumier, J. C., Currie, R. W., and Robertson, H. A. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 2638-2641

28. Butz, S., Fernandes-Chacon, R., Schmitz, F., Jahn, R., and Südhof, T. C. (1999) *J. Biol. Chem.* **274**, 18290-18296

29. Mizuta, M., Kurose, T., Miki, T., Shoji-Kasai, Y., Takahashi, M., Seino, S., and Matsukura, S. (1997) *Diabetes* **46**, 2002-2006

30. Mehrotra, B., Elliott, J. T., Chen, J., Olszewski, J. D., Profit, A. A., Chaudhary, A., Fukuda, M., Mikoshiba, K., and Prestwich, G. D. (1997) *J. Biol. Chem.* **272**, 4237-4244

31. Zhang, J. Z., Davletov, B. A., Südhof, T. C., and Anderson, R. G. W. (1994) *Cell* **78**, 751-760.

32. Mizutani, A., Fukuda, M., Niinobe, M., and Mikoshiba, K. (1997) *Biochem. Biophys. Res. Commun.* **240**, 128-131.

33. Sheng, Z. H., Yokoyama, C. T., and Catterall, W. A. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 5405-5410
FIG. 1. **Ca\(^{2+}\)**-dependent and -independent homo-oligomerization properties of synaptotagmins. **A**, schematic representation of Ca\(^{2+}\)-dependent oligomerization of T7- and FLAG-tagged synaptotagmins. T7-tag, FLAG-tag, transmembrane domain (TM), and C2 domains were shown by black, cross-hatched, open, and hatched boxes, respectively. In the left panel, Ca\(^{2+}\)-dependent oligomerization of T7- and FLAG-tagged synaptotagmins is restricted by the Ca\(^{2+}\)-independent oligomerization at the N-terminus (Syts I, II, III, V, VI, VIII, and X), whereas, in the right panel, Ca\(^{2+}\)-dependent oligomerization occurs independent of the N-termini (Syt VII) (see also Fig. 4A and 4C). A shaded region around the transmembrane domain in the left panel indicates the Ca\(^{2+}\)-independent self-oligomerization. “-S-S-” means disulfide bond (7). **B**, Ca\(^{2+}\)-dependent and -independent homo-oligomerization properties of Syts I, II, IV, VII, VIII, IX, and XI. pEF-T7-Syts and pEF-FLAG-Syts were co-transfected (left panel; Co-transfection) or separately transfected (right panel; Mix) into COS-7 cells, and recombinant proteins were immunoprecipitated in the presence or absence of 250 µM Ca\(^{2+}\) by anti-T7 tag antibody-conjugated agarose as described elsewhere (7). Co-immunoprecipitated FLAG-Syts were first detected by anti-FLAG antibody (Zymed; 5 µg/ml) using an Enhanced Chemiluminescence Kit (Amersham Pharmacia Biotech) (upper panel), and then the blots were stripped and reprobed with HRP-conjugated anti-T7 tag antibody to ensure that equivalent amounts of proteins were loaded (lower panel). The apparent molecular weight (Mr) of major bands of expressed T7-(or FLAG)-Syts corresponded to or were slightly higher than the Mr calculated from the cDNA sequences (7), indicating that some isoforms undergo the post-translational modifications [T7-Syt I (calculated Mr = 48.8 kDa); T7-Syt II (48.6); T7-Syt IV (49.0); T7-Syt VII (46.9); T7-Syt VIII (45.5); T7-Syt IX (44.5); and T7-Syt XI (49.7)]. The minor higher band of Syt VII is probably produced by partial post-translational modifications such as O-glycosylation or palmitoylation due to the transient expression. Note that only the Syt VII oligomerization showed strong Ca\(^{2+}\)-dependency regardless of the trnasfection method. In contrast,
oligomerization of other Syts could not be detected when T7- and FLAG-Syts were separately expressed in COS-7 cells (upper right panel), although very weak Ca\(^{2+}\)-dependent Syt II oligomerization was observed after prolonged exposure to X-ray film (data not shown). The positions of Mr markers (× 10\(^{-3}\)) are shown on the left. C, co-immunoprecipitated FLAG-Syts were quantified from the immunoreactive bands in B, normalized by the amount of T7-Syts, and expressed as arbitrary units. The results shown are representative of four independent experiments.

**Fig. 2. Ca\(^{2+}\)-dependent and -independent homo-oligomerization properties of cytoplasmic domains of synaptotagmins I-XI.** pEF-T7-Syts-cyto and pEF-FLAG-Syts-cyto were co-transfected into COS-7 cells, and recombinant proteins were immunoprecipitated in the presence (+) or absence (-) of 500 μM Ca\(^{2+}\) by anti-T7 tag antibody-conjugated agarose. Co-immunoprecipitated FLAG-Syt-cytos were first detected by anti-FLAG antibody in immunoblotting (upper panel), and then the blots were stripped and reprobed with HRP-conjugated anti-T7 tag antibody (lower panel). Note that FLAG-Syts II, V and VII cytoplasmic domains were easily detected but that only Syt VII homo-oligomerization was completely Ca\(^{2+}\)-dependent. The results shown are representative of three independent experiments. The positions of Mr markers (× 10\(^{-3}\)) are shown on the right. Mock indicates transfection of a control vector (pEF-BOS).

**Fig. 3. Ca\(^{2+}\)-dependent hetero-oligomerization of synaptotagmin VII with synaptotagmins V, VI, and X.** pEF-T7-Syt-cyto (or pEF-BOS) and pEF-FLAG-Syt VII-cyto were co-transfected into COS-7 cells, and recombinant proteins were immunoprecipitated in the presence or absence of 500 μM Ca\(^{2+}\) by anti-T7 tag antibody-conjugated agarose followed by immunoblotting. Co-immunoprecipitated FLAG-Syts were detected by anti-FLAG antibody in immunoblotting (upper panel). The lower panel indicates the immunoprecipitated T7-Syts visualized by HRP-conjugated anti-T7 tag antibody. Note that T7-Syt V-cyto, -Syt VI-cyto, -Syt VII-cyto, and -Syt X-cyto, but not T7-Syt III-cyto, bound FLAG-Syt VII-cyto only in the presence of Ca\(^{2+}\). The results
shown are representative of five independent experiments. The positions of Mr markers ($\times 10^{-3}$) are shown on the left.

**Fig. 4.** Functional relationship between Ca$^{2+}$-dependent and -independent oligomerization of synaptotagmin II or VII. pEF-T7-Syt II (or VII), pEF-FLAG-Syt II (or VII) and pEF-FLAG-Syt II-cyto (or VII-cyto) were co-transfected into COS-7 cells, and recombinant proteins were immunoprecipitated in the presence (+) or absence (-) of 500 $\mu$M Ca$^{2+}$ by anti-T7 tag antibody-conjugated agarose. A, co-immunoprecipitated FLAG-Syts were first detected by anti-FLAG antibody in immunoblotting (upper panel), and then the blots were stripped and reprobed with HRP-conjugated anti-T7 tag antibody (lower panel) to ensure that equivalent amounts of T7-tagged proteins were loaded. Immunoprecipitates of T7-Syt II (lanes 1 and 2) or T7-Syt VII (lanes 3 and 4) in the absence or presence of Ca$^{2+}$. Note that FLAG-Syt II-cyto could not be detected in lanes 1 and 2 in the upper panel but FLAG-Syt VII-cyto was detected in lane 4 in the upper panel (open arrowhead). B, expression of FLAG-Syts (upper panel) and T7-Syts (lower panel) in total homogenates of COS-7 cells. The 1/40 volume of total homogenates of COS-7 cells were used for immunoblotting to ensure that equivalent amounts of FLAG-Syt II (or VII) and FLAG-Syt II-cyto (or VII-cyto) were co-expressed. Open and closed arrows (in lane 1) indicate the positions of FLAG-Syt II-cyto and FLAG-Syt II, respectively. Open and closed arrowheads (in lane 2) indicate the positions of FLAG-Syt VII-cyto and FLAG-Syt VII, respectively. The results shown are representative of three independent experiments. The positions of Mr markers ($\times 10^{-3}$) are shown between A and B. C, schematic representation of Ca$^{2+}$-dependent oligomerization of T7-Syt and FLAG-Syt-cyto. Abbreviations are the same as those for Fig. 1A. In the left panel, FLAG-Syt II-cyto is unable to interact with full length T7-Syt II because T7-Syt II preferentially interacts with full length FLAG-Syt II pre-assembled at the N-terminal domain. In contrast, in the right panel, both FLAG-Syt VII-cyto and full length FLAG-Syt VII interact with full length T7-Syt VII in the presence of Ca$^{2+}$.
**Fig. 5.** **Ca**$^{2+}$-dependence of synaptotagmin VII oligomerization** pEF-T7-Syt VII-cyto (A) (or pEF-T7-Syt VI-cyto (C)) were co-transfected into COS-7 cells, and recombinant proteins were immunoprecipitated in the presence of 2 mM EGTA or various concentrations of Ca$^{2+}$ (50, 100, 200, 250, 500, 750 µM, or 1 mM) by anti-T7 tag antibody-conjugated agarose as described previously (7). Co-immunoprecipitated FLAG-Syts were detected by anti-FLAG antibody in immunoblotting (upper panels in A and C), and then the blots were stripped and reprobed with HRP-conjugated anti-T7 tag antibody (lower panels in A and C). The positions of Mr markers (× 10$^{-3}$) are shown on the right. A, Ca$^{2+}$-dependence of homo-oligomerization of Syt VII-cyto. B, immunoreactive bands in A were captured by Gel Print 2000i/VGA and quantified by Basic Quantifier software. The EC$_{50}$ value and Hill coefficient of Syt VII self-oligomerization were calculated to be 155 µM Ca$^{2+}$ and 3.1, respectively, by GraphPad PRISM software (Version 2.0). Bars indicate the S.E. of three independent experiments. C, Ca$^{2+}$-dependence of hetero-oligomerization of FLAG-Syt VII (upper panel) with T7-Syt VI-cyto (lower panel). The results shown are representative of two independent experiments.
Fukuda et al., Fig. 2, Top
Fukuda et al., Fig. 3, Top↑
A

anti-FLAG (Syt VII)

anti-T7

B

% of Maximal Binding

\[ \text{[Ca}^{2+}] \text{(M)} \]

C

anti-FLAG (Syt VII)

anti-T7 (Syt VI)

Fukuda et al., Fig. 5, Top↑
anti-FLAG (Syt VII)

anti-T7 (Syt VII)

1  2  3  4

1 mM Mg$^{2+}$ 500 μM Ca$^{2+}$ 500 μM Ca$^{2+}$ × 1 mM Mg$^{2+}$

2 mM EGTA
Distinct Self-dimerization Activities of Synaptotagmin Family: Unique Calcium-dependent Dimerization Properties of Synaptotagmin VII
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