The Calcium-binding Loops of the Tandem C2 Domains of Synaptotagmin VII Cooperatively Mediate Calcium-dependent Oligomerization

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Synaptotagmin VII (Syt VII), a proposed regulator for Ca\(^{2+}\)-dependent exocytosis, showed a robust Ca\(^{2+}\)-dependent oligomerization property via its two C2 domains (Fukuda, M., and Mikoshiba, K. (2001) J. Biol. Chem. 276, 27670–27676), but little is known about its structure or the critical residues directly involved in the oligomerization interface. In this study, site-directed mutagenesis and chimeric analysis between Syt I and Syt VII showed that three Asp residues in Ca\(^{2+}\)-binding loop 1 or 3 (Asp-172, Asp-303, and Asp-357) are crucial to robust Ca\(^{2+}\)-dependent oligomerization. Unlike Syt I, however, the polybasic sequence in the β4 strands of the C2 structures (so-called “C2 effector domain”) is not involved in the Ca\(^{2+}\)-dependent oligomerization of Syt VII. The results also showed that the Ca\(^{2+}\)-binding loops of the two C2 domains cooperatively mediate Syt VII oligomerization (i.e. the presence of redundant Ca\(^{2+}\)-binding sites) as well as the importance of Ca\(^{2+}\)-dependent oligomerization of Syt VII in Ca\(^{2+}\)-regulated secretion. Expression of wild-type tandem C2 domains of Syt VII in PC12 cells inhibited Ca\(^{2+}\)-dependent neuropeptide Y release, whereas mutant fragments lacking Ca\(^{2+}\)-dependent oligomerization activity had no effect. Finally, rotary-shadowing electron microscopy showed that the Ca\(^{2+}\)-dependent oligomer of Syt VII is “a large linear structure,” not an irregular aggregate. By contrast, in the absence of Ca\(^{2+}\), Syt VII molecules were observed to form a globular structure. Based on these results, we suggest that the linear Ca\(^{2+}\)-dependent oligomer may be aligned at the fusion site between vesicles and plasma membrane and modulate Ca\(^{2+}\)-regulated exocytosis by opening or dilating fusion pores.

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immunoprecipitation. Note that Ca²⁺-dependent self-oligomerization of Syt VII is sensitive to ionic strength. pEF-T7-Syt VII-cyto and pEF-FLAG-Syt VII-cyto were cotransfected into COS-7 cells. The proteins expressed were solubilized with 1% Triton X-100 and immunoprecipitated with anti-T7 tag antibody (1:10,000 dilution; IP: anti-T7) to ensure loading of the same amounts of T7-Syt proteins (1:10,000 dilution; input) used for immunoprecipitation. Note that Ca²⁺ selectively promoted oligomerization of Syt VII (in A) and that it was highly sensitive to ionic strength (NaCl concentration). The positions of the molecular weight markers (× 10⁻³) are shown on the left.

proposed to regulate several Ca²⁺-dependent processes (i.e. lysosomal exocytosis, insulin secretion in pancreatic β-cells, and norepinephrine release in PC12 cells) (27, 28, 30, 33, 34), little is known about the functional involvement of the Ca²⁺-dependent self-oligomerization of Syt VII in these Ca²⁺-regulated events, the structure of the Ca²⁺-dependent oligomer, or the critical residues directly involved in the oligomerization interface. In this study, we attempt to identify the residues (or an oligomerization interface) critical to the Ca²⁺-dependent oligomerization of Syt VII and the functional relationship between the two C2 domains. We show by site-directed mutagenesis that, unlike Syt I, Ca²⁺-dependent homo- and hetero-oligomerization of Syt VII are cooperatively mediated by the Ca²⁺-binding loops of the two C2 domains, not by the putative C2 effector domains. Moreover, whereas expression of the wild-type cytoplasmic tandem C2 domains in PC12 cells inhibited Ca²⁺-dependent neuropeptide Y (NPY) release, mutant proteins incapable of Ca²⁺-dependent oligomerization had no effect on NPY release. Furthermore, we show by rotary-shadowing electron microscopy that the Ca²⁺-dependent Syt VII oligomer has a linear structure and is not a random aggregate. Based on these findings, we discuss how the Ca²⁺-dependent Syt VII oligomer regulates vesicular exocytosis.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction and Site-directed Mutagenesis of Mouse Synaptotagmin VII**—Mutant Syt VII molecules carrying an Asp-to-Asn substitution at amino acid position 172 (D172N), a D305N substitution, a D357N substitution, or a Lys-to-Gln substitution in the C2A domain (named the AQ mutation; K183Q, K184Q, and K186Q) were produced by PCR. The following pairs of oligonucleotides were used for amplification with pGEM-T-Syt VII as a template (35): 5′-GTTCACTTAGAACCCCTTTTG-3′ (D172N primer; sense), 5′-GCCACGGAACCGGGGCACTATTTCCC-3′ (D183N primer; sense), 5′-CAGTTAGATCTTCCGATGACGGTGTTC-3′ (D305N primer; sense), and 5′-AGCTTCCTGCTTCCACCAACGAGCACCAC-3′ (AQ primer; sense). The PCR products were purified from an agarose gel on a Micro-Spin column (Amersham Biosciences, Inc., Buckinghamshire, UK) as described previously (35) and then directly inserted into the pGEM-T Easy vector (Promega, Madison, WI). After verifying the nucleotide sequences with a Hitachi 350-5500 DNA sequencer, full-length Syt VII DNA was constructed by replacement of appropriate inserts at the appropriate restriction enzyme sites (underlined above) on the pGEM-T Easy vector. A mutant Syt VII carrying a Lys-to-Gln substitution in the C2B domain (named the BQ mutation; K320Q, K321Q, and K325Q) was essentially produced by means of two-step PCR techniques as described previously (25) (using the following oligonucleotides): 5′-CTCTAAGGTTGGTCTTCATTT-3′ (BQ mutant primer 1; antisense) and 5′-GGGACACAGGAAGGCACGAAGCTGACAG-3′ (BQ mutant primer 2; sense). The resulting Syt VII mutant fragments were subcloned into a modified pBEF-Bos mammalian expression vector with an N-terminal T7 or FLAG tag (7, 18, 36). All constructs were verified by DNA sequencing as described above. pBEF-T7 (or FLAG)-VII-cyto and pBEF-FLAG-Syt VII-cyto were prepared as described previously (18, 19, 37). Plasmid DNA was prepared using Wizard-mini preps (Promega) or Qiagen Maxiprep kits.

**Purification of the Cytoplasmic Domain of Synaptotagmin VII**—A glutathione S-transferase (GST) tag was added to the N terminus of Syt VII-cyto by PCR using the pGEX-2T vector (Amersham Biosciences) as a template: 5′-CGAGATCTTATGGCTCCCTACTAGGTT-3′ (GST primer; sense) 5′-GGATCCACGCGGAACCAGGATATGACGTTGTT-3′ (BQ mutant primer 1; antisense) and 5′-GGGACACAGGAAGGCACGAAGCTGACAG-3′ (BQ mutant primer 2; sense). The purified PCR products were digested with BamHI and BglII (italiced and underlined above) and inserted into the BamHI site of pEF-T7-Syt VII-cyto. T7-GST-FLAG-Syt VII-cyto proteins expressed in COS-7 cells (10–cm-dish cultures) were solubilized with 1% Triton X-100, 50 mM HEPES-KOH, pH 7.2, and 250 mM NaCl and affinity-purified with glutathione-Sepharose (wet volume 20 ml) (Promega, Madison, WI). After verifying the nucleotide sequences with a Hitachi 350-5500 DNA sequencer, full-length Syt VII DNA was constructed by replacement of appropriate inserts at the appropriate restriction enzyme sites (underlined above) on the pGEM-T Easy vector. A mutant Syt VII carrying a Lys-to-Gln substitution in the C2B domain (named the BQ mutation; K320Q, K321Q, and K325Q) was essentially produced by means of two-step PCR techniques as described previously (25) (using the following oligonucleotides): 5′-CTCTAAGGTTGGTCTTCATTT-3′ (BQ mutant primer 1; antisense) and 5′-GGGACACAGGAAGGCACGAAGCTGACAG-3′ (BQ mutant primer 2; sense). The resulting Syt VII mutant fragments were subcloned into a modified pBEF-Bos mammalian expression vector with an N-terminal T7 or FLAG tag (7, 18, 36). All constructs were verified by DNA sequencing as described above. pBEF-T7 (or FLAG)-VII-cyto and pBEF-FLAG-Syt VII-cyto were prepared as described previously (18, 19, 37). Plasmid DNA was prepared using Wizard-mini preps (Promega) or Qiagen Maxiprep kits.
then stimulated with either low KCl buffer or high KCl buffer (56 mm KCl, 95 mm NaCl, 2.2 mm CaCl2, 0.5 mm MgCl2, 5.6 mm glucose, and 15 mm HEPES-KOH, pH 7.4) for 10 min at 37 °C. Released NPY-T7-GST was recovered by incubation with glutathione-Sepharose beads and analyzed by immunoblotting with horseradish peroxidase (HRP)-conjugated anti-T7 tag antibody. The intensity of the immunoreactive bands was quantified as described previously (18) and normalized to total expressed T7-Syt proteins (1:10,000 dilution; bottom panel in C). The top two panels indicate the total expressed T7-Syt VII-cyto and FLAG-Syt VII-cyto proteins (%input volume; input in C) used for immunoprecipitation. Note that the mutations in the putative C2 effector domains (AKQ and BKQ) have virtually no effect.

Electron Microscopy—The specimens for electron microscopy were prepared by rotary shadowing as described previously (41). In brief, an aliquot of the purified Syt VII cytoplasmic fragment (about 50–100 μg/ml) was mixed with four volumes of 25 mM ammonium acetate containing 50% glycerol. The mixture was immediately sprayed onto the surface of freshly cleaved mica. Following rotary shadowing with Pt/C (elevation angle: 6 or 8°) and backing with pure carbon, replicas were floated off and were picked up onto copper grids. The images were...
acceleration voltage. FLAG-Syt into COS-7 cells (7.5 respectively. cyto and FLAG-Syt VII(S1A)-cyto proteins (1 for immunoprecipitation. of at least three independent experiments. The blots shown in this paper are representative FLAG tag antibodies (Sigma) were also performed as described previously (18, 35, 42). The blots shown in this paper are representative concentrations of NaCl as described previously (35). SDS-PAGE and im-

we first investigated the effect of divalent cations (1 mM) on oligomerization of Syt VII via the two C2 domains is crucial to

self-oligomerization of Syt VII must be selectively stimulated by 

ions, whereas Sr²⁺ ions only marginally acti-

tions had no effect at all

self-oligomerization activity (Fig. 3

domain resulted in normal Ca²⁺-dependent self-oligomeriza-

neutralization of these basic residues (Lys-to-Gln or Lys-to-Ala substitution) completely abrogated the Ca²⁺-dependent self-oligomerization of Syts I and II (16, 20). However, neutralization of all three basic residues in the C2A domain (named the ARQ mutation) and C2B domain (BKQ mutation) had no effect on the Ca²⁺-dependent self-oligomerization activity (Fig. 2C, third panel), although the ARQ mutation slightly increased the Ca²⁺-independent self-oligomerization activity.

We next focused on the Asp residues in putative Ca²⁺-binding loops and the Putative C2 Effector Domains of Synaptotagmin VII—Site-directed mutagenesis was performed to define the oligomerization interface of the Syt VII C2 domains (Fig. 2, A and B). We initially focused on the Lys cluster in the putative C2 effector domains, which is located in the β4 strand (Lys-183, Lys-184, and Lys-186 in the C2A domain and Lys-320, Lys-321, and Lys-325 in the C2B domain, corresponding to the “C2B effector domain” of Syt I) of the C2 β-sandwich structure (16, 25, 43–46), because neutralization of these basic residues (Lys-to-Gln or Lys-to-Ala substitution) completely abrogated the Ca²⁺-dependent self-oligomerization of Syts I and II (16, 20). However, neutralization of all three basic residues in the C2A domain (named the ARQ mutation) and C2B domain (BKQ mutation) had no effect on the Ca²⁺-dependent self-oligomerization activity (Fig. 2C, third panel), although the ARQ mutation slightly increased the Ca²⁺-independent self-oligomerization activity.

We next focused on the Asp residues in putative Ca²⁺-bind-

loop 1 (Asp-172 in the C2A domain and Asp-303 in the C2B domain) and loop 3 (Asp-357 in the C2B domain) (43–45). The single Ca²⁺-binding loop mutations (D172N, D303N, or D357N) of the isolated C2 domain were sufficient to abolish the Ca²⁺-dependent self-oligomerization activity (Fig. 3A, third panel). To our surprise, however, the single Ca²⁺-binding loop mutations (D172N or D303N) of the full Syt VII cytoplasmic domain resulted in normal Ca²⁺-dependent self-oligomerization activity, whereas the double Ca²⁺-binding loop mutations (D172N and D303N) completely abrogated the Ca²⁺-dependent self-oligomerization activity (Fig. 3B, third panel). Similar results were obtained in regard to Ca²⁺-dependent hetero-oligomerization of Syt VII with Syt VI (compare Fig. 3, C and D, third panels); the single D172N mutation was neutral in regard

FIG. 4. Ca²⁺-dependent oligomerization property of the C2A chimera between Syt VII and Syt I, A, schematic representation of the C2A chimera between Syt I and Syt VII (named Syt VII(S1A)). The transmembrane domain (TM), Syt I C2A domain, and Syt VII C2B domain are represented by the open box, hatched box, and shaded box, respectively. B, Ca²⁺-dependent self-oligomerization of Syt VII(S1A) mutants. pEF-TT-Syt VII(S1A)-cyto and pEF-FLAG-Syt VII(S1A)-cyto were cotransfected into COS-7 cells. The proteins expressed were solubilized with 1% Triton X-100 and immunoprecipitated with anti-T7 tag antibody-conjugated agarose (IP) in the presence or absence of 1 mM Ca²⁺ as described previously (32, 35). Co-immunoprecipitated FLAG-Syts were first detected with HRP-conjugated anti-T7 tag antibody (1:10,000 dilution; third panel in B). The same blot was stripped and reprobed with HRP-conjugated anti-T7 tag antibody to ensure loading of the same amounts of T7-Syt proteins (1:10,000 dilution; bottom panel in B). The top two panels indicate the total expressed T7-Syt VII(S1A)-cyto and FLAG-Syt VII(S1A)-cyto proteins (% volume; input in B) used for immunoprecipitation.

recorded with a JEM-2000ES electron microscope (JEOL) at 80-kV acceleration voltage.

MISCELLANEOUS PROCEDURES—Cotransfection of pEF-TT-Syts and pEF-FLAG-Syts into COS-7 cells (7.5 × 10⁶ cells, the day before transfection, per 10-cm dish) was carried out with the LipofectAMINE Plus reagent according to the manufacturer’s instructions (Invitrogen) (42). Proteins were solubilized with a buffer containing 1% Triton X-100, 250 mM NaCl, 50 mM HEPES-KOH, pH 7.2, 0.1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, and 10 μM pepstatin A at 4 °C for 1 h. T7-Syts were immunoprecipitated with anti-T7 tag antibody-conjugated agarose (Novagen; Madison, WI) in the presence of 2 mM EGTA or 1 mM divalent cations (MgCl₂, CaCl₂, SrCl₂, and BaCl₂) and/or various concentrations of NaCl as described previously (35). SDS-PAGE and im-

FIG. 5. Ca²⁺-triggered NPY release requires an intact Ca²⁺-binding site in the tandem C2 domains of synaptotagmin VII. PC12 cells expressing Syt VII tandem C2 domains were stimulated by high KCl buffer, and the NPY-T7-GST released was measured by immu

nunoprecipitation as described under Experimental Procedures. The results are expressed as percentage of NPY-T7-GST release compared with control samples without expression of recombinant proteins. Note that expression of the wild-type fragment dramatically reduced the Ca²⁺-dependent NPY-T7-GST release (*, p < 0.01, Student’s unpaired t test (open bar), whereas expression of the mutant protein (D172N/ D303N) had no significant effect (closed bar). Bars indicate the mean ± S.E. of three determinations. The inset shows the similar expression levels of the wild-type and DN (D172N/D303N) mutant proteins visualized by HRP-conjugated anti-FLAG tag antibody. The arrowhead indicates the nonspecific interaction of anti-FLAG tag antibody. The results shown are representative of three different experiments.

RESULTS

Oligomerization of Synaptotagmin VII Is Selectively Stimulated by Ca²⁺ and Mediated by Electrostatic Interaction—If oligomerization of Syt VII via the two C2 domains is crucial to the Ca²⁺-regulated events previously described (27, 28, 30, 33, 34), oligomerization of Syt VII must be selectively stimulated by Ca²⁺ ions and not by other divalent cations. To confirm this, we first investigated the effect of divalent cations (1 mM) on self-oligomerization of Syt VII. The T7- and FLAG-tagged Syt VII cytoplasmic domains (Syt VII-cyto) were coexpressed in COS-7 cells, and the association between these two proteins was evaluated by the immunoprecipitation method (35, 42) in the presence of various divalent cations (1 mM). As expected, self-oligomerization of Syt VII was selectively promoted by Ca²⁺ ions, whereas Sr²⁺ and Ba²⁺ ions only marginally activated self-oligomerization. 1 mM Mg²⁺ ions had no effect at all (Fig. 1A, third panel). This divalent cation selectivity was quite similar to that of Syt I described previously (15, 16). Since the oligomerization of Syt VII was highly sensitive to ionic strength (failing to occur above 750 mM of NaCl), the Syt VII Ca²⁺-de-
to the interaction between Syt VII-cyto and Syt VI-cyto (Fig. 3D) but completely abrogated the interaction between Syt VII-C2A and Syt VI-cyto (Fig. 3C). In addition, the double Ca\(^{2+}\)-binding loop mutant (D172N/D303N) was incapable of interacting with Syt VI-cyto even in the presence of Ca\(^{2+}\) (Fig. 3D). This result markedly contrasts with those of Syt I, because neutralization of the corresponding acidic (Asp) residues of Syt I in the Ca\(^{2+}\)-binding loops enhanced the Ca\(^{2+}\)-independent self-oligomerization activity (47). These findings strongly indicated that the fundamental mechanism of Ca\(^{2+}\)-dependent self-oligomerization by Syt VII and Syt I is different, at least in terms of oligomerization interface, and we hypothesized that the two C2 domains of Syt VII cooperatively mediate Ca\(^{2+}\)-dependent self-oligomerization (i.e., existence of a redundant Ca\(^{2+}\)-binding site).

**Ca\(^{2+}\)-binding Loops of Two C2 Domains Cooperatively Mediate Ca\(^{2+}\)-dependent Oligomerization of Synaptotagmin VII**—If this hypothesis were true, pairing of the C2A domain and C2B domain should be critical for Ca\(^{2+}\)-dependent self-oligomerization of Syt VII, and to test this, we prepared a chimera between Syt VII and Syt I that contains the Syt I C2A domain (named Syt VII(S1A); see Fig. 4A). The Syt VII(S1A)-cyto protein showed weaker Ca\(^{2+}\)-dependent self-oligomerization activity than that of the wild-type Syt VII protein (Fig. 4B, third panel), probably as a result of the loss of one hand (i.e., the C2A domain of Syt VII), because the Syt I C2A domain did not show Ca\(^{2+}\)-dependent self-oligomerization activity (15, 16). It is noteworthy that the Syt VII(S1A)/D303N-cyto protein showed Ca\(^{2+}\)-independent self-oligomerization activity, the same as the Syt I Ca\(^{2+}\)-binding mutation (47) (Fig. 4B, third panel). Thus, pairing of the C2A and C2B domains is crucial to efficient Ca\(^{2+}\)-dependent self-oligomerization of Syt VII.

**Effect of Mutations in the Ca\(^{2+}\)-binding Loops on Ca\(^{2+}\)-dependent NPY Release in PC12 Cells**—We then investigated the involvement of Ca\(^{2+}\)-dependent oligomerization of Syt VII in Ca\(^{2+}\)-dependent NPY-T7-GST release by expression of wild-type or mutant (D172N/D303N) cytoplasmic fragments of Syt VII in PC12 cells. Expression of the wild-type Syt VII-cyto in PC12 cells resulted in about 50% inhibition of Ca\(^{2+}\)-induced exocytosis, whereas expression of Syt VII-cyto(D172N/D303N) completely reversed the inhibitory effect (Fig. 5). Ca\(^{2+}\)-independent NPY-T7-GST release was unaltered by the expression of recombinant proteins (data not shown). These results suggested a critical function of Ca\(^{2+}\)-dependent oligomerization of the C2 domains in Ca\(^{2+}\)-induced exocytosis.

**Structure of Ca\(^{2+}\)-dependent Oligomerization of Synaptotagmin VII Visualized by Rotary-shadowing Electron Microscopy**—Finally, we attempted to visualize the structure of the Ca\(^{2+}\)-dependent oligomer of Syt VII by rotary-shadowing electron microscopy. Since the bacterial recombinant Syt VII-cyto proteins were difficult to prepare due to inclusion bodies (data not shown), we used recombinant proteins from mammalian cultured cells for electron microscopy. The recombinant cytoplasmic domains of Syt VII fused to GST (Fig. 6A) were expressed in COS-7 cells and were affinity-purified as described under “Experimental Procedures.” Purified FLAG-Syt VII-cyto proteins were analyzed by SDS-PAGE and staining with Coomassie Brilliant Blue R-250. The authenticity of the bands (FLAG-Syt VII-cyto) was confirmed by immunoblotting with HRP-conjugated anti-FLAG tag antibody (data not shown). The positions of the molecular weight markers (MW) (× 10\(^{-3}\)) are shown on the left. WT, wild type.

**Fig. 6. Purification of cytoplasmic fragments of synaptotagmin VII**. A, schematic representation of the GST-tagged Syt VII cytoplasmic domain (amino acids 43–403). T7-tag, GST, and two C2 domains are represented by the black box, hatched box, and shaded boxes, respectively. B, pEF-T7-GST-FLAG-Syt VII-cyto was transfected into COS-7 cells. The proteins expressed were solubilized with 1% Triton X-100 and affinity-purified by glutathione-Sepharose beads. The GST tag was removed by thrombin digestion as described under “Experimental Procedures.” Purified FLAG-Syt VII-cyto proteins were analyzed by 10% SDS-PAGE and staining with Coomassie Brilliant Blue R-250. The authenticity of the bands (FLAG-Syt VII-cyto) was confirmed by immunoblotting with HRP-conjugated anti-FLAG tag antibody (data not shown). The positions of the molecular weight markers (MW) (× 10\(^{-3}\)) are shown on the left. WT, wild type.

**Fig. 7. Rotary-shadowing electron microscopy of Ca\(^{2+}\)-dependent synaptotagmin VII polymer. Left panel and insets, various lengths of Syt VII polymer (linear structures) in the presence of 1 mM Ca\(^{2+}\). Middle panel, globular structures (arrowheads) of Syt VII in the absence of Ca\(^{2+}\). Right panel, globular structures of Syt VII(D172N/D303N) mutants in the presence of Ca\(^{2+}\). Scale bar, 100 nm.**
These results together with the results of immunoprecipitation (Figs. 2–4 and Ref. 18) and gel filtration analyses (32) indicated that the Ca$^{2+}$-induced Syt VII oligomer is a large linear structure and not a random aggregate.

**DISCUSSION**

In our previous study, we showed that a single C2 domain of Syt VII is sufficient for Ca$^{2+}$-dependent homomultimerization and hetero-oligomerization with other Syt isoforms (18, 19, 32), but the functional relationship between the two C2 domains and the structure of the Syt VII oligomer had never been determined. In this paper, we have presented several lines of evidence indicating that the two C2 domains of Syt VII are not functionally independent and that the Ca$^{2+}$-binding loops of the two C2 domains cooperatively mediate Ca$^{2+}$-dependent oligomerization. First, mutation of the single Asp residue (D172N in the C2A domain or D303N in the C2B domain) in Ca$^{2+}$-binding loop 1 abrogated the Ca$^{2+}$-dependent self-oligomerization of the “isolated C2 domain” but was neutral in regard to the Ca$^{2+}$-dependent self-oligomerization of the “tandem C2 domains” (Figs. 2 and 3). The tandem C2 domains with the double mutation (D172N/D303N), however, did not show Ca$^{2+}$-dependent self-oligomerization, suggesting the presence of redundant Ca$^{2+}$-binding site(s). Second, chimeric analysis between Syt I and Syt VII showed that pairing of the C2A and C2B domains is an important factor for efficient Ca$^{2+}$-dependent oligomerization of Syt VII (Fig. 4). Based on these results, together with the recent crystallographic data showing that the Ca$^{2+}$-binding regions of the two C2 domains of Syt III face each other (45), we propose that the Ca$^{2+}$-binding loops of the two C2 domains directly contribute to formation of the oligomerization interface; Ca$^{2+}$-binding to the Asp residues in the loops of the C2 structures changes the electrostatic charges around the loop domains, which may directly form the oligomerization interface between two C2 domains. This model is completely different from the model of Syt I (or II), which also showed Ca$^{2+}$-dependent oligomerization mediated by the C2B effector domain but not the Ca$^{2+}$-binding loops themselves (16, 20, 47). The structure of the Ca$^{2+}$-dependent oligomer of Syt I is now under investigation in our laboratory, and it will be interesting to determine whether the Ca$^{2+}$-dependent Syt I oligomer exhibits the same linear structure.

We also demonstrated the physiological importance of the Ca$^{2+}$-dependent oligomerization of Syt VII by using the dominant negative approach (28, 30, 47). When the wild-type tandem C2 fragment was expressed in PC12 cells, Ca$^{2+}$-dependent NPY release was significantly inhibited, most likely by competing endogenous Syt proteins. By contrast, the mutants lacking Ca$^{2+}$-dependent oligomerization activity had no effect on NPY release (Fig. 5).

Although the exact role of the Ca$^{2+}$-dependent oligomer of Syt VII in vesicular exocytosis is unknown, based on the structure of the Ca$^{2+}$-dependent oligomer of Syt VII (i.e. the linear structure) visualized by rotary-shadowing electron microscopy (Fig. 7), it may be involved in dilation or opening of fusion pores by aligning to form a straight line at the fusion site between the vesicles and plasma membrane (48). Consistent with this hypothesis, Syt I and IV have recently been shown to modulate fusion pore kinetics in regulated exocytosis of PC12 cells (49).

Further work is necessary to examine the effect of wild-type or mutant (D172N/D303N) expression on fusion pore kinetics to clarify the relationship between Syt VII oligomerization and fusion pore formation.

In summary, site-directed mutagenesis and chimeric analysis in this study demonstrated that the Ca$^{2+}$-binding loops of the two C2 domains cooperatively mediate both Ca$^{2+}$-dependent oligomerization of Syt VII and Ca$^{2+}$-dependent NPY release. We have also shown that the Ca$^{2+}$-dependent Syt VII oligomer is a linear structure, not an irregular random aggregate.