Mechanism of the SDS-resistant Synaptotagmin Clustering Mediated by the Cysteine Cluster at the Interface between the Transmembrane and Spacer Domains*

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Synaptotagmin I (Syt I), a proposed major Ca$^{2+}$ sensor in the central nervous system, has been hypothesized as functioning in an oligomerized state during neurotransmitter release. We previously showed that Syts I, II, VII, and VIII form a stable SDS-resistant, β-mercaptoethanol-insensitive, and Ca$^{2+}$-independent oligomer surrounding the transmembrane domain (Fukuda, M., and Mikoshiba, K. (2000) J. Biol. Chem. 275, 28180–28185), but little is known about the molecular mechanism of the Ca$^{2+}$-independent oligomerization by the synaptotagmin family. In this study, we analyzed the Ca$^{2+}$-independent oligomerization properties of Syt I and found that it shows two distinct forms of self-oligomerization activity: stable SDS-resistant self-oligomerization activity and relatively unstable SDS-sensitive self-oligomerization activity. The former was found to be mediated by a post-translationally modified (i.e. fatty-acylated) cysteine (Cys) cluster (Cys-74, Cys-75, Cys-77, Cys-79, and Cys-82) at the interface between the transmembrane and spacer domains of Syt I. We also show that the number of Cys residues at the interface between the transmembrane and spacer domains determines the SDS-resistant oligomerizing capacity of each synaptotagmin isoform: Syt II, which contains seven Cys residues, showed the strongest SDS-resistant oligomerizing activity in the synaptotagmin family, whereas Syt XII, which has no Cys residues, did not form any SDS-resistant oligomers. The latter SDS-sensitive self-oligomerization of Syt I is mediated by the spacer domain, because deletion of the whole spacer domain, including the Cys cluster, abolished it, whereas a Syt I(CA) mutant carrying Cys to Ala substitutions still exhibited self-oligomerization. Based on these results, we propose that the oligomerization of the synaptotagmin family is regulated by two distinct mechanisms: the stable SDS-resistant oligomerization is mediated by the modified Cys cluster, whereas the relatively unstable (SDS-sensitive) oligomerization is mediated by the environment of the spacer domain.

Synaptotagmin (Syt)1 comprises a large family of type I membrane proteins present from nematodes to humans that are thought to regulate membrane trafficking (reviewed in Refs. 1–4). Syt consists of a single transmembrane domain and two Ca$^{2+}$-binding domains (the C2A domain and C2B domain) that are highly homologous to the C2 regulatory region of mammalian Ca$^{2+}$-dependent protein kinase C (5). To date, thirteen Syt isoforms have been identified in the mouse and rat (6, 7, and reviewed in Ref. 1), but, except for the role of Syt I in synaptic vesicle trafficking, the exact roles of the Syt isoforms largely remain to be elucidated.

Syt I is an abundant synaptic vesicle and multifunctional protein that regulates three distinct steps of the synaptic vesicle cycle (i.e. docking, fusion, and recycling). The WHXL motif in the carboxyl terminus of Syt I is involved in docking of synaptic vesicles to active zones in the squid giant synapse (8), the C2A domain is involved in the synaptic vesicle fusion step (9–11), and the C2B domain is involved in synaptic vesicle recycling, probably by binding to the clathrin assembly protein AP2 (12–17). In addition, genetic analysis of Drosophila syt I mutants has indicated that Syt I functions in an oligomerized state, because two independent Syt I mutants can partially complement each other’s phenotype (reviewed in Ref. 18). Consistent with this, Syt I (or II) forms SDS-resistant Ca$^{2+}$-independent oligomers by an unknown mechanism (19–22) and Ca$^{2+}$-dependent oligomers mediated by the C2B domain in vitro (21–30). Our previous study showed that the Ca$^{2+}$-dependent self-oligomerization via the C2B domain of Syt I (or II) occurs only when the two molecules are preassembled at the amino-terminal domain (21). Thus, the Ca$^{2+}$-independent oligomerization of Syt I is crucial for rapid Ca$^{2+}$-dependent clustering via the C2B domain in response to rapid increases in Ca$^{2+}$ ions entering through voltage-gated Ca$^{2+}$ channels during neurotransmitter release. Although the key amino acids responsible for Ca$^{2+}$-dependent multimerization (the so-called C2B effector domain) have been thoroughly investigated (25, 27–30), little is known about the molecular mechanism of Ca$^{2+}$-independent oligomerization of Syt I (e.g. which domain is involved in Ca$^{2+}$-independent oligomerization). However, this information is quite important in terms of identifying the number of Syt I clusters that need to cooperate during synaptic vesicle exocytosis.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) AB066804.

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1 The abbreviations used are: Syt(s), synaptotagmin(s); HRP, horse radish peroxidase; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; Sulfo-MBS, m-maleimidobenzoyl-N-hydroxysulfosuccinimide ester; Sulfo-BSOCOES, bis[2-(sulfosuccinimidylcarbonyloxy)ethyl]sulfone.
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EXPERIMENTAL PROCEDURES

Materials—Horseradish peroxidase (HRP)-conjugated anti-T7 tag antibody and anti-T7 tag antibody-conjugated agarose were from Novagen (Madison, WI). HRP-conjugated monoclonal (M2) antibody against FLAG peptide was obtained from Sigma Chemical Co. (St. Louis, MO). Sulfo-MBS (N-maleimidobenzoyl-N-hydroxysulfosuccinimide) ester) and Sulfo-BSO (N-benzoylsulfonimidomethyl oxyethyl)sulfone) were from Pierce (Rockford, IL). All other chemicals were commercial products of reagent grade. Solutions were made up in deionized water prepared with an Elix10 water purification system and Milli-Q Bioel A10 system (Millipore Corp. Bedford, MA).

Molecular Cloning of Mouse Synaptotagmin XII (Srg1)—cDNA encoding a full open reading frame of mouse brain Syt XII (also called synaptotagmin-related gene 1 (Srg1)) was amplified by the reverse transcriptase-polymerase chain reaction (PCR) using the following primers with restriction enzyme sites (underlined) that were designed on the basis of the rat sequence, as described previously (31–33): 5'-GAAGATCTATGGCAGCGACGACAG-3' (Met primer; sense; amino acid residues 1–7) and 5'-CCATATTCTTCTTTCGCCGGACGT-GA-3' (stop primer; antisense; amino acid residues 416–421). Reactions were carried out in the presence of a Perfect Match PCR enhancer (Stratagene, La Jolla, CA) for 30 cycles, each consisting of denaturation at 94 °C for 1 min, annealing at 50 °C for 2 min, and extension at 72 °C for 2 min. The PCR products were purified from agarose gel with a Microspin column (Amersham Pharmacia Biotech, Buckinghamshire, UK), as described previously (31), and directly inserted into the pGEM-T Easy vector (Promega, Madison, WI). Both strands were completely sequenced with the ThermoSequenase premixed cycle sequencing kit (Amersham Pharmacia Biotech) using a SQ-5500 DNA sequencer (Hitachi). Addition of the T7 tag to the amino terminus of Syt XII (pEF-T7-Syt XII) and construction of the expression vector were performed, as described previously (31).

Site-directed Mutagenesis of Mouse Synaptotagmins I and IX—A mutant Syt I carrying a Cys to Ala mutation (C75A, C77A, C79A, C82A, or CA) was cloned into the pEF-BOS mammalian expression vector (34, 35). All constructs were verified by DNA sequencing, as described above. T7-Syt I cDNA encodes amino acids 1–81 of mouse Syt I; T7-Syt IX cDNA encodes amino acids 1–137 of Syt I; Syt I C2A/B, amino acids 1–266 of Syt I; Syt IASpacer, deletion of amino acids 74–138 of Syt I; and Syt IAN, amino acids 47–421 of Syt I.

pEF-T7(or FLAG)-Syt I, II, VII, VIII, and IX were prepared, as described previously (21). Plasmid DNA was prepared using Wizard mini prep kits (Promega) or Qiagen (Chatsworth, CA) Maxi prep kits.

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In this study, we attempted to determine the structural basis of the SDS-resistant Ca2+-independent clustering of the Syt family. We show by site-directed mutagenesis that post-translational modification of the Cys cluster of Syt I at the interface between the transmembrane domain and the spacer domain is essential for stable SDS-resistant homo- and hetero-oligomerization. We also show that differences in oligomerization activity among the members of the synaptotagmin family result from the number of Cys residues in each isoform. Based on these findings, we discuss the mechanisms of Ca2+-independent homo- and hetero-oligomerization in the synaptotagmin family.

FIG. 1. Mapping of the domains responsible for Ca2+-independent self-oligomerization of synaptotagmin I. A, schematic representation of deletion mutants of Syt I. The T7 tag, transmembrane domain (TM), and two C2 domains are represented by the hatched box, black box, and shaded boxes, respectively. Systematic deletions were made from the amino and carboxyl termini. The relative oligomerizing activity (+ + +, +, or –) of each mutant is indicated after its name and was determined on the basis of the results shown in B. C2 indicates the number of Cys residues in each mutant. The sequence at the bottom indicates the region responsible for Ca2+-independent self-oligomerization (the five Cys residues are in boldface). Fatty-acylation is mediated from the amino and carboxyl termini. The relative oligomerizing activity (+ + +, +, or –) of each mutant is indicated after its name and was determined on the basis of the results shown in B. C2 indicates the number of Cys residues in each mutant. The sequence at the bottom indicates the region responsible for Ca2+-independent self-oligomerization (the five Cys residues are in boldface). Fatty-acylation is mediated from the amino and carboxyl termini.
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A

B

C

D

E

FIG. 2. Essential role of the Cys cluster in the SDS-resistant self-oligomerization properties of synaptotagmin I. A, the amino acid sequence (top) is the Cys cluster located at the interface between the transmembrane and spacer domains. "A" represents substitution of Ala for Cys. TM, transmembrane domain. B, T7-Syt I mutants were expressed in PC12 cells and homogenized in 1% SDS with a 27-gauge syringe. After addition of SDS sample buffer (1/10,000 dilution). Note that the apparent molecular weight of the CA mutants is significantly lower than that of the wild-type protein. The minor bands around 50 kDa may represent degradation products. C, T7-Syt IΔN/ΔC2AB mutants were expressed in COS-7 cells, and their SDS-resistent oligomerizing properties of synaptotagmin I. A, the amino acid sequence (top) is the Cys cluster located at the interface between the transmembrane and spacer domains. "A" represents substitution of Ala for Cys. TM, transmembrane domain. B, T7-Syt I mutants were expressed in PC12 cells and homogenized in 1% SDS with a 27-gauge syringe. After addition of SDS sample buffer (1/10,000 dilution). Note that the apparent molecular weight of the CA mutants is significantly lower than that of the wild-type protein. The minor bands around 50 kDa may represent degradation products.


differentiation of T7-Syt I mutants were prepared, as described previously (6, 32), and were suspended in 50 mM HEPES-KOH, pH 7.2, 150 mM NaCl, and 1 mM EDTA at room temperature. A 10% volume of water or 25 mM Sulfo-MBS and Sulfo-BSOOCES in 10 mM HEPES-KOH, pH 7.2 (final concentration 2.5 mM), was added, and the mixtures were incubated with gentle agitation at room temperature for 30 min. Reactions were terminated with the addition of 150 mM Tris to quench the cross-linking reagent. Proteins were homogenized in 1% SDS with a 27-gauge syringe, and insoluble materials were removed by centrifugation. Solubilized proteins were analyzed by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and then immunoblotted with HRP-conjugated anti-T7 tag antibody, as described previously (31).

**RESULTS**

Mapping of the Domain Responsible for Ca \(^{2+}\)-independent Self-oligomerization of Synaptotagmin I—We previously showed that a subclass of Syts (Syts III, V, VI, and X) form homo- and heterodimers via the conserved amino-terminal Cys motif in the extracellular domain and that Syts I, II, VII, and

potencies of the mutants were significantly lower than that of the wild-type protein (see the dimer band around 30 kDa in the right panel). Immunoreactive bands were captured by Gel Print 2000i/VGA (BioImage), and the ratio between monomer and dimer was analyzed with Basic Quantifier software (version 1.0, BioImage), as described previously (21). Long x-ray film exposures allowed detection of the pentamer of Syt I under our experimental conditions. The positions of the molecular weight markers (× 10^-3) are shown on the right. D, higher magnification of T7-Syt IΔN/ΔC2AB protein in C. Six bands (two major and four minor bands) were detected and probably correspond to the different modification states of the five Cys residues (arrowheads 0–5). E, cross-linking reagent of recombinant Syt IΔN/ΔC2AB (left panel) and Syt IΔN/ΔC2AB (right panel) in the membrane. Membrane fractions were treated with water (lanes 1 and 4), the cross-linking reagent Sulfo-BSOOCES (lanes 2 and 5), or Sulfo-MBS (lane 3), as described under "Experimental Procedures." After treatment, samples were suspended with SDS sample buffer, analyzed by SDS-PAGE, and then immunoblotted with HRP-conjugated anti-T7 tag antibody (31). Note that the Syt IΔN/ΔC2AB proteins shifted from the monomer range to the top of the gel (asterisk) but the Syt IΔN/ΔC2AB(CA) proteins form smaller oligomers (one to five molecules). The positions of the molecular weight markers (× 10^-3) are shown on the left.
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**Fig. 3. Alignment of the amino-terminal domains of the mouse synaptotagmin family (Syts I-XIII).** Cys residues are shown on a black background. The number signs indicate the conserved Cys residues only in the extracellular domain of Syts III, V, VI, and X (31), which are involved in disulfide bonding. The transmembrane domain (TM) is indicated by a box. The numbers in parentheses indicate the total number of Cys residues in the transmembrane domain and the amino-terminal spacer domain. Numbers of amino acid are indicated on the right.

**Fig. 4.** The Cys cluster at the interface between the transmembrane and spacer domains is involved in SDS-resistant oligomerization of the synaptotagmin family. A, T7-Syts II, I, VIII, VII, IV, and XII proteins were expressed in COS-7 cells and homogenized in 1% SDS with a 27-gauge syringe. After addition of SDS sample buffer (+β-mercaptoethanol), the solubilized proteins were boiled for 3 min, subjected to 7.5% SDS-PAGE, and immunoblotted with HRP-conjugated anti-T7 tag antibody (1/10,000 dilution). Note that the SDS-resistant dimerizing potencies closely correlated with the number of Cys residues (parentheses after their names). Prolonged exposure of the x-ray film also allowed detection of the SDS-resistant dimer of Syt IV, VIII form stable SDS- and β-mercaptoethanol-resistant homodimers on SDS-PAGE by unknown mechanisms (21, 31). The first step in understanding the structural basis of the Ca^{2+}-independent oligomerization of Syt I is to determine the key amino acids (or effector domain) for oligomerization. To identify the domain responsible for the SDS-resistant Ca^{2+}-independent self-oligomerization of Syt I, we produced various T7-tagged Syt I deletion mutants (Fig. 1A), then co-expressed each T7-tagged mutant with the FLAG-Syt I in COS-7 cells and evaluated the associations between the T7- and FLAG-tagged proteins by immunoprecipitation, as described previously (21, 31). In brief, T7-Syt proteins were immunoprecipitated with anti-T7 tag antibody-conjugated agarose in the presence of 2 mM EGTA. The co-immunoprecipitated FLAG-Syt I proteins were first detected by HRP-conjugated anti-FLAG antibodies (Fig. 1B, upper panel), and the same blot was then stripped and reprobed with HRP-conjugated anti-T7 tag antibodies (Fig. 1B, lower panel). With the exception of T7-Syt I-lspacer (deletion of the whole spacer domain; Fig. 1B, upper panel, lane 5), all the deletion mutants interacted with FLAG-Syt I, although the T7-Syt I-lcyto (deletion of the cytoplasmic domain) weakly interacted with FLAG-Syt I (Fig. 1B, upper panel, lane 2). Consistent with this, we could not detect any SDS-resistant homodimeric band of T7-Syt I-lspacer, whereas the other T7-tagged mutant proteins formed SDS-resistant homo-oligomers (Fig. 1B, lower panel, asterisks). This finding indicated that the amino-terminal spacer domain (or interface sequence between the transmembrane and spacer domains) is essential for Ca^{2+}-independent self-oligomerization of Syt I. Because five Cys residues are present in this region (Fig. 1A, boldface letters) and they are thought to be fatty-acylated (Fig. 1A, arrow and Refs. 37 and 38), we first focused on the possible involvement of these Cys residues in Ca^{2+}-independent self-oligomerization of Syt I, and indeed, found a close correlation between the number of Cys residues in the Syt I deletion mutants and their capacity for self-oligomerization (Fig. 1A).

The Cys Cluster of the Synaptotagmin Family Is Essential for SDS-resistant Ca^{2+}-independent Self-oligomerization—Ala-based site-directed mutagenesis was performed to investigate whether the Cys residues are directly involved in the Ca^{2+}-independent self-oligomerization capacity of Syt I (Fig. 2A). Substitution of Ala for the five Cys residues (CA mutant) resulted in a shift in molecular weight to a smaller value than that of the wild-type protein on SDS-PAGE (Fig. 2B, lanes 1 and 2), whereas the molecular weight shift in full-length Cys to Ala single mutant (C74A, C75A, C77A, C79A, or C82A) was so small that we could not determine whether each of the Cys residues was fatty-acylated (Fig. 2B, lanes 3–7). To clarify the post-translational modification of each Cys residue, T7-Syt IΔN/ΔC2AB proteins lacking both the amino-terminal domain and two C2 domains were used (Fig. 1A). As shown in Fig. 2C, T7-Syt IΔN/ΔC2AB(CA) mutant proteins yielded a single band (lane 2, arrowhead), whereas the other proteins showed broad bands (two major bands, see arrows and arrowhead). Because
the lowest band of the wild-type and the single Cys to Ala mutants correspond to the band of the CA mutant (arrowhead in Fig. 2C), they are probably unmodified proteins. The apparent molecular weight of the highest band of the single Cys to Ala mutants was clearly smaller than that of the wild-type protein (compare open and closed arrows in Fig. 2C). When the wild-type protein bands were analyzed in a higher magnification, six bands could be detected (two major and four minor bands; arrowheads in Fig. 2D), indicating that all five Cys residues are post-translationally modified (fatty-acylated), as described previously (37, 38). It is noteworthy that the SDS-resistant oligomer of CA mutants was not detected even when the x-ray film was overexposed (Fig. 2C, left panel) and that the SDS-resistant oligomerizing activity of single Cys to Ala mutants (dimer/monomer ratio is about 0.05) was significantly reduced as compared with the wild-type proteins (dimer/monomer ration, about 0.25). Under our experimental conditions, we could detect the SDS-resistant pentamer of Syt I on 10% SDS-polyacrylamide gel (Fig. 2C, left panel). These results strongly suggest that the strength of the SDS-resistant oligomer depends on the number of Cys residues that are fatty-acylated.

To further examine whether Syt I oligomers form in intact membranes, a cross-linking experiment was performed (see “Experimental Procedures” for details) (39). Following treatment of membrane fractions with either water-soluble, cleavable, homobifunctional cross-linker (Sulfo-BSOCOES) or water-soluble, noncleavable, heterobifunctional cross-linker (Sulfo-MBS), the Syt I(A)(C2AB(CA)), we could not detect such a large oligomer (Fig. 2E, lanes 1, 4, and 5). Thus, we concluded that Syt I proteins indeed form a large oligomer even in the membranes.

The Syt cluster at the interface between the transmembrane and spacer domains is known to be present in all Syt isoforms except Syt XII, but the number of Cys residues differs with each isoform (parentheses in Fig. 3). If the results obtained from Syt I Cys to Ala mutants are applied to other isoforms, SDS-resistant oligomer formation by other Syt isoforms should depend on the number of Cys residues at the interface between the transmembrane and spacer domains. As expected, we could easily detect the SDS-resistant dimer of Syt II (seven Cys), Syt I (five Cys), and Syt VIII (five Cys) (Fig. 4A). The SDS-resistant dimers of Syt VII (three Cys) also could be detected, but its activity was weaker than that of Syts I, II, and VIII (their apparent oligomerizing potencies were in the following order: Syt II > Syt I = Syt VIII > Syt VII >> Syt IV). By contrast, only a weak SDS-resistant dimer of Syt IV (two Cys) was detected.
even after prolonged exposure of the x-ray film, and no dimer band of Syt XII was detected (zero Cys).

To further confirm that the number of Cys residues determines SDS-resistant oligomer formation by the synaptotagmin family, we artificially introduced additional Cys residues into Syt IX at the interface between the transmembrane and spacer domains (named Syt IX+C1 and +C2; Fig. 4B). As expected, the potency of SDS-resistant dimer formation significantly increased as the number of Cys residues increased. It is noteworthy that an SDS-resistant trimer band was only observed with the Syt IX+C2 proteins (Fig. 4C, lane 3).

The Cys Cluster of Synaptotagmin I Is Essential for Stable SDS-resistant Ca\(^{2+}\)-independent Self-oligomerization and Hetero-oligomerization—In the next set of experiments, we investigated whether the Cys residues at the interface between the transmembrane and spacer domains of Syt I are the sole mechanism of oligomerization, because we could not rule out the possibility that Syt I also contains a second site that is involved in SDS-sensitive self-oligomerization. To determine whether it does, we performed a T7 and FLAG dual-tag co-immunoprecipitation assay, as described above. As shown in Fig. 5A, the self-oligomerization activity of the CA mutant was dramatically reduced as compared with the wild-type (lanes 1 and 3), but the CA mutant still showed oligomerization activity. Since the T7-Syt Δspacer proteins did not show Ca\(^{2+}\)-independent oligomerization (Fig. 1B), the spacer domain must also be involved in SDS-sensitive Ca\(^{2+}\)-independent oligomerization. We previously showed that Syt I hetero-oligomerizes with Syts I and VII when transiently co-expressed in COS-7 cells (16, 22). Since the Cys to Ala substitution of Syt I almost completely abolished the hetero-oligomerization of Syt I with Syts II and VII (Fig. 5B, third panel), the Cys cluster of Syt I was found to be essential for both stable Ca\(^{2+}\)-independent homo- and hetero-oligomerization.

Synaptotagmin I Forms an Oligomer on Gel Filtration Columns—In the final set of experiments, we used gel filtration column chromatography (see “Experimental Procedures” for details) to determine how many Syt I molecules assemble via the Cys cluster or around the spacer domain. As shown in Fig. 6 (upper panel), the Syt IΔN/ΔC2AB proteins were eluted at around 60–100 kDa. The SDS-resistant oligomer (dimer or trimer) on SDS-polyacrylamide gel seemed to be eluted faster than the monomer (arrows in upper panel), whereas the mutant Syt IΔN/ΔC2AB/CA proteins were eluted significantly slower than the wild-type protein on the same gel filtration column (Fig. 6, lower panel; around 80–40 kDa), consistent with the results of immunoprecipitation described above. Because the calculated molecular weight of both the wild-type and mutant Syt IΔN/ΔC2AB proteins is about 10,000, a maximum of six to ten molecules of Syt IΔN/ΔC2AB is estimated to have clustered, as opposed to a maximum of four to eight mutant proteins. These results, together with the immunoprecipitation results described above, strongly indicate that the spacer domain is essential for oligomerization of Syt I molecules (four to eight molecules) and that the fatty-acylated Cys cluster augments their oligomerization capacity, enabling them to form higher oligomers (six to ten molecules).

**DISCUSSION**

In the early 1990s, Syt I was shown to form an SDS-resistant oligomer, and it was hypothesized that it functioned in an oligomerized state during neurotransmitter release based on the results of a genetic analysis of Drosophila synaptotagmin mutants (18, 19). Several potential mechanisms for Syt I oligomerization (e.g. amphipathic α-helix in the spacer domain or disulfide bonding) (19, 40) were proposed, but the exact mechanism was never determined during the last decade. In this study, we first demonstrated that the post-translationally modified (i.e. fatty-acylated (37, 38)) Cys residues (Cys-74, Cys-75, Cys-77, Cys-79, and Cys-82) at the interface between the transmembrane and spacer domains of Syt I are essential for stable SDS-resistant self-oligomerization by Ala-based site-directed mutagenesis (Figs. 2 and 5). Our finding, that the number of modified Cys residues determines the strength of SDS-resistant oligomerization by Syt I, provides an explanation for the distinct self-oligomerization activity of the synaptotagmin family, as described previously (21, 22). The number of Cys residues in the mouse synaptotagmin isoforms (Syts I-XIII) at the interface between the transmembrane and spacer domains differs from zero to seven (Fig. 3). Syts I, II, VII, and VIII form stable SDS-resistant Ca\(^{2+}\)-independent oligomers via the modified Cys clusters (more than three Cys residues), whereas Syts IV, IX, and XI form rather weak SDS-resistant Ca\(^{2+}\)-independent oligomers, because they contain only two or three Cys residues, and Syt XII, which completely lacks Cys residues, did not form any SDS-resistant oligomers. Syts III, V, VI, and X are exceptional, because Syts III, V, and VI stably oligomerize by forming disulfide bonds in the extracellular domain rather than by their few fatty-acylated Cys residues (only two Cys residues) (31), and both disulfide bonding and three Cys residues at the interface between the transmembrane and spacer domains equally contribute to oligomer formation by Syt X.\(^2\)

We also demonstrated that the spacer domain of Syt I is involved in SDS-sensitive oligomerization by gel filtration column chromatography (Fig. 6). Due to the relatively weak interaction, such SDS-sensitive oligomers probably dissociate into monomers after extensive washing of the immunoprecipitants (Fig. 5A). Because the spacer domain is a highly diversified region among the synaptotagmin family (1), we speculate that it is unlikely to be a common module that contributes to homo- and hetero-oligomerization in the synaptotagmin family.

Because the synaptotagmin isoforms, except Syt XII, have Cys clusters at the interface between the transmembrane and spacer domains, are they (Syts I-XI, and XIII) able to form stable hetero-oligomers via the modified Cys residues in all possible combinations? Although certainly possible even in vivo, we noted certain limitations to hetero-oligomerization in the synaptotagmin family. First, once oligomerization occurs via fatty-acylated Cys clusters, it is highly stable, because T7-Syt and FLAG-Syt associations are only observed by cotransfection assay, not in mixtures in which they are expressed separately (21). Because of this, hetero-oligomerization via the fatty-acylated Cys cluster should occur in the endoplasmic reticulum membrane immediately after two newly synthesized proteins are inserted into the endoplasmic reticulum membrane and are fatty-acylated (41). However, because the spatio-temporal expression of several synaptotagmins is different (42-44), hetero-oligomerization of the isoforms may be limited in vivo. Second, synaptotagmin isoforms containing a high number of Cys residues seem to preferentially assemble with their own isoforms rather than with synaptotagmin isoforms having a low number of Cys residues. For instance, hetero-oligomerization of Syt I (five Cys) with Syt II (seven Cys) can easily be detected both in the brain (26) and as a result of overexpression in COS-7 cells (22), whereas hetero-oligomerization of Syt I (five Cys) with Syt IV (two Cys) was difficult to detect even in the overexpression study (22). Third, relative expression of each isoform is another important factor for hetero-oligomerization, because oligomerization is a concentration-dependent reaction. In our preliminary experiments, hetero-oligomerization of the synaptotagmin family proved to be more limited

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\(^2\) M. Fukuda, unpublished observations.
than we expected (22, 27), because Syts I and II are the predominant isoforms in the brain and the others are minor components. 2 Further work is necessary to elucidate whether hetero-oligomerization of Syt I with other isoforms (e.g., Syt VII) can modulate the Ca\textsuperscript{2+}-sensing function of Syt I.

In summary, we have shown that fatty-acylation of Cys clusters at the interface between the transmembrane and spacer domains of Syt I is essential for stable homo- and hetero-oligomerization and that the number of the Cys residues is the primary determinant of the SDS-resistant oligomerization activities of the synaptotagmin family. This stable oligomerization enables efficient Ca\textsuperscript{2+}-dependent C2B clustering in response to rapid increases in Ca\textsuperscript{2+} ions.

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Mechanism of the SDS-resistant Synaptotagmin Clustering Mediated by the Cysteine Cluster at the Interface between the Transmembrane and Spacer Domains
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