J. Biochem. 128, 637–645 (2000)

Calcium-Dependent and -Independent Hetero-Oligomerization in the Synaptotagmin Family[1]

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Received June 29, 2000; accepted August 2, 2000

Synaptotagmins constitute a family of membrane proteins that are characterized by one transmembrane region and two C2 domains. Recent genetic and biochemical studies have indicated that oligomerization of synaptotagmin (Syt) I is important for expression of function during exocytosis of synaptic vesicles. However, little is known about hetero-oligomerization in the synaptotagmin family. In this study, we showed that the synaptotagmin family is a type I membrane protein ($N_{lumen}/C_{cytoplasm}$) by introducing an artificial *N*-glycosylation site at the N-terminal domain, and systematically examined all the possible combinations of hetero-oligomerization among synaptotagmin family proteins (Syts I–XI). We classified the synaptotagmin family into four distinct groups based on differences in Ca^{2+}-dependent and -independent oligomerization activity. Group A Syts (III, V, VI, and X) form strong homo- and hetero-oligomers by disulfide bonds at an N-terminal cysteine motif irrespective of the presence of Ca^{2+} [Fukuda, M., Kanno, E., and Mikoshiba, K. (1999) *J. Biol. Chem.* 274, 31421–31427]. Group B Syts (I, II, VIII, and XI) show moderate homo-oligomerization irrespective of the presence of Ca^{2+}. Group C synaptotagmins are characterized by weak Ca^{2+}-dependent (Syts IX) or no homo-oligomerization activity (Syt IV). Syt VII (Group D) has unique Ca^{2+}-dependent homo-oligomerization properties with EC_{50} values of about 150 μM Ca^{2+} [Fukuda, M., and Mikoshiba, K. (2000) *J. Biol. Chem.* 275, 28180–28185]. Syts IV, VIII, and XI did not show any apparent hetero-oligomerization activity, but some sets of synaptotagmin isoforms can hetero-oligomerize in a Ca^{2+}-dependent and/or -independent manner. Our data suggest that Ca^{2+}-dependent and -independent hetero-oligomerization of synaptotagmins may create a variety of Ca^{2+}-sensors.

Key words: Ca^{2+} sensor, C2 domain, exocytosis, self-oligomerization, synaptotagmin.

The synaptotagmin (Syt) family comprises evolutionarily conserved proteins that are thought to be involved in vesicular trafficking including synaptic vesicle exocytosis (reviewed in Refs. *1–3*). They are characterized by a short amino (N)-terminus, a single transmembrane region, and two highly conserved C2 domains (named the C2A and C2B domains) in the large carboxyl terminus. The C2 domains of Syt I, a well characterized isoform, are essential for synaptic vesicle exocytosis and endocytosis (*4–9* and reviewed in Refs. *1–3*) and this region is likely to be functional in all synaptotagmin family proteins. To date, 12 synaptotagmin isoforms have been described in rat and mouse (Ref. *10* and references therein), and 7 and 5 isoforms respecfively in *Drosophila* and *Caenorhabditis elegans* (*11, 12*). These isoforms can be divided into subclasses based on the phylogenetic relationship (*10*), gene structures (*13*) and differences in the biochemical natures of the C2 domains, such as Ca^{2+}-dependent phospholipid or syntaxin binding to the C2A domain (*14–16*), and inositol 1,3,4,5-tetrakisphosphate binding to the C2B domain (*17–19*).

Syts III, V, VI, and X form a small branch in the phylogenetic tree (*10*). This class of synaptotagmins form hetero-oligomers through the N-terminal cysteine motif by disulfide bonding (*10*) and, except for Syt III, interact with Syt VII in a Ca^{2+}-dependent manner at the cytoplasmic domain (*20*). Syts III, V, VI, and X are also characterized by weak or no inositol 1,3,4,5-tetrakisphosphate binding activity (*19*). Syts IV and XI are another class of synaptotagmins, characterized by deficient phospholipid (phosphatidylcholine/phosphatidylserine, 1/1 liposome) binding due to the substitution of Ser for Asp at position 244 (or 247) of Syt IV (or XI) (*14, 16*). Syts I, II and IX also form a small branch in the phylogenetic tree (*10*). Although the functions of the other classes of synaptotagmins largely remain unknown,

[1] This work was supported in part by grants from the Science and Technology Agency of Japan (to K.M.) and from the Ministry of Education, Science, Sports and Culture of Japan [to K.M., and M.F. (11780571 and 12053274)].

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Abbreviations: cyto, cytoplasm; HRP, horseradish peroxidase; lum, lumen; M_r, molecular weight; N, amino; *N*-Gly, *N*-glycosylation; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; Syt(s), synaptotagmin(s).

Syt I (or II) is thought to function in an oligomerized state, probably as a Ca^{2+}-sensor for neurotransmitter release on the basis of the genetic analysis of *Drosophila* synaptotagmin mutants (reviewed in Ref. *21*) and *in vitro* biochemical studies (*20*, *22–26*). Syt I (or II) shows two self-oligomerization properties: A SDS-resistant Ca^{2+}-independent multimerization probably mediated by a region just downstream of the transmembrane region (*10*, *20*, *27*, *28*) and a Ca^{2+}-dependent oligomerization mediated by the C2B domain (*22–26*). Since several other isoforms also showed Ca^{2+}-dependent and -independent homo-oligomerization properties (*10*, *20*), we thought that it would be interesting to determine whether Syt I or II can hetero-oligomerize with other isoforms and whether the hetero-oligomerization of different classes of synaptotagmins would create a variety of Ca^{2+}-sensors.

In the present study, we determined the membrane topology of synaptotagmin isoforms (Syts III–XI), and then systematically examined the homo- and hetero-oligomerization properties of synaptotagmin family (Syts I–XI) by using two different epitope (T7 and FLAG)-tagged synaptotagmins.

EXPERIMENTAL PROCEDURES

Materials—AmpliTaq DNA polymerase and restriction enzymes were obtained from PE Biosystems (Foster City, CA, USA) and Toyobo Biochemicals (Tokyo), respectively. Polyclonal and monoclonal antibodies (M2) against FLAG peptide were obtained from Zymed Laboratories (San Francisco, CA, USA) and Sigma (St. Louis, MO, USA), respectively. Horseradish peroxidase (HRP)–conjugated anti-T7 tag antibody was from Novagen (Madison, WI, USA). 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 Biocel A10 System (Millipore Bedford, MA, USA).

Construction of T7-N-Gly–Tagged Synaptotagmins III–XI—cDNA encoding the T7-*N*-Gly-tag [MASMTGGQQMG-**RNGS**; T7-tag underlined and *N*-glycosylation (*N*-Gly) site in bold letters] was amplified from pGEM-T-T7-Syt IX as a template (*10*) by polymerase chain reaction (PCR) using the following oligonucleotides: T7 primer (sense) and 5′-*CGGATCCGTTGCGACCCATTTGCTGTCC-3′* (*antisense*). Reactions were carried out for 25 cycles, each consisting of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. The PCR products, purified from an agarose gel by MicroSpin Column (Amersham Pharmacia Biotech; Buckinghamshire, UK), were digested with *Apa*I and *Bam*HI (italics above) and then substituted for the *Apa*I–*Bam*HI insert of pGEM-T-T7-Syt IX (*10*). The resulting pGEM-T-T7-*N*-Gly-Syt IX plasmid was verified by DNA sequencing using a Hitachi SQ-5500 DNA sequencer. Other plasmids encoding the T7-*N*-Gly–tagged Syts III-VIII, X, and XI were similarly constructed by this method. The full length synaptotagmin inserts with a T7-*N*-Gly tag were excised from the pGEM-T Easy vector (Promega; Madison, WI, USA) by *Not*I digestion and subcloned into the *Not*I site of pEF-BOS (*29*, *30*) (pEF-T7-*N*-Gly-Syts III–XI). pEF-T7(or FLAG)-Syts I–XI and -Syts I–XI-cyto were prepared as described previously (*10*, *20*). The plasmid DNA was prepared by using Wizard-mini preps (Promega) or a QIAGEN Maxi prep kits.

N-Glycosidase F Digestion of T7-Synaptotagmins I, II, and T7-N-Gly-Synaptotagmins III–XI—Recombinant T7-Syts I, II, and T7-*N*-Gly-Syts III–XI expressed in COS-7 cells (5×10^5 cells, the day before transfection/10 cm dish) were immunoprecipitated by anti-T7 tag antibody–conjugated agarose (wet volume 30 μl, Novagen) as described previously (*10*). Beads were resuspended in 100 μl of 20 mM HEPES-KOH, pH 7.2, 0.1% SDS, 0.5% IGEPAL CA-630 (Sigma), 20 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin and 10 μM pepstatin A, and then divided into two microtubes. After denaturation by boiling for 3 min, and cooling to 37°C, one unit of *N*-glycosidase F (Roche Diagnostics, Mannheim, Germany) was added to one tube, and the mixtures were incubated for 1 h at 37°C. Reactions were stopped by adding SDS sample buffer and boiling for 3 min. Proteins were subjected to 10% SDS–polyacrylamide gel electrophoresis (PAGE) and immunoblotting as described elsewhere (*10*).

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 (*10*). Three days after transfection, cells were harvested, and proteins were solubilized with 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. Immunoprecipitation of T7-Syts by anti–T7 tag antibody–conjugated agarose, SDS-PAGE, and immunoblotting analyses were also performed as described previously (*10*).

RESULTS

Membrane Topology of Mouse Synaptotagmins I–XI—Synaptotagmin family proteins have a single transmembrane region with a short N-terminus and a large carboxyl terminus containing tandem C2 domains. In vertebrates, Syts I and II have a single potential *N*-glycosylation site at the N-terminus, and this site has been shown to be *N*-glycosylated by *N*-glycosidase F digestion (*27*, *29*) or site-directed mutagenesis (*31*). In addition, antibodies against the N-terminal domain of Syt I were incorporated into hippocampal neurons by endocytosis after membrane depolarization (*32–34*), and microinjection of antibodies against the C2A domain of Syt I into squid giant presynapse visualized synaptic vesicles (*4*). Based on these results, the synaptotagmin family is thought to comprise type I membrane proteins, showing $N_{lumen\ (or\ extracellular\ domain)}/C_{cytoplasm}$ (N_{lum}/C_{cyto}) orientation. However, the Syt isoforms other than Syts I and II do not contain an *N*-glycosylation site at the N-terminus upstream of the transmembrane region (*10*), and therefore there is no direct evidence that other isoforms also show the same N_{lum}/C_{cyto} orientation. To address this issue, we introduced an artificial *N*-glycosylation site (Asn-Gly-Ser) into the N-terminus of each synaptotagmin isoform (T7-*N*-Gly-Syts III–XI) (Fig. 1A). We first confirmed that addition of a short tag (T7, T7-*N*-Gly or FLAG) to the N-terminus did not affect the subcellular localization of several isoforms in PC12 cells (Fukuda, M., unpublished data), so that such N-terminal tagging is unlikely to affect the membrane topology. T7-Syts I, II, and T7-*N*-Gly-Syts III–XI proteins were transiently expressed in COS-7 cells and immunoprecipitated by anti–T7 tag antibody–conju-

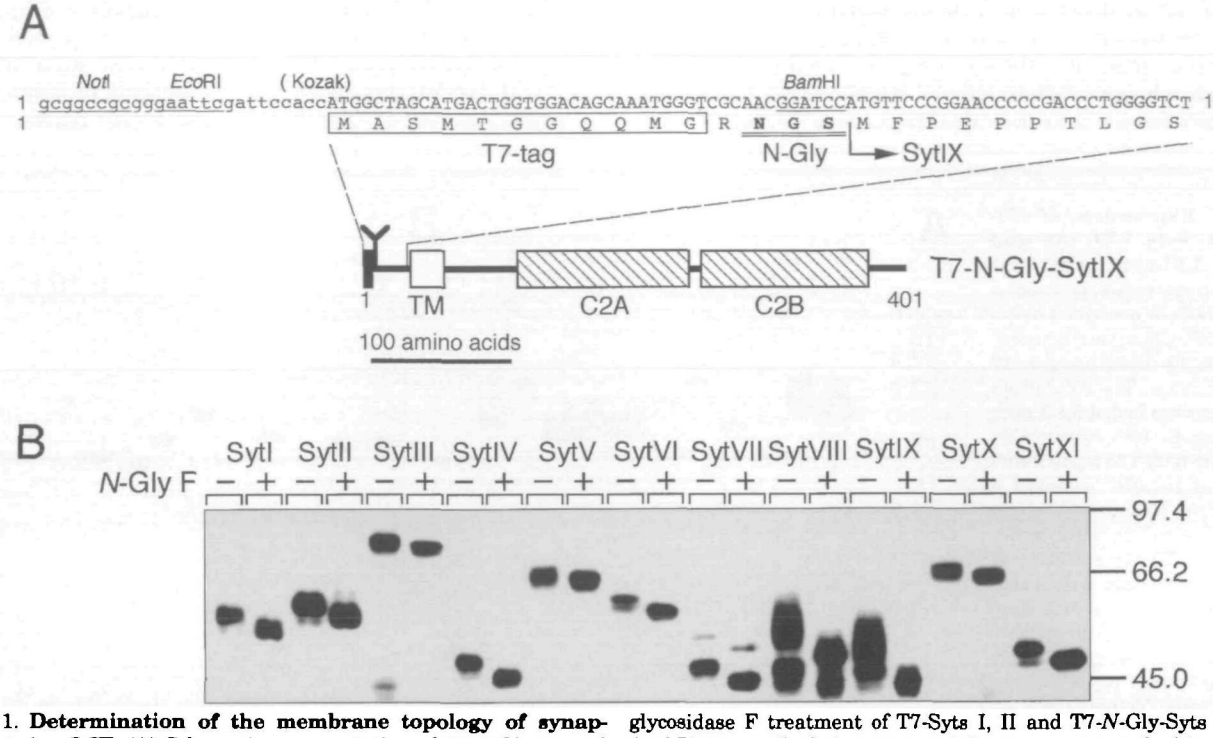

Fig. 1. **Determination of the membrane topology of synaptotagmins I–XI.** (A) Schematic representation of T7-*N*-Gly–tagged Syt IX. Top indicates the nucleotide and amino acid sequences around the T7-*N*-Gly-tag. T7-tag and *N*-glycosylation sites (*N*-Gly) are shown by open box and double lines, respectively. Non-coding regions and the major restriction enzyme sites are shown by small letters and underlining, respectively. Nucleotide and amino acid numbers are given on both sides. Bottom is a schematic representation of T7-*N*-Gly-Syt IX: T7-tag (black box), *N*-glycosylation (Y), transmembrane region (TM; open box), and two C2 domains (C2A and C2B; hatched boxes). (B) *N*-glycosidase F treatment of T7-Syts I, II and T7-*N*-Gly-Syts III–XI. Anti-T7 tag antibody immunoprecipitants were treated with or without *N*-glycosidase F and analyzed by immunoblotting with HRP-conjugated anti-T7 tag antibody (1:1,000 dilution). Note that the apparent M_r of all synaptotagmin isoforms was decreased by *N*-glycosidase F treatment. A proportion of *N*-linked sugars of T7-*N*-Gly-Syts VIII and IX were further converted to a complex form because they showed broad bands and were resistant to endoglycosidase H treatment (data not shown). The positions of M_r markers ($\times 10^{-3}$) are shown on the right.

gated agarose. Although several isoforms (*e.g.*, Syt VII) showed heterogeneous bands which probably reflect posttranslational modifications, the apparent molecular weight (M_r) of expressed T7-*N*-Gly-Syts III–XI was larger than that of T7-Syts III–XI (data not shown), suggesting that T7-*N*-Gly-Syts III–XI were *N*-glycosylated. To confirm this, the beads were then treated with *N*-glycosidase F, which cleaves the *N*-linked sugar, and the M_r of each isoform with or without *N*-glycosidase F treatment was compared by immunoblotting. As shown in Fig. 1B, the apparent M_r of all isoforms decreased when they were treated with *N*-glycosidase F, indicating that all of the synaptotagmins show N_{lum}/C_{cyto} orientation. These results indicate that studies on the hetero-oligomerization between synaptotagmin isoforms *via* C2 domains are meaningful because all isoforms show the same membrane topology.

Ca^{2+}-Dependent and -Independent Hetero-Oligomerization of Synaptotagmin Isoforms—To examine the Ca^{2+}-dependent and -independent hetero-oligomerization of synaptotagmin isoforms, we used a T7- and FLAG-tagged Syts co-expression assay as described previously (*10*). Briefly, T7- and FLAG-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 500 μM Ca^{2+}. This assay system has three merits. First, only *in vivo* interactions in the cells seem to be detected, because when T7- and FLAG-Syts were separately expressed and mixed after solubilization, T7- and FLAG-Syts interactions were in many cases not detectable (*10*, *20*). Second, since native synaptotagmins undergo posttranslational modifications such as palmitoylation (*35*, *36*) or disulfide bond formation (*10*), it would be better to use a mammalian rather than bacterial expression system. Third, significant levels of endogenous Syts I–XI are not expressed in COS-7 cells (Fukuda, M., unpublished data), so that the involvement of endogenously expressed Syts in oligomerization need not be considered. The only problem with this assay is that the expression levels of the two isoforms may be very different. The amounts of each immunoprecipitant from each isoform were different even when the same amount of plasmid was transfected (Fig. 2). For instance, Syts I, II, VIII, and IX were expressed at relatively high levels in COS-7 cells, Syts VII and XI were moderately expressed, but the expression levels of Syts III–VI, and X were at least 5–10 times lower than that of Syt I (Fig. 2A). To overcome this, we initially analyzed 1/10 the volume of immunoprecipitants by 10% SDS-PAGE and normalized them based on the immunoreactivity of anti-T7 tag antibodies in immunoblotting. Then, equivalent amounts of anti-T7 tag antibody immunoprecipitants were subjected to 10% SDS-PAGE and analyzed by immunoblotting using anti-FLAG rabbit antibody (Fig. 3, upper four panels and summarized in Table I). To confirm the amounts of anti-T7 tag antibody immunoprecipitants, the blots were stripped and reprobed with HRP-conjugated

anti-T7 tag antibody (Fig. 3, bottom panel).

The results are summarized in Table I, and representative immunoblots are shown in Fig. 3. Based on the differences in oligomerization activity, we classified the synaptotagmin family into four distinct groups. Group A Syts (III, V, VI, and X) formed stable hetero-oligomers with each other irrespective of the presence of Ca^{2+} (*10*). In addition, except for Syt III, group A synaptotagmins strongly associated with Syt VII (group D) in a Ca^{2+}-dependent manner as described previously (Fig. 3, third and fourth panels) (*20*).

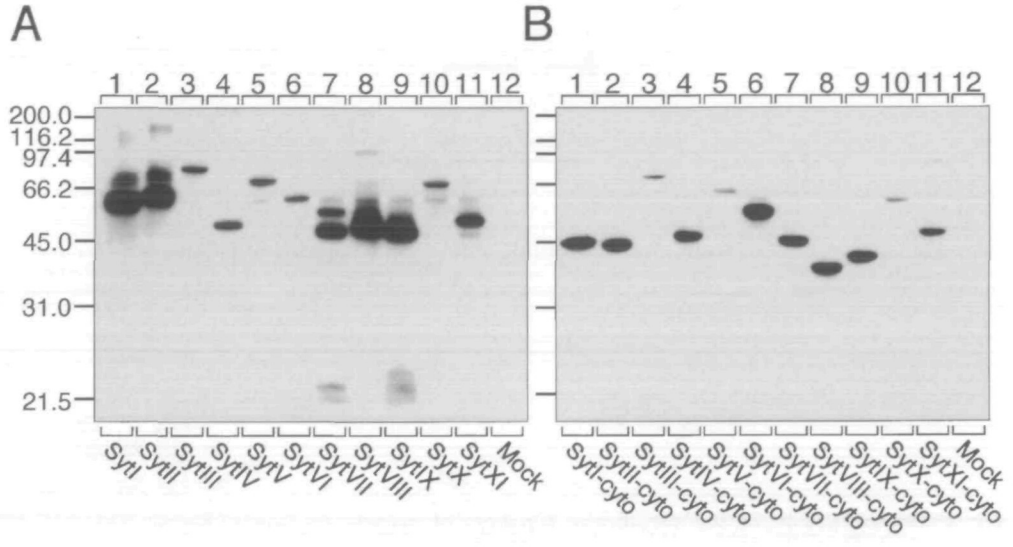

Fig. 2. **Expression of T7-tagged Syts I-XI (A) and -Syts I-XI-cyto (B).** T7-tagged Syts were expressed in COS-7 cells as described previously (*10*). Cells were homogenized in 1% SDS using a 27 gauge syringe. The solubilized proteins were boiled for 3 min, subjected to 10% SDS-PAGE, and transferred to a polyvinylidene difluoride membrane (Millipore). Expressed T7-Syts were detected by HRP-conjugated anti-T7 tag antibody (1/1,000 dilution). Since the exposure time of (B) is less than half of that in (A), comparable amounts of Syt cytoplasmic domains were expressed in COS-7 cells. Notably, Syts IV and VI cytoplasmic domains were more highly expressed than full length proteins. Similar protein expression levels were observed in the case of FLAG-Syts I-XI (data not shown). The positions of M_r markers ($\times 10^{-3}$) are shown on the left. Lanes 1–11, T7-Syts I–XI (or I–XI-cyto) and lane 12, vector (pEF-BOS) transfected control.

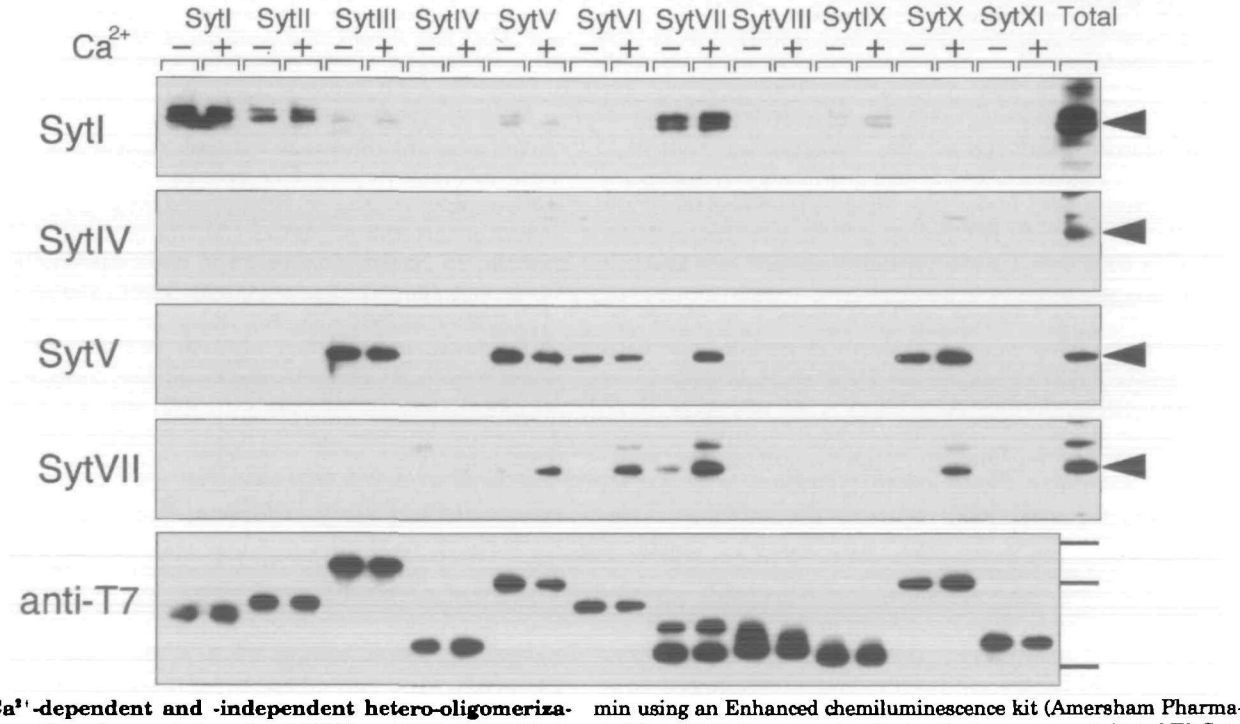

Fig. 3. **Ca^{2+}-dependent and -independent hetero-oligomerization properties of synaptotagmins I, IV, V, and VII.** pEF-T7-Syts I–XI and pEF-FLAG-Syt I (Group B), -Syt IV (group C), -Syt V (Group A), or -Syt VII (Group D) were co-transfected into COS-7 cells, and recombinant proteins were immunoprecipitated 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 exposure to X-ray film (X-OMATAR, Kodak) for 2.5 min using an Enhanced chemiluminescence kit (Amersham Pharmacia Biotech). Bottom panel indicates the immunoprecipitated T7-Syts I-XI of the third panel from the top visualized by HRP-conjugated anti-T7 tag antibody. Total (right most lane) includes 1/40 volume of reaction mixtures used for immunoprecipitation. Arrowheads indicate the positions of the FLAG-Syt I, IV, V, or VII. Bars shown on the right are M_r markers, corresponding to 97.4, 66.2, and 45.0 kDa from the top, respectively.

T7-Syt V was shown to interact well with FLAG-Syt II irrespective of the presence of Ca^{2+} (Table I). In contrast, T7-Syt II–FLAG-Syt V interactions were difficult to detect unless a large amount of T7-Syt II immunoprecipitant was loaded on SDS-PAGE. This was probably due to the difference in expression levels of the two molecules; the amount of Syt II expressed was at least 10 times greater than that of Syt V (Fig. 2A), and accordingly T7-Syt II homo-oligomers were predominantly immunoprecipitated rather than T7-Syt II–FLAG-Syt V hetero-oligomers. Group B was composed of Syts I, II, VIII, and XI, which form homo-oligomers irrespective of the presence of Ca^{2+}. Syts I and II associated with each other irrespective of the presence of Ca^{2+} (Fig. 3, upper panel), consistent with the previous report that

TABLE I. **Summary of Ca^{2+}-dependent and -independent oligomerization between synaptotagmins I–XI.**

	T7-Syt I		T7-Syt II		T7-Syt III		T7-Syt IV		T7-Syt V		T7-Syt VI	
	–	+	–	+	–	+	–	+	–	+	–	+
FLAG-Syt I	++±	++±	++	++±	+	+	–	–	+	+	–	–
FLAG-Syt II	+++	+++	+++	+++±	++	++	++	++	++++	++++	+++	+++
FLAG-Syt III	–	–	–	–	++++	++++	–	–	++++	++++	++++	++++
FLAG-Syt IV	–	–	–	–	–	–	–	–	–	–	–	–
FLAG-Syt V	–	–	–	–	++++	++++	–	–	++++	++++	++++	++++
FLAG-Syt VI	–	–	–	–	++++	++++	–	–	++++	++++	++++	++++
FLAG-Syt VII	–	–	–	–	+	+	+	+	+	+++	+	+++
FLAG-Syt VIII	–	–	–	–	+	+	+	+	+	+	+	+
FLAG-Syt IX	–	+	–	+	+	+	+	+	+	+±	+	+
FLAG-Syt X	–	–	–	–	++++	++++	–	–	++++	++++	++++	++++
FLAG-Syt XI	–	–	–	–	+	+	+	+	+	+	+	+

	T7-Syt VII		T7-Syt VIII		T7-Syt IX		T7-Syt X		T7-Syt XI	
	–	+	–	+	–	+	–	+	–	+
FLAG-Syt I	+++	+++±	+	+	+	+±	–	–	–	–
FLAG-Syt II	+++	++++	+	+	+	+++	++	++	+±	+±
FLAG-Syt III	–	–	–	–	–	–	++++	++++	–	–
FLAG-Syt IV	–	–	–	–	–	–	–	–	–	–
FLAG-Syt V	–	++++	–	–	–	–	++++	++++	–	–
FLAG-Syt VI	–	+++	–	–	–	–	++++	++++	–	–
FLAG-Syt VII	++±	++++	–	+	–	+	+	+++	+	+
FLAG-Syt VIII	–	+	+++	+++	–	+	+	+	–	–
FLAG-Syt IX	+	+±	+	+	+	+±	+	+	–	–
FLAG-Syt X	–	+++	–	–	–	–	++++	++++	–	–
FLAG-Syt XI	–	+	+	+	–	–	+	+	++	++

Ca^{2+}-dependent (500 μM) and -independent (2 mM EGTA) association of FLAG-Syts and T7-Syts were judged from at least two or three independent experiments (see immunoblots of Fig. 3). The FLAG-Syts background level was determined by using the immunoprecipitants from the cell extracts co-transfected with pEF-FLAG-Syts and a vector control. Oligomerization activities were mainly determined from X-ray films by eye, but in some cases, bands were captured by Gel Print 2000i/VGA (Bio Image) and quantified by Basic Quantifier software (Version 1.0) (Bio Image) as described previously (*20*). ++++, strong association, like group A Syts (Syts III, V, VI, and X). +++±, between "++++" and "+++." +++, moderate association like group B Syts (Syts II and VIII). ++±, between "+++" and "++", like Syt I homo-oligomerization. ++, weak association, like Syt XI homo-oligomerization. +±, between "++" and "+", like Syt IX homo-oligomerization in the presence of Ca^{2+}. +, very weak association, which was detected only after prolonged exposure to X-ray film. –, undetectable under our binding conditions, like Syt IV homo-oligomerization.

TABLE II. **Summary of Ca^{2+}-dependent oligomerization between the cytoplasmic domains of synaptotagmins I–XI.**

	T7-Syt I	T7-Syt II	T7-Syt III	T7-Syt IV	T7-Syt V	T7-Syt VI	T7-Syt VII	T7-Syt VIII	T7-Syt IX	T7-Syt X	T7-Syt XI
FLAG-Syt I	±	+	–	–	–	–	–	–	+	–	–
FLAG-Syt II	+	++[a]	–	–	–	–	+	–	±	–	–
FLAG-Syt III	ND	ND	±[a]	ND	++	±	±	ND	ND	±	ND
FLAG-Syt IV	ND	ND	ND	–	ND	ND	ND	ND	ND	ND	ND
FLAG-Syt V	–	–	+	–	+++[a]	±	++	–	±	+	–
FLAG-Syt VI	ND	ND	ND	ND	ND	±[a]	±	ND	ND	ND	ND
FLAG-Syt VII	±	+	–	–	+++	+++	+++	–	±	+++	–
FLAG-Syt VIII	ND	ND	ND	ND	ND	ND	ND	–	ND	ND	ND
FLAG-Syt IX	±	±	–	–	±	–	±	–	±	–	–
FLAG-Syt X	ND	ND	±	ND	±	–	±	ND	ND	±[a]	ND
FLAG-Syt XI	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	–

Ca^{2+}-dependent (500 μM) and -independent (2 mM EGTA) association of FLAG-Syts-cyto and T7-Syts-cyto were judged from at least two or three independent experiments. The FLAG-Syts-cyto background level was determined by using immunoprecipitants from cell extracts co-transfected with pEF-FLAG-Syts-cyto and a vector control. Oligomerization activities were mainly determined from X-ray films by eye, but in some cases, bands were captured by Gel Print 2000i/VGA (Bio Image) and quantified by Basic Quantifier software (Version 1.0) (Bio Image) as described previously (*20*). +++, strong Ca^{2+}-dependent association, like Syt VII homo-oligomerization. ++, moderate Ca^{2+}-dependent association, like Syt II homo-oligomerization. +, weak Ca^{2+}-dependent association, like Syt IX homo-oligomerization which was detected only after prolonged exposure to X-ray film. ±, very weak association, only when a large amount of immunoprecipitant was loaded on SDS-PAGE. –, undetectable under our binding conditions like Syt IV homo-oligomerization. ND, not determined because the full-length proteins did not show apparent hetero-oligomerization (see Table I). a, some fractions of oligomerization are Ca^{2+}-independent.

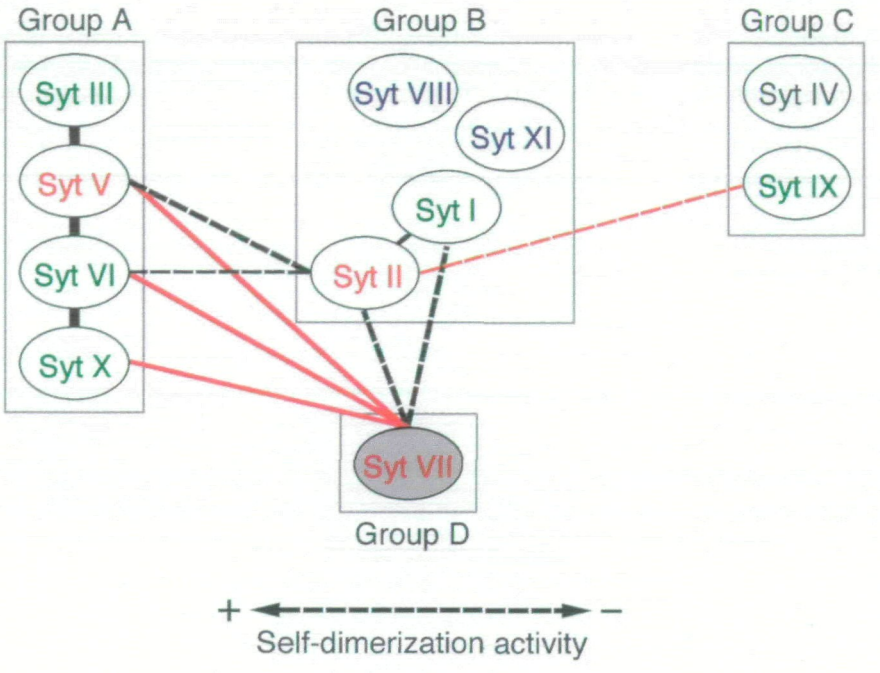

Fig. 4. Summary of Ca^{2+}-dependent and -independent oligomerization properties of synaptotagmins in COS-7 cells. Synaptotagmin isoforms are classified into four distinct groups according to their ability to self-oligomerize (Group A > D ≥ B > C; see text and Tables I and II). Isoforms that show strong or weak Ca^{2+}-dependent homo-oligomerization mediated by cytoplasmic domains are indicated by red or green letters, respectively. Syt VII is further shaded because it shows robust Ca^{2+}-dependent oligomerization even when the full-length proteins are used. Isoforms that do not essentially show hetero-oligomerization are indicated by blue letters (Syts VIII and XI). An isoform that does not essentially show homo-oligomerization is indicated by black letters (Syt IV). Lines indicate the hetero-oligomerization of two molecules and their thickness indicates the relative strength of the oligomerization, although the weak hetero-oligomerizations (*e.g.* "++" or "+" in Table I) are omitted in this figure. Hetero-oligomerization that was activated by Ca^{2+} is colored in red. Broken lines indicate that the interaction of two isoforms was detected in only one way (*e.g.*, T7-Syt V–FLAG-Syt II interaction).

native Syts I and II proteins from brain associate with each other (*26*). Syts I and II also associated with Syt VII, and these associations seemed to be slightly activated by Ca^{2+}. Compared to FLAG-Syt I, FLAG-Syt II interacted more broadly with other T7-tagged isoforms with various affinities (Table I). However, these interactions were not detected in reverse, probably due to the differences in expression level (see Fig. 2A). In contrast, Syts VIII and XI could self-oligomerize, but did not essentially interact with other isoforms. The group C Syt IV was mainly present as a monomer, though we cannot rule out the possibility that Syt IV oligomerization is sensitive to 1% Triton X-100 or 250 mM NaCl used for solubilization. Although the self-oligomerizing activity of Syt IX was quite weak, its association showed weak Ca^{2+}-dependency, which was evident at 500 μM Ca^{2+}, but not 250 μM Ca^{2+}. Syt IX also weakly interacted with Syts I, II, V, and VII at the cytoplasmic domain in a Ca^{2+}-dependent manner.

Ca^{2+}-Dependent Hetero-Oligomerization Properties of Cytoplasmic Domains from Synaptotagmins I–XI—In the final set of experiments, we examined the Ca^{2+}-dependent hetero-oligomerization properties of synaptotagmin cytoplasmic domains as demonstrated by the interaction of recombinant Syt I or II expressed in bacteria with detergent-solubilized native Syt I protein from brain (*22–26*). As compared to the full length protein, comparable or greater amounts of T7-(or FLAG)–Syts I–XI cytoplasmic domains were expressed in COS-7 cells (Fig. 2B). The results are summarized in Table II. Syt II could hetero-oligomerize with Syts I, VII, and IX, but not Syt V, though the full-length Syts II and V were strongly associated irrespective of the presence of Ca^{2+}. Syt V seemed to hetero-oligomerize equally well with Syt VII and weakly with Syts III, VI, IX, and X in a Ca^{2+}-dependent manner. The Syt VII cytoplasmic domain showed the broadest specificity and bound various Syts (I, II, V, VI, IX, and X) with different affinities.

DISCUSSION

In this study, we have demonstrated that synaptotagmin family proteins show type I membrane topology (N_{lum}/C_{cyto}) by introducing an artificial *N*-glycosylation site at the N-terminal domain, and that synaptotagmin isoforms show complex hetero-oligomerization properties by using two different epitope-tags (T7 and FLAG). As illustrated in Fig. 4, we classified the synaptotagmin family into four distinct groups based on their homo-oligomerization properties. Group A Syts (III, V, VI, and X) strongly associated with each other *via* the N-terminal cysteine motif by disulfide bonding (*10*), and accordingly their associations were Ca^{2+}-independent. At the cytoplasmic domain, however, these synaptotagmins can hetero-oligomerize with each other in a Ca^{2+}-dependent manner with differing affinities. This grouping is consistent with the previous phylogenetic analysis (*10*) and the weakness or absence of inositol 1,3,4,5-tetrakisphosphate binding ability (*19*).

Syts I, II, VIII, and XI were classed as group B and were characterized by Ca^{2+}-independent homo-oligomerization, which was insensitive to β-mercaptoethanol (*10, 20*). Although Syts I and II hetero-oligomerize at the N-terminus (Ca^{2+}-independent) as well as at the cytoplasmic domain (Ca^{2+}-dependent), Syt I itself showed very weak Ca^{2+}-dependent activity in our binding conditions. In addition, we found that Syt II can associate with a number of different isoforms, whereas Syt I only associated with a limited number of isoforms (*e.g.*, Syts II, VII, and IX). This finding is surprising because Syts I and II are thought to be functionally equivalent based on high sequence similarity (*10*), the fact that Syts I and II show almost complimentary distribution in brain (*37*), and the fact that the biochemical properties of the C2 domains tested so far are quite similar (*15–17, 38*). Since Syts I and II are thought to be Ca^{2+}-sensors

for neurotransmitter release (*1–3*), our results raise the possibility that their hetero-oligomerization with other isoforms having distinct biochemical activities may produce the variety of Ca^{2+}-sensors. In contrast, Syts VIII and XI are thought to be present in the homo-oligomer or monomer state.

Is Ca^{2+}-dependent oligomerization of Syt I C2B domain indeed essential for synaptic vesicle exocytosis *in vivo*? Although the importance of the Syt I C2B domain in synaptic vesicle exocytosis and endocytosis has been well demonstrated by antibody-loading experiments (*5–7*) and analysis of *Drosophila syt* mutants (*21*), there is no direct evidence that Ca^{2+}-dependent oligomerization of the Syt I C2B domain itself is essential for synaptic vesicle exocytosis *in vivo*. Recently, however, we found that a Syt II mutant carrying a Tyr-312 to Asn substitution in the C2B domain, which corresponds to the *Drosophila* AD3 mutation, had completely impaired self-oligomerization activity but could weakly hetero-oligomerize with wild-type proteins in a Ca^{2+}-dependent manner (Fukuda, M., unpublished data). This result is consistent with the fact that this *syt* allele could complement another mutant phenotype, although it is homozygous lethal (*21*). Under our binding conditions, however, the Syt I cytoplasmic domain showed only very weak Ca^{2+}-dependent self-oligomerization activity. However, given that Syt I proteins are enriched on synaptic vesicles at the preterminal (*i.e.*, local concentrations of this protein are relatively high), it is possible that Ca^{2+}-dependent oligomerization of the two C2B domains that are preassembled at the N-terminal domain may occur in intact preterminals (*20*).

Group C synaptotagmins were characterized by weak (Syt IX) or no homo-oligomerization activity (Syt IV) under our binding conditions. Because of the weak oligomerization activity, we could not detect their SDS-insensitive homo-oligomerization on SDS-PAGE (*10*). Although the Ca^{2+}-independent homo-oligomerization activity of Syt IX was very weak, Syt IX weakly associated with Syts I, II, V, and VII in a Ca^{2+}-dependent manner. In contrast, Syt IV is probably present mainly as a monomer irrespective of the presence of Ca^{2+}. When Syt IV is expressed in COS-7 cells, it is predominantly localized at the Golgi-like perinuclear compartment, whereas other Syt isoforms are localized at tubular or vesicular structures and/or plasma membranes (*39*, *40* and Fukuda, M., unpublished data). Thus, this different compartmentalization of Syt IV to other isoforms in intact cells may restrict the hetero-oligomerization of Syt IV with other informs (Fig. 3, 2nd panel). In contrast to our findings, however, Chapman *et al.* reported that Syts I and IV hetero-oligomerize in a Ca^{2+}-dependent manner by using recombinant proteins from bacteria (*25*). This discrepancy may be due to differences in binding conditions, such as different recombinant proteins, amount of proteins or incubation time. Alternatively, *in vitro* oligomerization of recombinant synaptotagmin cytoplasmic domains from bacteria may be non-selective as in the case of the SNARE [SNAP (soluble NSF attachment protein) receptors] complex (*41*, *42*).

Syt VII, the only member of group D, showed unique Ca^{2+}-dependent oligomerization properties. The Syt VII cytoplasmic domain oligomerizes in a Ca^{2+}-dependent manner without being tethered at the N-terminal domain (*20*). Since Syt VII is ubiquitously expressed (*15*), Syt VII can modulate the function of other neuronal (I, II, V, X) and non-neuronal (VI, IX) isoforms without tethering at the N-terminal domain. While we were preparing this manuscript, Martinez *et al.* reported that Syt VII is involved in Ca^{2+}-dependent exocytosis of lysosomes in fibroblasts (*43*). Therefore, it will be interesting to see whether Syt I–Syt VII hetero-oligomers are involved in neurotransmitter release in the future.

Among the synaptotagmin isoforms, Syts I and II are known to be localized at synaptic vesicles as well as secretory vesicles of some endocrine cells (*44*). Recently, several synaptotagmin isoforms other than Syts I and II in brain have been reported to have different subcellular localization: Syt III is mainly localized at the synaptic plasma membrane (*45*); Syt VIΔTM, which lacks the transmembrane domain, is associated with various membrane fractions (*45*, *46*); and Syt IV is localized at the Golgi and distal part of neurites in nerve growth factor–differentiated PC12 cells and cultured hippocampal neurons (*39*, *40*). These observations are consistent with our results that Syt I did not essentially interact with Syts III, IV, and VI even in the presence of Ca^{2+}. At this stage, we cannot completely rule out the possibility that certain adaptor proteins [*e.g.*, syntaxin IA, which binds Syt I in a Ca^{2+}-dependent manner (*15*)] bridges different Syt isoforms. However, this possibility is unlikely, because we could only detect Syts and IgG used for immunoprecipitation, when immunoprecipitants were visualized by Coomassie Brilliant Blue R-250 staining (data not shown). Furthermore, the T7- and FLAG-Syts cotransfection assay needs to be performed in other types of cells, such as PC12 cells, in the future, because of the possibility that subcelluar localization of Syt isoforms may differ between cell types.

In summary, we investigated the Ca^{2+}-dependent and -independent homo- and hetero-oligomerization of synaptotagmin family proteins and showed that some isoforms can oligomerize *via* the N-terminal domain (Ca^{2+}-independent) and/or the cytoplasmic domain (Ca^{2+}-dependent). Our results and heterologous expression of synaptotagmin family in brain (*37*, *47*, *48*) suggest that hetero-oligomerization of a possible neuronal Ca^{2+}-sensor, Syt I (or II), with other isoforms that have different biochemical properties, may create a variety of Ca^{2+}-sensors.

We thank Dr. Shigekazu Nagata for the expression vector, Dr. Masao Sakaguchi for helpful advice, Eiko Kanno for technical assistance, Hiroyuki Kabayama, Keiji Ibata, and Fumiaki Hamazato for valuable discussions, and Akihiro Mizutani for critical comments on the manuscript.

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