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Molecular Cloning, Expression, and Characterization of a Novel Class of Synaptotagmin (Syt XIV) Conserved from *Drosophila* to Humans

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Synaptotagmins (Syts) represent a large family of putative membrane trafficking proteins found in various species from different phyla. In this study, I identified a novel class of Syt (named Syt XIV) conserved from *Drosophila* to humans and its highly related molecule, Strep14 (Syt XIV-related protein). Although both Syt XIV and Strep14 belong to the C-terminal-type (C-type) tandem C2 protein family, only Syt XIV has a single transmembrane domain at the N-terminus and a putative fatty-acylation site just downstream of the transmembrane domain. Biochemical analyses have indicated that Syt XIV is a Ca^{2+}-independent Syt (*e.g.*, Syts VIII, XII, and XIII) and that, like other Syt family proteins, it is capable of forming a Ca^{2+}-independent oligomer. Unlike other Syt isoforms, however, expression of Syt XIV and Strep14 mRNA is highly restricted to mouse heart and testis and absent in the brain, where most other Syts are abundantly expressed, suggesting that Syt XIV and Strep14 may be involved in membrane trafficking in specific tissues outside the brain. I also identified all of the C-type tandem C2 proteins in humans, the mouse, the fruit fly, a nematode, a plant, and a yeast and discuss the molecular evolution of the C-type tandem C2 protein families, including the Syt family, the Syt-like protein (Slp) family, and the Doc2 family.

Key words: C2 domain, C-type tandem C2 protein, membrane trafficking, phospholipid binding, synaptotagmin.

Abbreviations: C-type, C-terminal-type; En, embryonic day n; GST, glutathione *S*-transferase; HRP, horseradish peroxidase; Mid, Munc13-1 interacting domain; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase; SHD, Slp homology domain; Slp(s), synaptotagmin-like protein(s); Strep, synaptotagmin-related protein; Syt(s), synaptotagmins.

Over the past five years it has become increasingly evident that several members of the synaptotagmin (Syt) family are involved in the regulation of Ca^{2+}-triggered cellular events through Ca^{2+}-regulated membrane trafficking, including communication between neurons (*i.e.*, synaptic vesicle exocytosis) (reviewed in Refs. *1–5*), peptide hormone secretion (*i.e.*, dense-core vesicle exocytosis) (*6–13*), neurite outgrowth (*i.e.*, growth cone vesicle exocytosis) (*14*, *15*), fertilization (*i.e.*, acrosome reaction) (*16*), and plasma membrane repair (*e.g.*, lysosomal exocytosis) (*17–19*). All Syt family members consist of an N-terminal single transmembrane domain and C-terminal tandem C2 domains (known as the C2A domain and the C2B domain) that are homologous to the C2 regulatory region of Ca^{2+}-dependent protein kinase C (*1–5*). Ca^{2+}-binding to the two C2 domains of the Syt family is widely believed to be essential for Ca^{2+}-regulated exocytosis, and the two C2 domains of Syt I, the best characterized Syt isoform, which is abundant on synaptic vesicles, have been shown to regulate neurotransmitter release (*20–24*). By contrast, however, the exact function of other Syt isoforms largely remains to be determined and is still a matter of controversy.

The Syt family is the largest of the C-terminal-type (C-type) tandem C2 protein families (*25–33*). To date, 13 distinct *syt* genes in mice and humans and several corresponding *syt* genes have been found in invertebrates (*2*, *34–37*). A recent genomic analysis by BLAST search indicated the presence of six additional putative *syt* genes that encode fragments of tandem C2 domains in the human genome (*36*). However, whether these putative *syt* genes are indeed transcribed and belong to the *syt* gene family has never been elucidated.

In the present study, I identified a novel class of Syt (named Syt XIV) conserved from *Drosophila* to humans and its highly related molecule (named Strep14, Syt XIV-related protein), and demonstrated their Ca^{2+}-independent phospholipid binding properties and their tissue distributions. I also searched for *syt*-related genes in humans, the mouse, the fruit fly (*Drosophila*), a nematode (*Caenorhabditis elegans*), a plant (*Arabidopthis thaliana*), and a yeast (*Saccharomyces cerevisiae*), classified them into several groups, and discuss the molecular evolution of the C-type tandem C2 protein family proteins (*e.g.*, the Doc2 family, the Syt-like protein (Slp) family, and Rabphilin).

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MATERIALS AND METHODS

Molecular Cloning of Mouse Syt XIV and Strep14—cDNAs encoding the N-terminal (or C-terminal) region of mouse Syt XIV and Strep14 were amplified from Marathon-Ready mouse spleen (or heart) cDNA by 5′-(or 3′-) rapid amplification of cDNA ends (RACE) (Clonetech Laboratories, Inc., Palo Alto, CA, USA), essentially as described previously (*34*). The first 5′-RACE reactions were carried out by using adapter primer 1 (5′-CCATCCTAATACGACTCACTATAGGGC-3′) and the following primers designed on the basis of the mouse genome sequence (similar to synaptotagmin; mouse chromosomal positions 1 H6 and 12 C3) (http://www.ncbi.nlm.nih.gov/genome/guide/mouse/): 5′-CTATGGATCAAAAGGTTTAG-3′ (Syt XIV-ΔC2 primer; antisense), 5′-CTACAAAAGCCTGTGTGGTA-3′ (Strep14-ΔC2 primer; antisense), or 5′-AAGCAATTCAAGATCTCA-3′ (Strep14-N1 primer; sense). The second RACE reactions were carried out by using internal adapter primer 2 (5′-ACTCACTATAGGGCTCGAGCGGC-3′) and the following primers designed on the basis of the mouse genome resource: 5′-CCTCATTCTCTGAATGCA-3′ (Syt XIV-C1 primer; antisense), 5′-TGAGATCTTGAATTGCTT-3′ (Strep14-C1 primer; antisense), or 5′-CAGGTACCACACAGGCTTTT-3′ (Strep14-N2 primer; sense). Both PCR reactions were carried out in the presence of Perfect Match PCR Enhancers (Stratagene, La Jolla, CA, USA) for 40 cycles, each consisting of denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 2 min. The purified second PCR products were directly inserted into the pGEM-T Easy vector (Promega, Madison, WI, USA), and both strands were completely sequenced as described previously (*34*). cDNAs encoding the open reading frame of the mouse Syt XIV and Strep14 were similarly amplified by reverse transcriptase (RT)-PCR from Marathon-Ready mouse spleen cDNA using the following pairs of primers with a *Bam*HI site (underlined) or a stop codon (bold letters): 5′-CGGATCCATGGCGATCGAAGGTGGAGA-3′ (Syt XIV-Met primer; sense) and 5′-**TCA**GGACTCCAGCAGTGCGT-3′ (Syt XIV-stop primer; antisense), or 5′-CGGATCCATGGTGTTGACCATGGCATCTCAGGATGTCCAG-3′ (Strep14-Met primer; sense) and 5′-**CTA**GGATTCCAACAGCGTAT-3′ (Strep14-stop primer; antisense). The purified PCR products were subcloned into the pGEM-T Easy vector (named pGEM-T-Syt XIV or pGEM-T-Strep14) and verified by DNA sequencing. The human Syt XIV and Strep14 cDNAs were determined by database searching (standard BLAST search) using the mouse Syt XIV sequence and Strep14 sequence, respectively, as a query.

Molecular Cloning of Arabidopthis thaliana Syt A and Syt C—cDNAs encoding the open reading frame of the *A. thaliana* Syt A and Syt C were similarly amplified by RT-PCR from the Superscript™ *Arabidopthis* cDNA library (Invitrogen; Carlsbad, CA, USA) using the following pairs of primers with a *Bam*HI site (underlined) or a stop codon (bold letters) designed on the basis of the *A. thaliana* genome resource: 5′-CGGATCCATGGGCTTTTTCAGTACGAT-3′ (at-Syt A-Met primer, sense), 5′-**TCA**AGAGGCAGTTCGCCACT-3′ (at-Syt A-stop primer, antisense), 5′-CGGATCCATGGGTTTCTTCACCAGTGT-3′ (at-Syt C-Met primer, sense), and 5′-**TTA**ACTAGTTGTCCAACGGA-3′ (at-Syt C-stop primer, antisense). The purified PCR products were subcloned into the pGEM-T Easy vector and were completely sequenced.

Expression Constructs, Transfection, and Immunoprecipitation—Addition of T7 tag (or FLAG tag) to the N terminus of Syt XIV or Strep14 and construction of mammalian expression vectors (named pEF-T7-Syt XIV, pEF-FLAG-Syt XIV, pEF-T7-Strep14, and pEF-FLAG-Strep14) were performed as described previously (*38–40*). Plasmid DNA was prepared using Qiagen (Chatsworth, CA, USA) Maxi prep kits. Cotransfection of pEF-T7-Syt XIV (or -Strep14) and pEF-FLAG-Syt XIV (or -Strep14) into COS-7 cells (7.5×10^5 cells, the day before transfection/10 cm dish) was performed as described previously (*41*). Proteins were solubilized at 4°C for 1 h with a buffer containing 1% Triton X-100, 250 mM NaCl, 1 mM $MgCl_2$, 50 mM HEPES-KOH, pH 7.2, 0.1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, and 10 μM pepstatin A. T7-tagged proteins were immunoprecipitated with anti-T7 tag antibody-conjugated agarose (Novagen; Madison, WI, USA) as described previously (*34*). SDS-PAGE and immunoblotting analyses with HRP (horseradish peroxidase)-conjugated anti-FLAG tag (Sigma Chemical, St. Louis, MO, USA) and anti-T7 tag antibodies (Novagen) were also performed as described previously (*34*). The blots shown in this paper are representative of two independent experiments.

Phospholipid-Binding Assay—Construction of pGEX-4T-3 vector (Amersham Biosciences, Tokyo) carrying each C2 domain of the mouse Syt XIV or Strep14 was essentially performed by PCR (*39*) using pGEM-T-Syt XIV or pGEM-T-Strep14, respectively, as a template. The following primers with a *Bam*HI site (underlined) or a stop codon (bold letter) were used for amplification: Syt XIV-C2A upper primer (5′-CGGATCCGAGCCAGAAGCTAAATATGG-3′), Syt XIV-C2A lower primer (5′-**CTA**ACAGCCAGAAGGATTGT-3′), Syt XIV-C2B upper primer (5′-CGGATCCGACAGTACATCCTCCTGTCA-3′), Syt XIV-stop primer, Strep14-C2A upper primer (5′-CGGATCCGAGCCTATCTCAAAATGCGG-3′), Strep14-C2A lower primer (5′-**CTA**TCCACTGCTCAGATTAC-3′), Strep14-C2B upper primer (5′-CGGATCCGACAGTGCTTCATCCACGCA-3′), and Strep14-stop primer. Preparation of GST (glutathione *S*-transferase) fusion proteins containing the single C2 domain of Syt XIV (or Strep14) was performed as described previously (*39*). GST-Syt XIV-C2A contains amino acid residues 256–390 of mouse Syt XIV; GST-Syt XIV-C2B, amino acid residues 403–555 of mouse Syt XIV; GST-Strep14-C2A, amino acid residues 340–474 of mouse Strep14; and GST-Strep14-C2B, amino acid residues 487–639 of mouse Strep14. Liposome co-sedimentation assay was also performed as described previously (*42*, *43*). The gels shown in this paper are representative of three independent experiments.

Sequence Analyses—Multiple sequence alignment was performed with the CLUSTALW program (http://hypernig.nig.ac.jp/homology/clustalw.shtml) set at the default parameters and then modified manually to optimize similarity. Depiction of the phylogenetic tree was also performed by use of the CLUSTALW program (*25*).

RT-PCR Analysis—Mouse first-strand cDNAs prepared from various tissues and developmental stages (whole embryo) were obtained from Clontech Laboratories, Inc. (mouse MTC Panel I) (*25*, *26*). PCRs were carried out in

the presence of Perfect Match PCR enhancer for 28 cycles (for G3PDH) or 40 cycles (for Syt XIV and Strep14), each consisting of denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 2 min. The Syt XIV-C2B upper primer and Syt XIV-stop primer, and the Strep14-C2B upper primer and Strep14-stop primer were used for amplification. The PCR products were analyzed by 1% agarose gel electrophoresis followed by ethidium bromide staining. The authenticity of the products was verified by subcloning into a pGEM-T Easy vector and DNA sequencing as described previously (*34*).

RESULTS

Identification of a Novel Class of Synaptotagmin (Syt XIV) Conserved from Drosophila to Humans—To identify a novel gene that encodes a C-type tandem C2 protein, I searched for tandem C2 proteins homologous to mouse Syts I-XIII C2 domains in human and mouse genome databases by use of protein BLAST or SMART (simple modular architecture research tool; http://smart.embl-heidelberg.de/). Two distinct genes that potentially encode novel C-type tandem C2 proteins were found in the human and mouse genomes (human chromosomal positions 4q13.1 and 14q23.1 and mouse chromosomal positions 1 H6 and 12 C3), and their full open reading frame was cloned from the mouse cDNA library by 5′- and 3′-RACE (see Materials and Methods for details). The 1,731 nucleotide sequence and 3,058 nucleotide sequence contain a single open reading frame encoding 555 and 639 amino acids with a calculated molecular weight of 62,041 and 71,251, respectively (Fig. 1A). These two proteins contain tandem C2 proteins (*i.e.*, C2A domain and C2B domain) at the C terminus, and therefore they should be classified as members of the C-type tandem C2 protein family (*25*). Interestingly, these two proteins show significant homology with each other, especially in the two C2 domains (66.7% similarity in the C2A domain, 83.8% similarity in the C2B domain, and 46.4% similarity in the entire proteins) (Fig. 2), but relatively weak similarity to other Syt isoforms reported thus far (*34–37*). According to the results of a hydrophobicity analysis (*44*), the 555 amino acid protein contains a single hydrophobic region sufficient for a transmembrane domain at the N terminus, but the 639 amino acid protein has no hydrophobic region (Fig. 1A and data not shown). I therefore named the former protein the 14th isoform of Syt (Syt XIV) and the latter protein Strep14 (Syt XIV-related protein). Interestingly, homology search analysis revealed that the orthologue of mammalian Syt XIV is found in *Drosophila* (named dm-Syt XIV) (63.2% similarity in the transmembrane domain, 78.6% similarity in the Syt XIV homology domain (see below), 45.7% similarity in the C2A domain and 71.4% similarity in the C2B domain), but not in *C. elegans*, the plant, or the yeast (Figs. 2 and 3), suggesting that Syt XIV may have an important role in membrane trafficking conserved from *Drosophila* to humans.

The mouse, human, and *Drosophila* Syt XIV proteins share several features characteristic of Syt family members. First, they contain several Cys residues at the interface between the transmembrane domain and the spacer

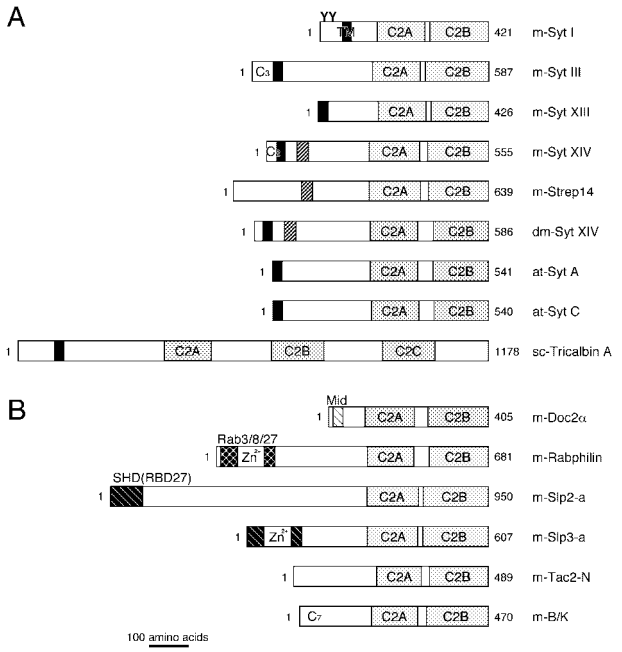

Fig. 1. **Schematic representation of C-type tandem C2 protein families.** (A) Syts from mouse (m), *Drosophila* (dm), and plant (at) and yeast (sc) Tricalbin. (B) Other C-type tandem C2 protein families from mice. The C-type tandem C2 protein is defined as a protein that contains tandem C2 domains (shaded boxes) at the C terminus. Mammalian Syt XIV, *Drosophila* Syt XIV, and Strep14 contain a Syt XIV homology domain (hatched boxes; see also Fig. 2, solid bars). Amino acid numbers are given on both sides. Y, glycosylation sites of Syt I; TM, transmembrane domain (black boxes); C_x, numbers of cysteine residues; Mid, Munc13-1 interacting domain; Rab3/8/27, Rab3/8/27 binding domain; Zn^{2+}, zinc finger motif; SHD, Slp homology domain; RBD27, Rab binding domain specific for Rab27 isoforms.

domain (# in Fig. 2), and such Cys residues often undergo fatty-acylation, which is involved in SDS-insensitive oligomerization of Syt proteins (*41*). The putative extracellular domain of the Syt XIV protein was very short, as are those of Syts I-XII, and the mammalian Syt XIV protein contains two Cys residues in this region (small boxes in Fig. 2). Similar Cys residues were also found in the extracellular domain of Syts III, V, VI, and X (Fig. 1A), and they are involved in dimer formation through disulfide bonding (*34*, *40*, *45*). Unlike Syts I and II, the Syt XIV protein lacks a putative *N*-glycosylation site and di-Thr/Ser residues (*i.e.*, putative *O*-glycosylation site) (*46*). Although the mouse and human Strep14 protein lacks a transmembrane domain as well as the known protein motifs of the C-type tandem C2 proteins, including Mid (Munc13-1 interacting domain) (*47*), Slp homology domain (SHD) (*48*, *49*), and Rab3/8/27 binding domain (*31*, *48*) (see Fig. 1B), it contains a short sequence (Thr/Ser- and Glu/Asp-rich region) highly conserved between Syt XIV and Strep14 (named Syt XIV homology domain; Fig. 2, solid bars). The physiological meaning of this sequence is currently unknown, but it should have an important role(s) in the expression of Syt XIV function, because it has been retained during evolution. In addition, both the Syt XIV and Strep14 proteins contain a WHXL motif at the C terminus, which may be crucial for

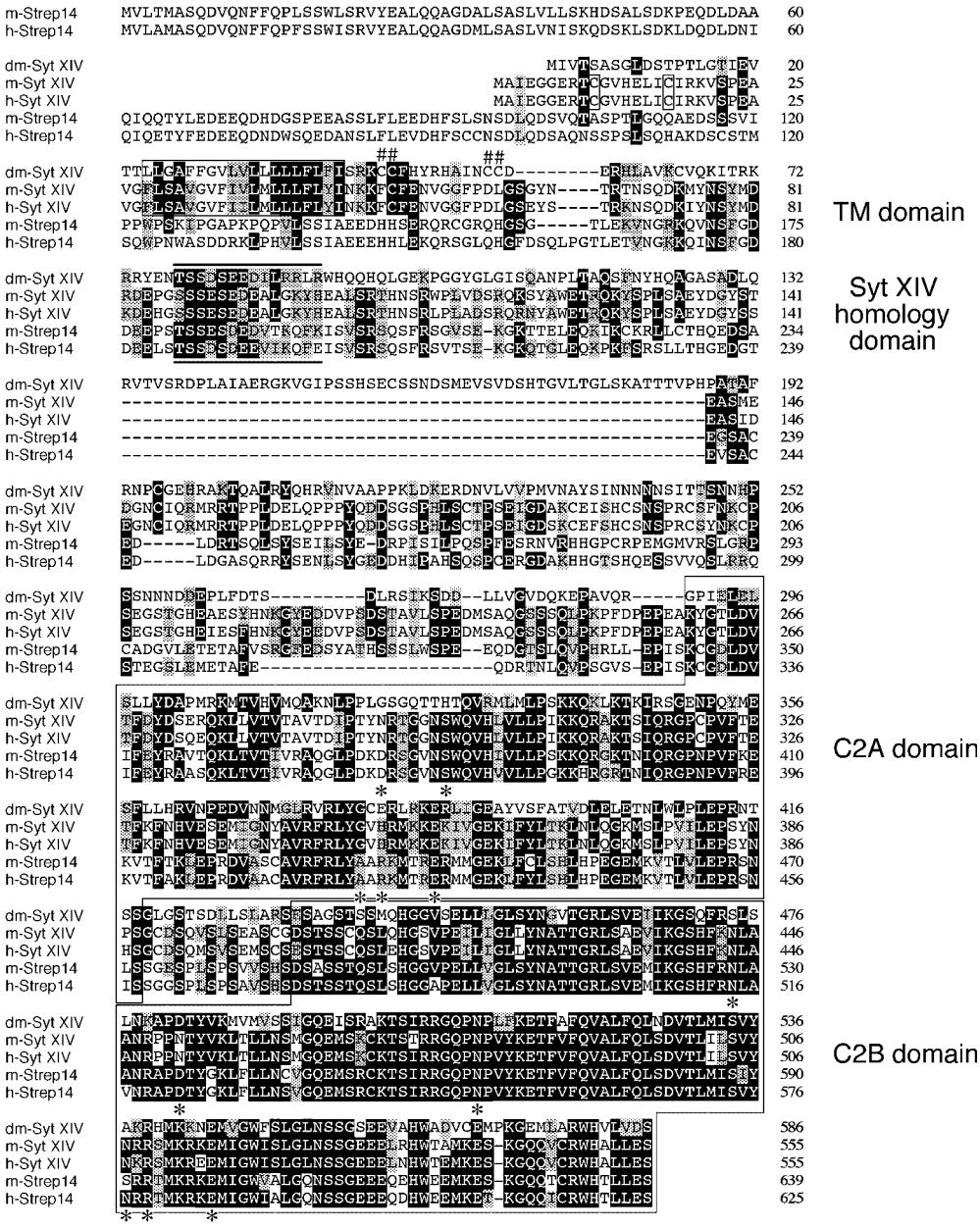

Fig. 2. **Sequence alignment of the mouse, human, and *Drosophila* Syt XIV and its related molecule, Strep14.** Residues conserved and similar between sequences are shown on a black background and shaded background, respectively. Asterisks indicate the five conserved aspartate or glutamate residues in the C2 domains, which may be crucial for Ca^{2+} binding by analogy with the Syt I-C2A domain (*68*, *69*). The symbol # indicates the Cys residues that may be fatty-acylated between the transmembrane domain (large box) and spacer domains (*41*). The cysteine residues located at the extracellular domain of mammalian Syt XIV are indicated by small boxes. The solid lines indicate the Syt XIV homology domain conserved across phylogeny. The protein IDs for newly named proteins in this paper (see http://smart.embl-heidelberg.de/) are indicated in parentheses: ce-Syt VII (Q9TZL3), ce-Syt A (Q22372), ce-Syt B (O16700), ce-Strep9 (Q20354), ce-Tricalbin (O76835), dm-Syt A (Q9I7M6), dm-Syt B (Q9VUJ3), dm-Syt XII (Q9VYE1), dm-Syt XIV (Q9VN04), dm-Slp (Q9VFF9), dm-Tricalbin (Q9VC62), at-Syt B (Q9LNT5), at-Syt D (Q9FY55), at-Syt E (Q8L706), sc-Tricalbin A (YNI7_YEAST), sc-Tricalbin B (Q12466), and sc-Tricalbin C (YMH2_YEAST). The GenBank™ accession numbers of mouse and human Syt XIV and Strep14 and of plant Syts A and C are AB102947- AB102952, respectively.

docking to the plasma membrane and correct folding of the C2B domain (*50–52*).

Ca^{2+}-Independent Oligomerization Property of Mouse Syt XIV—Since the Syt family proteins form Ca^{2+}-independent and -dependent oligomers (*34*, *40*, *41*, *45*, *53*) and Syt I is thought to function as an oligomer during neurotransmitter release (*1*, *37*, *54*, *55*), I investigated the oligomerization property of Syt XIV and Strep14 by dual-tag coexpression assay (*40*). In brief, T7- and FLAG-tagged Syt XIV were coexpressed in COS-7 cells, and their association was determined by immunoprecipitation assay. As shown in Fig. 4A (lanes 1 and 2), FLAG-Syt XIV protein was efficiently co-immunoprecipitated with T7-Syt XIV protein irrespective of the presence of Ca^{2+}, in

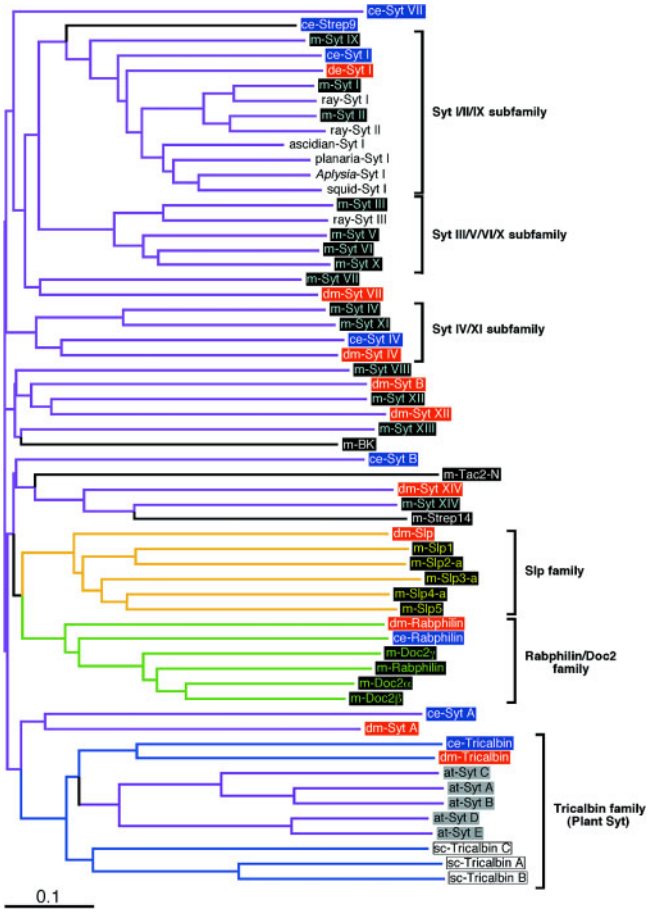

Fig. 3. **Phylogenetic tree of the C-type tandem C2 protein families from various species.** The phylogenetic tree is depicted as described under "MATERIALS AND METHODS." Note that the plant Syt family and the Tricalbin family forms a branch distinct from the animal Syt family (blue letters, mouse Syts), the Doc2/Rabphilin family (green letters), and the Slp family (yellow letters). The animal Syt family is further classified into several subfamilies (*i.e.*, Syt I/II/IX subfamily, Syt III/V/VI/X subfamily, and Syt IV/XI subfamily). Proteins from mouse (m), *Drosophila* (dm), *C. elegans* (ce), the plant (at), and the yeast (sc) are indicated by a black background, red background, blue background, gray background, and boxes, respectively.

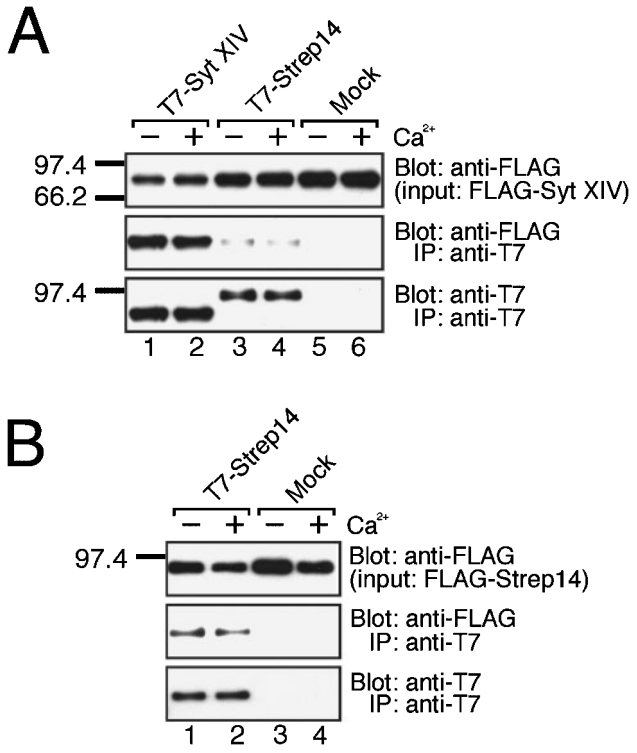

Fig. 4. **Oligomerization properties of mouse Syt XIV and Strep14.** (A) Homo- and hetero-oligomerization property of Syt XIV in COS-7 cells as revealed by coexpression assay. (B) Oligomerization of Strep14 in COS-7 cells as revealed by coexpression assay. pEF-T7-Syt XIV (or pEF-T7-Strep14) and pEF-FLAG-Syt XIV (or pEF-FLAG-Strep14) were cotransfected into COS-7 cells. Expressed proteins were solubilized with 1% Triton X-100 and immunoprecipitated by anti-T7 tag antibody-conjugated agarose as described under "MATERIALS AND METHODS." Co-immunoprecipitated FLAG-tagged proteins were first detected with HRP-conjugated anti-FLAG tag antibody (1:10,000 dilution) (middle panels, Blot: anti-FLAG and IP: anti-T7). Then the same blots were stripped and reprobed with HRP-conjugated anti-T7 tag antibody to ensure that equivalent amounts of T7-tagged proteins had been loaded (1:10,000 dilution) (bottom panels, Blot: anti-T7 and IP: anti-T7). The upper panels indicate the total expressed FLAG-tagged proteins (1/80 volume of the reaction mixture) used for immunoprecipitation. Note that Syt XIV and Strep14 formed a weak Ca^{2+}-independent hetero-oligomer (lanes 3 and 4 in A). The positions of the molecular weight markers ($\times 10^{-3}$) are shown on the left.

the same way as the group B Syts (*e.g.*, Syts I, II, and VIII) (*45*). The Ca^{2+}-independent oligomerization of Syt XIV was mainly mediated by the fatty-acylated Cys residues between the transmembrane domain and spacer domain (*41*), rather than by a disulfide-bond at the extracellular domain (*34*, *40*, *45*), because the SDS-insensitive dimer band of Syt XIV on SDS-PAGE was unaffected by the presence of β-mercaptoethanol, which cleaves disulfide bonds (data not shown). Interestingly, Strep14 also showed relatively weak homo-oligomerization and hetero-oligomerization with Syt XIV irrespective of the presence of Ca^{2+} (Fig. 4B and Fig. 4A, lane 3 and 4), suggesting that Syt XIV and Strep14 may function as a complex in membrane transport.

Phospholipid-Binding Property of C2 Domains of Mouse Syt XIV and Strep14—Although the C2 domain was originally described as a domain responsible for Ca^{2+}-dependent translocation of proteins to phospholipid membranes (*56*), some C2 domains from C-type tandem C2 proteins have been shown to lack such Ca^{2+}/phospholipid-binding activity (*29*, *30*, *33*, *35*, *57*, *58*). I recently found that the presence of five conserved acidic residues in loops 1 and 3 of the C2 structure is a good marker for the Ca^{2+}-dependent phospholipid-binding ability of the C2A domain of C-type tandem C2 proteins, except for Slp3-a and Slp5 (*27*, *57*). In contrast to the Syt I C2A domain, the Syt XIV and Strep14 C2 domains lack such acidic residues (*i.e.*, only one Glu residue is found in loop 3 of the C2 structure) (Fig. 2, asterisks), suggesting that the Syt XIV and Strep14 proteins should be classified as Ca^{2+}-independent tandem C2 proteins. As expected, both C2 domains of Syt XIV and Strep14 fused to GST bound liposomes (phosphatidylcholine and phosphatidylserine, 1 : 1, w/w)

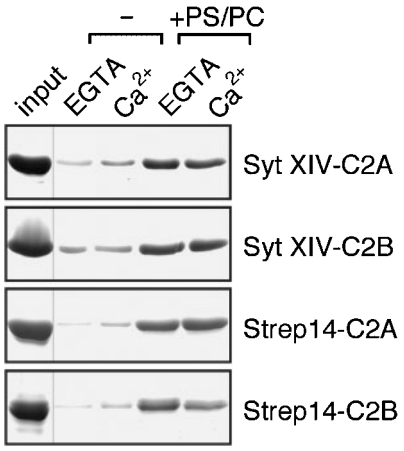

Fig. 5. **Phospholipid-binding properties of C2 domains of mouse Syt XIV and Strep14.** Liposomes and GST fusion proteins were incubated in 50 mM HEPES-KOH, pH 7.2, in the presence of 2 mM EGTA or 1 mM Ca^{2+} for 15 min at room temperature. After centrifugation at 12,000 ×*g* for 10 min, the supernatants (non-binding fraction) and pellets (phospholipid-binding fraction) were separated as described previously (*42*, *43*). The pelleted portion of the input samples was subjected to 10% SDS-PAGE and then stained with Coomassie Brilliant Blue R-250. Note that the C2 domains of Syt XIV (or Strep14) were recovered in the pelleted portion only in the presence of liposomes, but regardless of the presence of Ca^{2+}. The results shown are representative of three independent experiments.

in a Ca^{2+}-independent manner (Fig. 5). Under the same experimental conditions, GST-Syt I-C2A binds PS/PC liposomes in a Ca^{2+}-dependent manner, whereas GST alone did not show liposome binding activity at all (data not shown) (*27*, *33*, *42*, *43*).

Tissue Distribution of Syt XIV and Strep14—RT-PCR with specific primers was performed to investigate the tissue distribution of mouse Syt XIV and Strep14 (Fig. 6, top and middle panels). Syt XIV mRNA and Strep14 mRNA were found in almost the same tissues and at similar developmental stages, suggesting that the Syt XIV·Strep14 complex described above may be formed under physiological conditions. Their expression levels were highest in the heart and testis and moderate in the kidney. In addition, expression of Syt XIV and Strep14 mRNA was very weak (or absent) on embryonic day 7 (E7) and remained constant from E11 to E17. It is of great interest that mRNA expression of Syt XIV and Strep14 is almost absent in brain, because the mRNAs of other Syt isoforms (I-XIII) are easily detected in brain by RT-PCR analysis (*35*, *58*, *59*) and most of the Syt proteins (even non-neuronal or ubiquitous type Syt proteins) are abundant in brain (*11*, *12*, *19*, *60–63*). As far as I know, Syt XIV is the first Syt isoform that is only expressed in selected non-neuronal tissues outside the brain.

DISCUSSION

In the present study, I isolated and characterized a novel Syt isoform, Syt XIV, and its related molecule, Srep14. The mammalian and *Drosophila* Syt XIV and Strep14 form a small branch of the phylogenetic tree of C-type tandem C2 protein families, and they possess a unique Thr/Ser- and Glu/Asp-rich sequence (named Syt XIV homology domain) at the N-terminal domain (downstream of the transmembrane domain of Syt XIV) (Figs. 1–3). Biochemical analyses indicated that both Syt XIV and Strep14 associate with phospholipids irrespective of the presence of Ca^{2+} and that Syt XIV and Strep14 can form homo- and hetero-oligomers even in the absence of Ca^{2+} (Figs. 4 and 5). Moreover, the mRNA expression profiles of Syt XIV and Strep14 in mouse tissues were almost identical, with both mRNAs being highly expressed in the heart, kidney, and testis, but not being expressed in the brain (Fig. 6). These results imply that Syt XIV, or possibly a Syt XIV·Strep14 complex, may be involved in non-neuronal-type membrane trafficking. Further work is necessary to elucidate the exact functions of Syt XIV and Strep14.

A previous study by Craxton (*36*) identified six additional putative *syt* genes in the human genome (*chr10 syt*, *chr11 syt*, *chr2 syt*, *chr3 syt*, *chr14 syt*, and *chr415 syt*), and sequence comparison revealed that the human Syt XIV and Strep14 correspond to the products of *chr415 syt* and *chr14 syt*, respectively. Further database searching revealed the products of *chr10 syt*, *chr11 syt*, *chr2 syt*, and *chr3 syt* to be a single C2 domain-containing protein with a single N-terminal transmembrane domain (GenBank™ accession no., XM_089553), Slp2-a (*26*), KIAA1228, which contains three C2 domains with a single N-terminal transmembrane domain (*64*), and a pro-

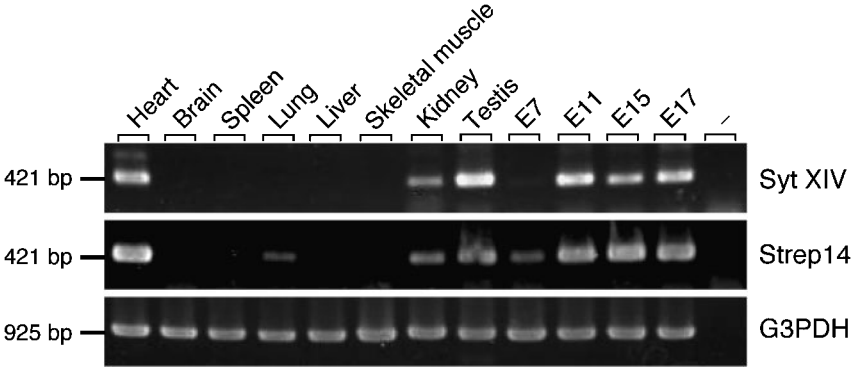

Fig. 6. **Tissue distribution of mouse Syt XIV and Strep14.** RT-PCR analysis of Syt XIV (or Strep14) expression in various mouse tissues (heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis) and on embryonic days 7 (E7), 11, 15, and 17 (upper two panels). RT-PCR analysis of G3PDH expression was also performed (bottom panel) to ensure that equivalent amounts of first strand cDNA were used for RT-PCR analysis. Note that tissue distributions of mouse Syt XIV and Strep14 mRNA were almost identical. Abundant expression was found in heart and testis, and moderate expression in kidney. "–" means without templates as a negative control. The size of the molecular weight markers (λ/StyI) is shown at the left of the panel. The results shown are representative of two independent experiments.

tein highly homologous to the mouse MBC2 (membrane bound C2 domain containing protein) (GenBank™ accession no., XM_125941) or the rat GLUT4 vesicle protein (*65*), respectively. Therefore, the Syt XIV identified in this study is most likely to be the final member of the Syt family that is conserved from *Drosophila* to mammals.

Phylogenetic relationships of the tandem C2 proteins from the mouse (black background), *Drosophila* (red background), *C. elegans* (blue background), *A. thaliana* (gray background), and yeast (boxed) are summarized in Fig. 3. The Syt family (magenta trees) is the largest family among the tandem C2 protein families, and 14 Syt proteins are found in the mouse (Syts I-XIV), 5 in *C. elegans* (Syts I, IV, VII, A, and B), 7 in *Drosophila* (Syts I, IV, VII, XII, XIV, A, and B), and 5 in the plant (at-Syts A-E). Mammalian Syts I, IV, and VII homologues are found in both *Drosophila* and *C. elegans* (*66*), but mammalian Syts XII and XIV homologues are found only in *Drosophila*. By contrast, no vertebrate homologues of the Syt III/V/VI/X subfamily (*34*, *45*, *67*) are found in invertebrates, and no invertebrate homologues of Syts A and B are found in vertebrates, suggesting that some Syt members may have a species-specific role(s). Interestingly, five members of the plant Syt family form a branch completely different from the animal Syt family (*i.e.*, having a different origin from the animal *syt* genes), even though they have C-terminal tandem C2 domains and an N-terminal single transmembrane domain without an extracellular domain, the same structure as mouse Syt XIII (Fig. 1A). By careful database searching, I found that the plant Syt proteins are highly homologous to yeast Tricalbin proteins, which have three C2 domains and an N-terminal single transmembrane domain (Fig. 1A). As judged from the phylogenetic tree and sequence alignment (blue trees in Fig. 3), the plant Syt proteins probably evolved from the yeast Tricalbin proteins by losing a third C2 domain (*i.e.*, C2C of Fig. 1A). Since most of the C2 domains of plant Syt and yeast Tricalbin contain four or five conserved Asp/Glu residues, which may be crucial for Ca^{2+}-binding (*56*, *68*, *69*), they may function as Ca^{2+}-dependent regulators of membrane trafficking, in the same way as the animal Syt proteins, although no research has been attempted on the plant Syt proteins and yeast Tricalbin proteins. Further work is necessary to determine whether they are indeed involved in membrane trafficking.

Both *Drosophila* and *C. elegans* have one Rabphilin homologue, a putative Rab3/8/27 effector (*31*, *48*, *70*), but they do not have any Doc2 homologue (green trees in Fig. 3). Five Slp members (Slp1-5) are present in the mouse and humans, (*26*, *27*) (yellow trees in Fig. 3), and they are thought to function as effectors for Rab27, one of the small GTP-binding proteins, by directly interacting with the N-terminal SHD (*48*, *49*). Although one *Drosophila* Slp (named dm-Slp) has been found, it lacks an N-terminal SHD, as do the mouse Slp2-b and Slp3-b (*26*), and thus the dm-Slp is unlikely to function as a Rab27 effector. Since Rab27 is highly conserved from *C. elegans* to *Drosophila* and to humans (*71*), while other Rab27 effectors, Slps and Slac2s (*48*, *72*, *73*), are not found in invertebrates, what the Rab27 effector(s) are in invertebrates is an open question.

In summary, I first identified a novel class of Syt proteins (Syt XIV), presumably the final Syt in mammals, and analyzed the phylogenetic relationships of the C-type tandem C2 proteins from five different species. The results indicate that the Syt family is the largest C-type tandem C2 protein family and that several Syt isoforms (*e.g.*, Syts I, IV, VII, XII, and XIV) are evolutionarily conserved from *Drosophila* to humans, suggesting important roles of the Syt family proteins in membrane trafficking conserved across phylogeny. Future studies will clarify the function of individual Syt isoforms in membrane trafficking.

Note Added in Proof: After the acceptance of this paper we found that *chr10 syt* also encoded a tandem C2 domain–containing protein with a single N-terminal transmembrane domain. We refer to the product of *chr10 syt* as synaptotagmin XV (Syt XV) [*Biochem. Biophys. Res. Commun.* (2003) **306**, 64–71].

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