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Molecular cloning and characterization of human, rat, and mouse synaptotagmin XV☆,☆☆

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Abstract

Synaptotagmin (Syt) constitutes a large family of putative membrane trafficking proteins that share a short extracellular domain, a single N-terminal transmembrane domain, and C-terminal tandem C2 domains. In this study, I identified and characterized a novel member of the Syt family (named Syt XV-a) in the mouse, the rat, and humans. Although Syt XV-a protein has a short hydrophobic region at the very end of the N terminus (i.e., lacks a putative extracellular domain), biochemical and cellular analyses have indicated that the short hydrophobic region (amino acids 5–22) is sufficient for producing type I membrane topology in cultured cells, the same as in other Syt family proteins. Unlike other Syt isoforms, however, the mouse and human Syt XV have an alternative splicing isoform that lacks the C-terminal portion of the C2B domain (named Syt XV-b). Since the expression of Syt XV-a/b mRNA was mainly found in non-neuronal tissues (e.g., lung and testis) and Syt XV-a C2 domains lack Ca^{2+}-dependent phospholipid binding activity, Syt XV-a is classified as a non-neuronal, Ca^{2+}-independent Syt.

Keywords: Synaptotagmin; C2 domain; C-type tandem C2 protein; Phospholipid binding; Membrane traffic

The synaptotagmin (Syt) family belongs to the C-terminal-type (C-type) tandem C2 protein families [1,2] and is found in various species from different phyla, including invertebrates and vertebrates [3–5]. The member of the Syt family was originally defined as a protein containing a single N-terminal transmembrane domain and C-terminal tandem C2 domains (known as the C2A domain and the C2B domain) that often bind several Ca^{2+} ions [6–10]. The Syt family constitutes the largest C-type tandem C2 protein family across phyla [3–5] and 14 distinct Syt isoforms (Syts I–XIV) have been identified in mice to date [5,11,12]. Several members of the Syt family (e.g., Syts I, VI, VII, and IX) have been shown to regulate Ca^{2+}-dependent membrane trafficking, including synaptic vesicle exocytosis [6–10, 13–18], endocrine exocytosis [19–27], plasma membrane repair [28,29], and acrosome reaction [30]. Ca^{2+}-binding to the two Syt C2 domains is widely believed to activate Syt molecules and to promote fusion of transport vesicles to plasma membranes. Although the function of the abundant synaptic vesicle protein Syt I in neurotransmitter release has been well documented [6–10], the functions and subcellular localizations of most of other Syt isoforms largely remain to be determined. Accordingly, it remains unclear whether Syt family proteins regulate all types of Ca^{2+}-dependent membrane trafficking or only specific type(s) of Ca^{2+}-dependent membrane trafficking. A recent genomic analysis revealed that Syts form a very large family in mice and humans [3,5], the same as SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) family proteins, which are general components of the membrane fusion machinery (reviewed in [31]), suggesting that Syt proteins may regulate various types of membrane trafficking.

☆ *Abbreviations:* C-type, C-terminal-type; Ex, embryonic day x; GST, glutathione *S*-transferase; HRP, horseradish peroxidase; PC, phosphatidylcholine; PCR, polymerase chain reaction; PS, phosphatidylserine; RT, reverse transcriptase; Syt(s), synaptotagmin(s).

☆☆ The nucleotide sequence(s) reported in this paper is deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases with Accession Number(s) of AB109019-23.

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In this study, I report a 15th member of the Syt family, designated as Syt XV-a, in the mouse, the rat, and humans, and its alternatively spliced isoform, Syt XV-b, which is unusual because it lacks the C-terminal portion of the C2B domain. Like other members of the Syt family, Syt XV-a contains a single transmembrane domain with type I membrane topology. Based on the biochemical properties of the Syt XV-a C2 domains and tissue distribution of Syt XV-a mRNA, Syt XV-a is classified as a non-neuronal, Ca^{2+}-independent Syt.

Materials and methods

Molecular cloning of mouse, rat, and human Syt XV cDNA. cDNA encoding the open reading frame of mouse, rat, and human Syt XV was amplified from first-strand cDNAs prepared from spleen, lung, or heart (Clontech Laboratories; Palo Alto, CA, USA) by the polymerase chain reaction (PCR), as described previously [11], using the following primers with restriction enzyme sites (underlined) or stop codons (bold letters) designed on the basis of the mouse, rat, and human Syt XV genome sequences (GenBank Accession Nos. NT_039598, NW_043031, and AL356056, respectively) and/or the mouse and human cDNA sequence (XM_147817, AK042641, and AI971263) in the public database: 5′-GGATCCATGGCAGAGCAGCTGGCCTT-3′ (mouse/rat Syt XV-Met primer; sense), 5′-GGC**TCA**GGGCTCCG TGGGGC-3′ (mouse Syt XV-a-stop primer; antisense), 5′-CACT TGAGGGTAGCCATT-3′ (mouse Syt XV-b-C1 primer; antisense), 5′-**TCA**GGGCTCCATGGGGCGGC-3′ (rat Syt XV-a-stop primer; antisense), 5′-GGATCCATGGCGGAGCAGCTGGCCC-3′ (human Syt XV-Met primer; sense), 5′-**TCA**GGGCTCCGTGGTGCGGC-3′ (human Syt XV-a-stop primer; antisense), and 5′-GAATGTGGGGG TGGCTGGCA-3′ (human Syt XV-b-C1 primer; antisense). PCR was carried out in the presence of Perfect Match PCR Enhancer (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 PCR products were purified from an agarose gel on a Micro-Spin column (Amersham Biosciences; Buckinghamshire, UK) as described previously [11] and directly inserted into the pGEM-T Easy vector (pGEM-T-Syt XV) (Promega; Madison, WI, USA). Both strands of the cDNA inserts were completely sequenced. Addition of the T7 tag (or FLAG tag) to the N terminus of mouse Syt XV (pEF-T7-Syt XV or pEF-FLAG-Syt XV) and construction of the expression vectors were performed as described previously [11,32–34]. pEF-T7-Syt XVΔTM (or pEF-FLAG-Syt XVΔTM) was constructed by conventional PCR [11,34] using the following primers: 5′-GGATCCAGC TGCTGTCTGTGGAGGAG-3′ (mouse Syt XVΔTM primer; sense) and the mouse Syt XV-a-stop primer described above. T7-Syt XVΔTM protein completely lacked the N-terminal hydrophobic region (amino acids 1–22).

Preparation of glutathione S-transferase fusion proteins. Construction of pGEX-4T-3 vector (Amersham Biosciences) carrying fragments of the mouse Syt XV-C2A domain or -C2B domain was essentially performed by PCR [33] with pGEM-T-Syt XV-a as a template. The following pairs of oligonucleotides with appropriate restriction enzyme sites (underlined) and/or termination codons (bold letters) were used for amplification: Syt XV-C2A-5′ primer (sense) 5′-GGATCC CCTGATGGCTGCCTGGGCCG-3′ and Syt XV-C2A-3′ primer (antisense) 5′-**CTA**CAGGTTCTTAGCTTCCA-3′; Syt XV-C2B-5′ primer (sense) 5′-GGATCCCCCCCCTCGGAGTTTGGTGA-3′ and the mouse Syt XV-a-stop primer described above. The resulting pGEX-Syt XV-C2A and -C2B were confirmed by DNA sequencing and transformed into *Escherichia coli* JM109. Glutathione *S*-transferase (GST) fusion proteins were expressed and purified on glutathione–Sepharose (Amersham Biosciences) by the standard method [35]. GST-Syt XV-C2A and -C2B encoded amino acids 141–270 and 272–418 of mouse Syt XV, respectively.

RT-PCR analysis. Mouse first-strand cDNAs prepared from various tissues and at various stages of development were obtained from Clontech Laboratories (Mouse MTC Panel I) [1,2]. The PCRs were carried out in the presence of Perfect Match PCR Enhancer for 40 (for Syt XV-a), 37 (for Syt XV-b), or 30 cycles (for G3PDH), 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. Syt XV-Met primer and Syt XV-a-stop primer (or Syt XV-C2A-5′ primer and Syt XV-b-stop primer; 5′-GAGTCCTGC**CTA**CAATTGAG-3′) were used for amplification. The PCR products were analyzed by 1% agarose gel electrophoresis and ethidium bromide staining. The authenticity of the products was verified by subcloning into a pGEM-T Easy vector and DNA sequencing.

Miscellaneous procedures. Transfection of plasmids into COS-7 cells, subcellular fractionation, SDS–PAGE, and immunoblotting with horseradish peroxidase (HRP)-conjugated anti-T7 tag antibody (Novagen; Madison, WI, USA) was performed as described previously [11,12,36]. Transfection of plasmids into PC12 cells, immunocytochemistry, and antibody-uptake experiments was performed as described previously [12,26,37,38]. Preparation of liposomes consisting of phosphatidylcholine (PC) and phosphatidylserine (PS) (1:1 w/w) and the phospholipid binding assay was also performed as described previously [39,40]. The protein concentrations were determined with a Bio-Rad protein assay kit (Bio-Rad Laboratories; Hercules, CA, USA) using bovine serum albumin as the standard. Multiple sequence alignment and depiction of the phylogenetic tree of the Syt family proteins were performed by using the CLUSTALW program (http://hypernig.nig.ac.jp/homology/clustalw.shtml) set at the default parameters (gapdist = 8 and maxdiv = 40) as described previously [1,2].

Results and discussion

Molecular cloning of Syt XV, a novel member of the synaptotagmin family

To delineate the molecular evolution of C-type tandem C2 proteins involved in membrane trafficking, I previously searched and characterized C-type tandem C2 protein families in humans, the mouse, the fruit fly, a nematode, a plant, and a yeast [1,2,5,41–43]. Although that search yielded 14 distinct *syt* genes and 5 *slp* (or *sytl*) genes in the mouse and human genome [5,44], a recent update of the rat genome sequencing project indicated the presence of an additional gene that encodes a putative C-type tandem C2 protein similar to Syt I (rat chromosome 16; gene model name, LOC306285). Database searching revealed that this rat gene corresponds to human *chr10 syt* [3], although I previously characterized the human *chr10 syt* gene product as a single C2 domain-containing protein with a single N-terminal transmembrane domain [5]. cDNA cloning revealed that the human *chr10 syt* encoded two different proteins having a different C terminus (Fig. 1A). Since the longer form (421 amino acids) contains a short N-terminal hydrophobic region (i.e., a putative transmembrane domain; Fig. 2B), putative fatty-acylation sites (# in Fig. 2A) [36], and two C-terminal tandem C2 domains, I

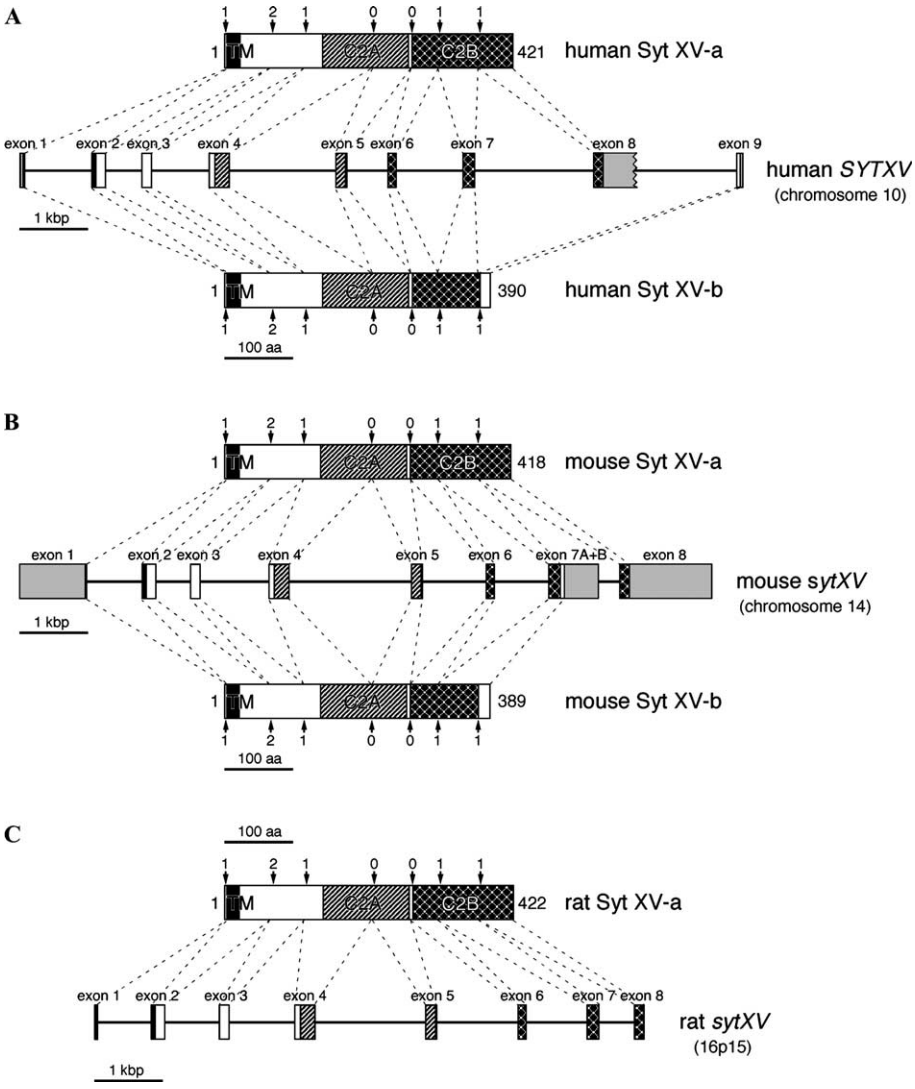

Fig. 1. Organization of the human *SYTXV* (A), mouse *sytXV* (B), and rat *sytXV* genes (C). Exons and introns are represented by boxes and solid bars. Exons encoding the transmembrane domain (TM), C2A and C2B domains, and the non-coding (untranslated) regions are represented by black, hatched, cross-hatched, and shaded boxes, respectively. The protein structure of mouse, human, and rat Syt XV is indicated above (Syt XV-a) or below (Syt XV-b) the corresponding genes. Amino acid numbers are given on both sides. The exon/intron boundaries are indicated by arrows, and the numbers above and below the arrows describe the position in the codon at which the coding sequence is separated by an intron (0 = at the codon junction; 1 and 2 = after the first codon and second codon position, respectively). Note that the gene structure of the mouse, human, and rat Syt XV-a proteins (i.e., pattern of exon/intron divisions) is identical. aa, amino acids.

tentatively named it Syt XV-a, a 15th member of the Syt family (Fig. 1A, top). By contrast, the 390 amino acid shorter form of Syt XV (named Syt XV-b) lacks the C-terminal portion of the C2B domain (arrow in Fig. 2A), corresponding to the β-7 and β-8 strands of the C2 structure by analogy to the Syt I C2B domain [45], and short sequences (18 amino acids) are linked to the truncated C2B domain (Fig. 1A, bottom). The Syt C2 domains are composed of an eight-stranded anti-parallel β-sandwich consisting of four-stranded β-sheets [45,46], and the last β-8 stand containing WHXL motif (or pairing of the β-8 strand with the β-1 strand) has been shown to be essential for correct folding of the Syt C2 domain [47]. Therefore, the truncated C2B domain of human Syt XV-b is most unlikely to function as a C2 domain, indicating that Syt XV-b behaves as a single C2 domain-containing protein, consistent with the findings in my previous study [5]. To our knowledge this type of alternative splicing has never been reported in other Syt isoforms [37,48–51].

Comparison of the gene structures of the human, mouse, and rat synaptotagmin XV

Based on the human Syt XV-a/b cDNA sequences, I also determined the full open reading frame of mouse Syt XV-a/b and rat Syt XV-a cDNA sequences by RT-PCR in combination with database searching (Fig. 1B

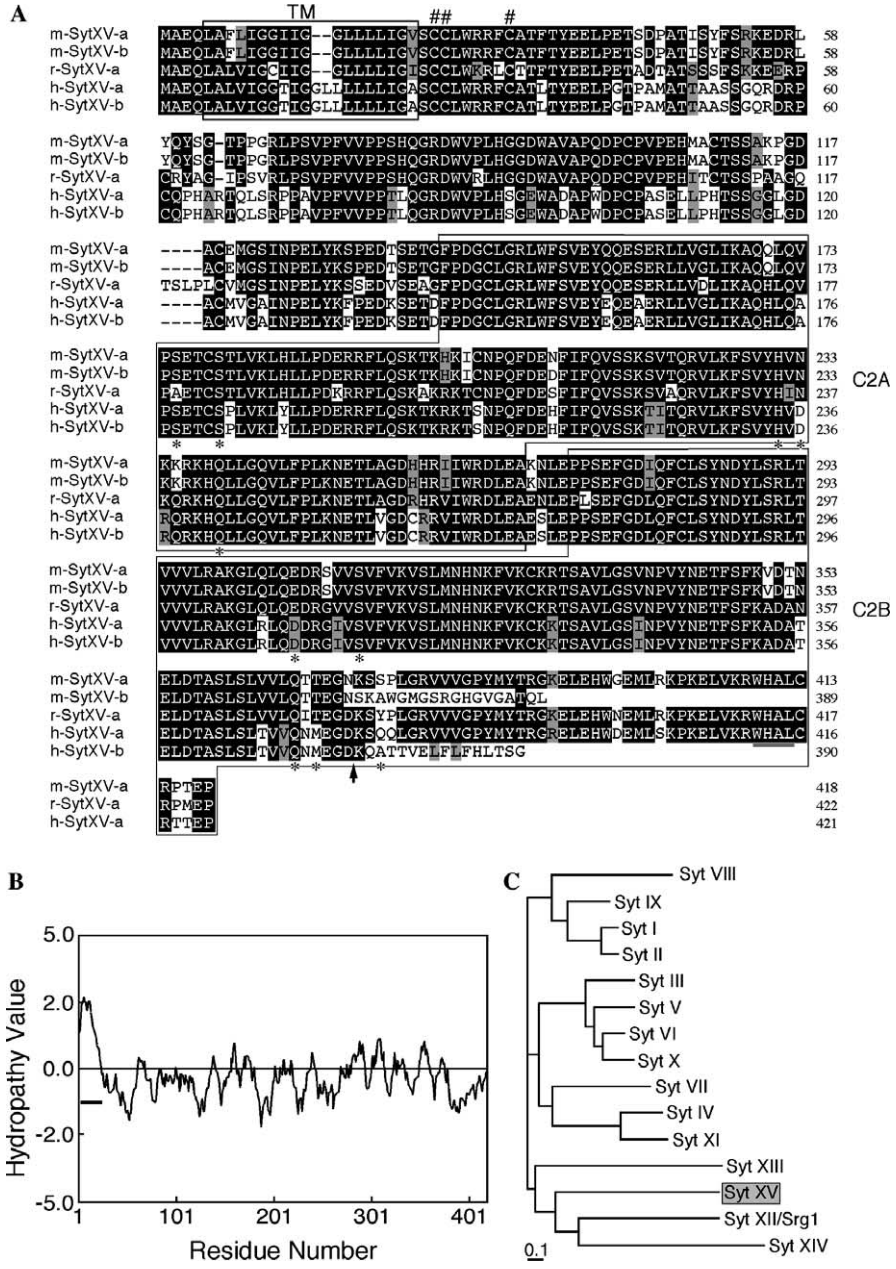

Fig. 2. Comparison of the Syt XV proteins of the mouse, the rat, and humans. (A) Sequence alignment of the mouse Syt XV-a/b, rat Syt XV-a, and human Syt XV-a/b. Residues in the sequences that are conserved and similar are shown against a black background and a shaded background, respectively. The arrow indicates the position of the alternative splicing site (see also Fig. 1). Open boxes indicate the putative transmembrane domain (TM) and two C2 domains (C2A and C2B domains). Asterisks indicate the positions of the five Asp or Glu residues in the C2 domain, which may be crucial for Ca^{2+}-binding by analogy with the Syt I-C2A domain [46]. The double underlined sequence is the WHXL motif, which may be crucial for docking to the plasma membrane and correct folding of the C2B domain [15,37,47]. The symbol # indicates the Cys residues that may be fatty-acylated between the transmembrane domain and spacer domains [36]. Amino acid numbers are indicated at the right of each line. (B) Hydropathy profile of mouse Syt XV-a, obtained according to Kyte and Doolittle [55]. Note that the mouse Syt XV-a has one hydrophobic region at the N-terminal domain, which may be responsible for the transmembrane domain. (C) Phylogenetic tree of the mouse Syt family proteins reported to date. The phylogenetic tree is depicted as described under "Materials and methods." Note that Syt XV-a is a distantly related isoform of Syt I (a putative neuronal Ca^{2+}-sensor), the same as Syt XII–XIV.

and C and Fig. 2A). The predicted mouse Syt XV-a, Syt XV-b, and rat Syt XV-a proteins consisted of 418, 389, and 422 amino acids having a calculated molecular weight of 47,268, 43,522, and 47,590, respectively. The mouse Syt XV-a protein showed 88.6% identity with the rat Syt XV-a protein and 78.6% identity with the human Syt XV-a protein. The N-terminal sequence of the rat Syt XV-a protein was different from that of the model protein (487 amino acids) predicted from the rat genome in the database (GenBank Accession No. XP_224703),

and the difference may be attributable to the connection of a wrong exon upstream of the real exon 1. Interestingly, the most C-terminal region of the mouse Syt XV-b and the human Syt XV-b did not show any significant homology (Fig. 2A). The genomic structures of the mouse, rat, and human Syt XV proteins were determined in public databases (http://www.ncbi.nlm.nih.gov/genome/seq/MmBlast.html, /RnBlast.html, or /HsBlast.html, respectively) by a BLAST search using default parameters. The mouse, rat, and human *syt XV* genes were mapped to chromosome 14, 16p15, and 10, respectively, and spanned about 10–12 kb. The exon–intron boundaries of each *syt XV* gene (Fig. 1, middle) were determined based on the cDNA sequence and AG-GT rule (i.e., splice acceptor and donor sequences). The genomic structure of Syt XV-a protein was exactly the same in mice, rats, and humans. Their Syt XV-a coding regions consist of 8 exons with introns of various lengths. The same as in other Syt isoforms, exon 2 encoded the transmembrane domain, and the C2A and C2B domains were encoded by two (exons 4–5) and three exons (exons 6–8), respectively [3,7,52]. The overall genomic structures of Syt XV-a protein resemble those of Syt VIIα (data not shown), although the positions of the introns are not exactly the same.

To my surprise, the exon–intron organization of the mouse and human Syt XV-b proteins was clearly different (Figs. 1A and B, bottom), although mouse and human Syt XV-a and Syt XV-b share exons 1–7. The human Syt XV-b protein was produced by connection of the exon 7 with exon 9, not with exon 8, which encodes the C-terminus of the C2B domain (Fig. 1A). By contrast, the mouse Syt XV-b protein was produced by connection of exon 7A with exon 7B, not with exon 8 (Fig. 1B), and as a result the C terminus of mouse and human Syt XV-b is completely different (Fig. 2A). Since a putative exon corresponding to the mouse exon 7B was also found in the rat genome (data not shown), it is possible that rat Syt XV-b protein is produced by a similar alternative splicing event. At present, however, there is no report of expression of rat Syt XV-b mRNA in the database. Further work is necessary to resolve this issue.

Characterization of the mouse Syt XV as a type I membrane protein

To determine whether the short N-terminal hydrophobic region of Syt XV (Fig. 2B, solid bar) indeed functions as a transmembrane domain in living cells, the mouse Syt XV-a protein was expressed in COS-7 cells, and its membrane association was evaluated by subcellular fractionation. As expected, the Syt XV-a protein was recovered only from the membrane fraction, and this association was resistant to treatment with 1 M NaCl and 0.1 M Na_2CO_3, pH 11 (Fig. 3B, lanes 4 and 5 in upper panel), as was Syt I protein (data not shown and [12]). Under these conditions, most peripheral membrane proteins (e.g., Syt VIΔTM, an alternative splicing isoform lacking a transmembrane domain; see [37]) were expected to be released from membranes. By contrast, when the hydrophobic region of Syt XV-a (amino acids 5–22) was deleted, the membrane association of Syt XVΔTM protein was highly sensitive to 1M NaCl and 0.1 M Na_2CO_3, pH 11 (Fig. 3B, lanes 4 and 5 in lower panel). The membrane association of Syt XVΔTM protein may be attributable to the WHXL

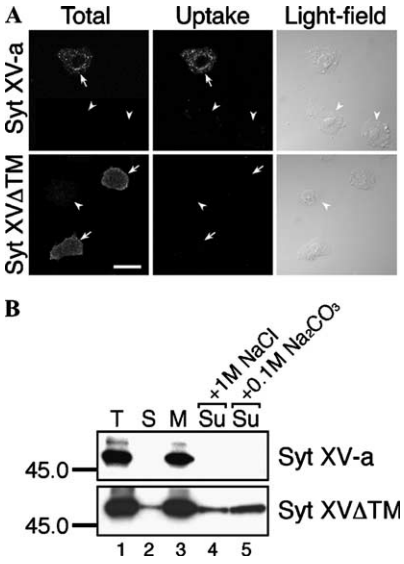

Fig. 3. Characterization of Syt XV-a as a new member of the synaptotagmin family. (A) Characterization of Syt XV-a as a type I membrane protein. FLAG-Syt XV-a and FALG-Syt XVΔTM were expressed in PC12 cells. PC12 cells were cultured in medium containing anti-FLAG mouse monoclonal antibody (Sigma Chemical; St. Louis, MO, USA). After washing with PBS to remove the unincorporated antibodies, PC12 cells were fixed and permeabilized, and the antibodies incorporated were visualized with anti-mouse Alexa Fluor 568 antibody (middle panels). Total expressed FLAG-tagged proteins were differently visualized by anti-FLAG rabbit polyclonal antibody and anti-rabbit Alexa Fluor 488 antibody (left panels). Note that FLAG-Syt XV-a-expressing cells (arrow in upper middle panel), but not FLAG-Syt XVΔTM-expressing cells (arrows in lower middle panel), took up antibodies in the cell body, as evidenced by the numerous dots. The arrowheads point to non-transfected cells. Scale bar = 20 μm. (B) Subcellular fractionation of COS-7 cells expressing T7-Syt XV-a (upper panel) and T7-Syt XVΔTM (lower panel). Membrane (M) and soluble (S) fractions were separated as described previously [12]. The membrane fraction was then resuspended in a buffer containing 1 M NaCl or 0.1 M Na_2CO_3, pH 11, and incubated for 1 h at 4 °C. After centrifugation at 100,000*g* for 1 h, the supernatants (Su) were recovered, and equal proportions of total (T), soluble, membrane, and supernatant fractions treated with 1 M NaCl or 0.1 M Na_2CO_3, pH 11, were subjected to 10% SDS–PAGE. The proteins were then transferred to a polyvinylidene difluoride membrane (Millipore; Bedford, MA, USA) and immunoblotted with HRP-conjugated anti-T7 tag antibody (1:10,000 dilution). Note that membrane association of Syt XV-a was insensitive to both treatments, suggesting that Syt XV-a is an integral membrane protein rather than a peripheral membrane protein. The positions of the molecular mass markers (kDa) are shown on the left. The results shown are representatives of two independent experiments.

motif of the C2B domain [15,37] rather than the phospholipid binding activity of the C2 domains (see below). These results indicate that the N-terminal hydrophobic region of the mouse Syt XV-a is essential for tight membrane association.

Anti-FLAG antibody-uptake experiments were performed to further characterize the Syt XV-a protein as a type-I integral membrane protein (Fig. 3A). In brief, PC12 cells expressing either FLAG-Syt XV-a or FLAG-Syt XVΔTM were cultured in the presence of anti-FLAG antibody for 2 h. The antibodies incorporated were visualized by anti-mouse Alexa Fluor 568 antibody (middle panels), and total FLAG-Syt XV proteins were stained separately with anti-FLAG rabbit polyclonal antibody and anti-rabbit Alexa Fluor 488 antibody (left panels) [12]. If the mouse Syt XV-a protein is a type-I membrane protein, the N-terminal FLAG tag should be exposed to the extracellular domain, and the FLAG·antibody complex would be incorporated into the cells via endocytosis. As expected, PC12 cells expressing FLAG-Syt XV-a protein took up antibody as evidenced by many dots in the cell body, especially in the peri-nuclear region (Fig. 3A, arrow in upper middle panel). By contrast, cells expressing FLAG-Syt XVΔTM and non-transfected cells (arrowheads) contained no antibody signals, although FLAG-Syt XVΔTM protein was also present near the plasma membrane (Fig. 3A, compare lower left and lower middle panels). I therefore concluded that mouse Syt XV-a is indeed a type-I membrane protein lacking an extracellular N-terminal domain and that Syt XV-a is a genuine member of the Syt family.

Phospholipid binding properties of the mouse Syt XV-a C2 domains

Members of the Syt family are often divided into two types in terms of their phospholipid binding ability [5,12,39,53,54]: a Ca^{2+}-dependent type (Syts I–VII, IX, and X) and a Ca^{2+}-independent type (Syts VIII and XII–XIV). I recently found that the presence of five conserved acidic residues (Asp or Glu) in the top loops 1 and 3 of the C2 structure is a good marker for the Ca^{2+}-dependent phospholipid binding ability of the Syt C2A domain [53]. Judging from their sequence alignment, the Syt XV-a C2 domains lack such acidic residues, in contrast to the Syt I C2A domain. Consistent with this, neither GST-Syt XV-C2A nor GST-Syt XV-C2B showed any Ca^{2+}-dependent or -independent PS/PC liposome (or PC alone) binding activity (data not shown), indicating that Syt XV should be classified as a Ca^{2+}-independent Syt.

Tissue distribution of the mouse Syt XV mRNA

The tissue distribution of mouse Syt XV-a and Syt XV-b mRNA was investigated by RT-PCR with specific primers (Fig. 4, top and middle panels, respectively). Syt XV-a mRNA and Syt XV-b mRNA were found in almost the same tissues (i.e., heart, lung, skeletal muscle, and testis) and at similar developmental stages (i.e., embryonic day 7 (E7)). Unlike the mRNAs of other Syt isoforms (Syts I–XIII), mRNA expression of Syt XV-a/b was almost absent in the brain, and it was highest on E7 [5,12,54].

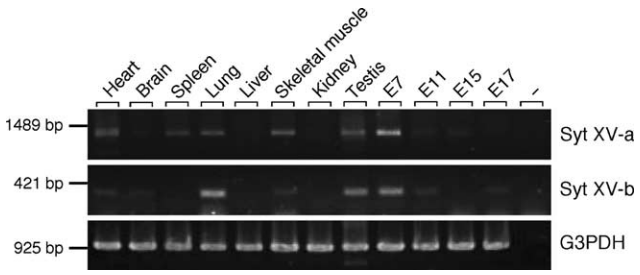

Fig. 4. Tissue distribution of mouse Syt XV. RT-PCR analysis of Syt XV-a mRNA and Syt XV-b mRNA in various tissues (heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis) and on E7, 11, 15, and 17 (top and middle panels, respectively). Note that the Syt XV-a mRNA and Syt XV-b mRNA are found in almost the same tissues and at similar developmental stages (i.e., E7). 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. "–" means without templates, as a negative control. The size of the molecular weight markers (λ/*Sty*I) is shown at the left of the panel. The results shown are representatives of two independent experiments.

Conclusion

In summary, I cloned and characterized a novel member of the Syt family, Syt XV-a, from mice, rats, and humans. Since no putative homologue of Syt XV-a was found in invertebrates (*Caenorhabditis elegans* and *Drosophila*), Syt XV-a is presumably retained only in vertebrates. Unlike other Syt family members, Syt XV-a/b mRNA is expressed outside the brain, and the Syt XV-a C2 domains lack Ca^{2+}-dependent phospholipid binding activity. These results suggest that Syt XV-a may be involved in constitutive membrane trafficking in selected non-neuronal tissues.

Acknowledgments

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