Characterization of KIAA1427 protein as an atypical synaptotagmin (Syt XIII)

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Synaptotagmin (Syt) belongs to a family of type-I membrane proteins and is a protein that consists of a short extracellular N-terminus, a single transmembrane domain, two C2 domains and a short C-terminus. Here, we cloned and characterized a mouse orthologue of human KIAA1427 protein as an atypical Syt (named Syt XIII). Subcellular fractionation and antibody-uptake experiments indicate that Syt XIII is indeed a type-I membrane protein, but, unlike other Syt isoforms, lacks an N-terminal extracellular domain. Syt XIII C2 domains show relatively little similarity to Syt I (less than 35% identity at the amino acid level), and lack key amino acids responsible for Ca^{2+} binding. Because of these substitutions, the Syt XIII C2 domains did not show Ca^{2+}-dependent phospholipid-binding activity, and Syt XIII is thus classified as a Ca^{2+}-independent isoform. By contrast, the Syt XIII C-terminal domain is highly homologous with other Syt isoforms and can function as a common receptor for neurexin Iα *in vitro*. Since Syt XIII is expressed in various tissues outside the brain, Syt XIII may be involved in constitutive vesicle transport.

Key words: C2 domain, oligomerization, phospholipid binding, vesicle docking, vesicle transport.

INTRODUCTION

Neurotransmitter release from nerve terminals in the brain is regulated by several families of Ca^{2+}-binding proteins having tandem C2 domains (named the C2A domain and C2B domain; reviewed in [1]). These domains were originally found in the Ca^{2+}-activated type of protein kinase C, and they are thought to function as a Ca^{2+}-dependent phospholipid-binding site (reviewed in [2]). Three proteins, synaptotagmin (Syt) I [1,3–5], rabphilin-3A (downstream target of rab3A) [6] and Doc2α [7], have been identified on synaptic vesicles to date. Syt I, an integral membrane protein of synaptic vesicles, is essential for the fusion step between the synaptic vesicle and the presynaptic plasma membrane, probably functioning as a Ca^{2+} sensor via the C2A domain [8–18]. By contrast, the latter two proteins play a regulatory role in a step before the final fusion step of the synaptic vesicle with the presynaptic plasma membrane [19,20].

Syts now constitute a large protein family in both vertebrates and invertebrates (reviewed in [4,5]). All members show type-I membrane topology ($N_{extracellular}/C_{cytoplasmic}$ orientation) and lack a signal-peptide sequence [21]. They consist of a short extracellular N-terminus, a single transmembrane region and tandem C2 domains in the cytoplasmic domain. In some cases, however, alternatively spliced variants (e.g. Syt VIΔTM) have been reported [22,23]. Involvement of Syt family members in vesicular traffic, other than in synaptic vesicle exocytosis, was reported recently. For instance, Syt I is also involved in Ca^{2+}-regulated exocytosis in some endocrine cells [16–18], neurite outgrowth of chick dorsal root ganglion neurons [24] and axonal repair in squid giant axons and PC12 cells [25,26]. Syt III or Syt VII is involved in insulin secretion by pancreatic β-cells [27–29]. Syt VII regulates Ca^{2+}-dependent lysosomal exocytosis in fibroblasts [30]. These findings suggest that the Syt family regulates various types of vesicular traffic, although the exact subcellular localization of most of the isoforms remains largely unknown.

Herein, we report the cDNA sequence of a mouse orthologue of human KIAA1427 protein [31]. We characterized this protein as an atypical type of Syt, and designated it Syt XIII. Syt XIII also shows type-I membrane topology, but, unlike other Syt isoforms, it lacks an extracellular domain at its N-terminus. We also examined the biochemical properties of Syt XIII and the tissue distribution of Syt XIII mRNA.

MATERIALS AND METHODS

Materials

Ex *Taq* and recombinant *Taq* DNA polymerases were obtained from Takara Shuzo Co. (Shiga, Japan) and Toyobo Biochemicals (Tokyo, Japan), respectively. Polyclonal and monoclonal antibodies (M2) against FLAG peptide were obtained from Zymed Laboratories (San Francisco, CA, U.S.A.) and Sigma (St. Louis, MO, U.S.A.), respectively. Horseradish peroxidase (HRP)-conjugated anti-(T7 tag) antibody and anti-(T7 tag) antibody-conjugated agarose were from Novagen (Madison, WI, U.S.A.). Dipalmitoyl L-α-phosphatidylcholine (PC) and dioleoyl L-α-phosphatidylserine (PS) were from Sigma. All other chemicals were commercial products of reagent grade. Solutions were made up in deionized water prepared with an Elix10 Water Purification

Abbreviations used: GST, glutathione S-transferase; HRP, horseradish peroxidase; Nxlα, neurexin Iα; PC, dipalmitoyl L-α-phosphatidylcholine; PS, dioleoyl L-α-phosphatidylserine; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase; Syt, synaptotagmin.

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The nucleotide sequence reported here is deposited in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases under accession number AB048947.

System and Milli-Q Biocel A10 System (Millipore Corp., Bedford, MA, U.S.A.).

Molecular cloning of mouse Syt XIII

cDNAs encoding the open reading frame of mouse Syt XIII were amplified from Marathon-Ready mouse adult brain cDNA by 5′-rapid amplification of cDNA ends (RACE; Clontech Laboratories, Palo Alto, CA, U.S.A.) as described previously [32]. The first 5′-RACE reactions were carried out using an adapter primer 1 (5′-CCATCCTAATACGACTCACTATAG-GGC-3′) and the following primer designed on the basis of the mouse EST sequence database (accession number AI050367); 5′-CTACAGGTGCAGTTGGTGCCACAT-3′ (C1 primer, antisense; amino acid residues 420–426). The second 5′-RACE reactions were carried out using an internal adapter primer 2 (5′-ACTCACTATAGGGCTCGAGCGGC-3′) and the following primer designed on the basis of the mouse EST sequence; 5′-AGCAAGTCATCCGGTAGCTC-3′ (C2 primer, antisense; amino acid residues 363–369). Both PCRs were carried out in the presence of Perfect Match PCR Enhancer (Stratagene, La Jolla, CA, U.S.A.) for 35 cycles, each consisting of denaturation at 94 °C for 1 min, annealing at 55 °C for 2 min and extension at 72 °C for 3 min. The first and second PCR products were purified from an agarose gel on a Micro-Spin column (Amersham Pharmacia Biotech, Little Chalfont, Bucks., U.K.) as described previously [33], and they were inserted directly into the pGEM-T Easy vector (Promega, Madison, WI, U.S.A.). Both strands of the cDNA inserts were randomly sequenced with a Hitachi SQ-5500 DNA sequencer until a cDNA clone including the putative initiation methionine was obtained.

Expression constructs

Full-length Syt XIII cDNA including the open reading frame was constructed using appropriate restriction-enzyme sites on the pGEM-T Easy vector (named pGEM-T-Syt XIII). pEF-T7 (or -FLAG in place of -T7) -Syt XIII, -Syt XIIIΔN and -*N*-Gly-Syt XIII were constructed by PCR using Met primer (5′-GCGGA-TCCATGGTGCTGTCGGTGCCT-3′; amino acid residues 1–6) or ΔN primer (5′-GCGGATCCCGACACATGCACCC-CAAGAA-3′; amino acid residues 31–37) and C1 primer as described previously [33]. Other expression constructs (pEF-T7-Syt I, -Syt VIΔTM, -FLAG-Syt VIΔTM and -Syt XI) were prepared as described previously [21,22,33]. Plasmid DNA was prepared using Wizard mini preps (Promega) or QIAGEN Maxi prep kits.

Preparation of glutathione S-transferase (GST) fusion proteins

The pGEX-4T-3 vector (Amersham Pharmacia Biotech), carrying fragments of the Syt XIII C2A or C2B domains, was essentially constructed by PCR [34] using pGEM-T-Syt XIII as a template. The following pairs of oligonucleotides with appropriate restriction-enzyme sites (underlined) and/or termination codons (bold letters) were used for amplification: C2A upper primer (sense), 5′-GCGGATCCGAGAAGGCTGCCA-GCTGGAA-3′ and C2A lower primer (antisense), 5′-GCACTAG**TCA**CTTTGCTGTGGTCTTCAGCT-3′; and C2B upper primer (sense), 5′-GCGGATCCGCAAAGGAGCCAT-CTGCAGG-3′ and SP6 primer. The resulting pGEX-Syt XIII-C2A and -C2B were confirmed by DNA sequencing and transformed into *Escherichia coli* JM109. GST fusion proteins were expressed and purified on glutathione–Sepharose (Amersham Pharmacia Biotech) by the standard method [35]. GST-Syt XIII-C2A and GST-Syt XIII-C2B encoded amino acids 151–282 and 281–426 of mouse Syt XIII, respectively. GST-Syt I-C2A was prepared as described previously [34].

Subcellular fractionation

COS-7 cells transfected with pEF-Syt constructs (5×10^5 cells/10 cm dish, the day before transfection) were harvested 3 days after transfection and homogenized in 1 ml of 0.32 M sucrose/1 mM EDTA/0.1 mM PMSF/10 μM leupeptin/10 μM pepstatin A/1 mM β-mercaptoethanol/5 mM Tris/HCl, at pH 7.5, in a glass-Teflon Potter homogenizer with 10 strokes at 900–1000 rev./min. The homogenate was centrifuged at 1000 ***g*** for 10 min at 4 °C. The supernatant (300 μl) was further centrifuged at 100000 ***g*** for 1 h at 4 °C to precipitate membrane fractions. The membrane fractions were resuspended in 300 μl of 1 M NaCl or 0.1 M Na_2CO_3, at pH 11, containing protease inhibitors. After incubation for 1 h at 4 °C, the membrane fractions were precipitated by centrifugation at 100 000 ***g*** for 1 h at 4 °C, and were dissolved in 300 μl of SDS sample buffer. Then, 10 μl each of supernatants and membrane fractions were subjected to SDS/PAGE (10% gels) and analysed by immunoblotting as described previously [22,33].

Uptake of anti-FLAG antibody

Transfection of pEF-FLAG-Syts into PC12 cells [$(0.8–1) \times 10^5$ cells/35 mm dish, the day before transfection; MatTek Corp., Ashland, MA, U.S.A.] was performed as described previously [22]. Before fixation (3 h), PC12 cells expressing pEF-FLAG-Syt XIII, pEF-FLAG-Syt VIΔTM or pEF-FLAG-Syt XI were incubated in medium containing anti-FLAG (M2) antibody (3 μg/ml). After being washed twice with PBS, the cells were fixed and the incorporated antibodies were visualized by anti-mouse Alexa 568 antibody (Molecular Probes, Eugene, OR, U.S.A.). Total FLAG-Syt proteins were stained separately with anti-FLAG (0.5 μg/ml) rabbit polyclonal antibody and anti-rabbit Alexa 488 antibody (Molecular Probes) as described previously [22,36]. Immunoreactivity was analysed using a fluorescence microscope (TE300, Nikon, Tokyo, Japan) attached to a laser confocal scanner unit CSU 10 (Yokogawa Electric Corp., Tokyo, Japan) and a HiSCA CCD camera (C6790, Hamamatsu Photonics, Hamamatsu, Japan). Images were pseudo-coloured and superimposed using Adobe Photoshop version 4.0.

Neurexin Iα (NxIα) binding assay

cDNA encoding the cytoplasmic portion of the mouse NxIα was amplified by reverse transcriptase (RT)-PCR from mouse cerebellum cDNAs [34] using the following set of primers designed on the basis of previously reported rat sequences [37]: 5′-GCGGATCCATGTACAAGTACAGAAACCGG-3′ (sense, amino acids 1452–1458) and 5′-GCGAATTCTCAGACATAA-TACTCCTTAT-3′ (antisense, amino acids 1502–1507). Reactions were carried out in the presence of Perfect Match PCR Enhancer for 30 cycles, each consisting of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min. The PCR products were digested with *Bam*HI/*Eco*RI (underlined above), and subcloned into the *Bam*HI/*Eco*RI site of the pGEX-2T vector (Amersham Pharmacia Biotech). The resulting pGEX-2T-NxIα-C vector was confirmed by DNA sequencing. GST fusion proteins were prepared as described above.

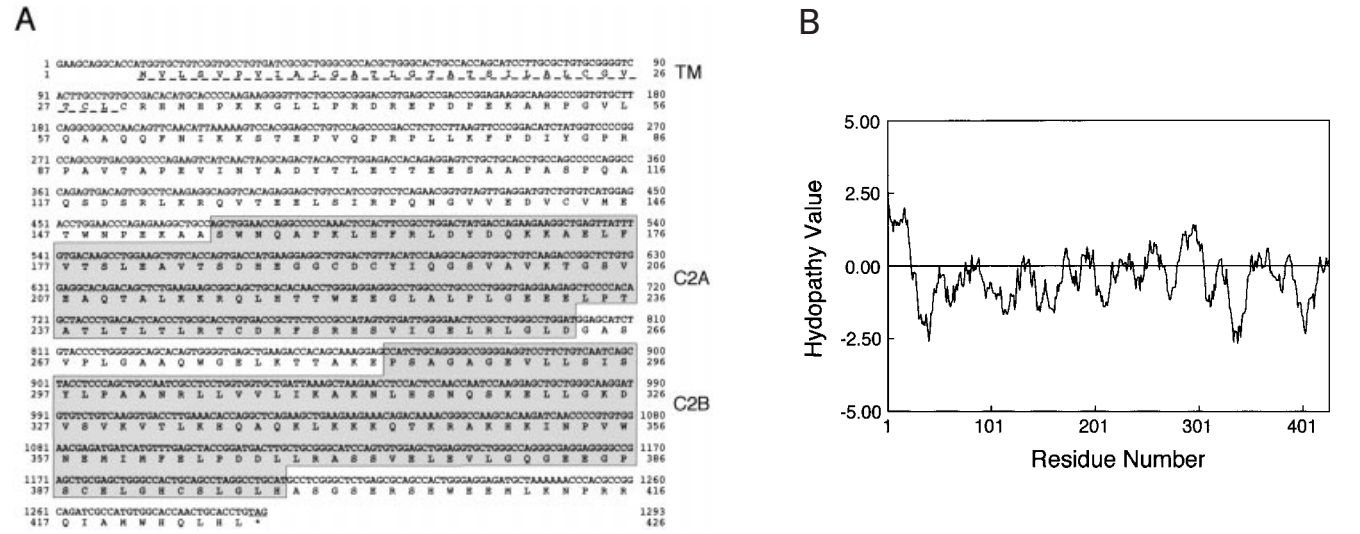

Figure 1 Nucleotide and deduced amino acid sequences and hydrophobic profile of mouse Syt XIII

(**A**) The deduced amino acid sequence is represented by the single-letter code below the nucleotide sequence. The nucleotide and deduced amino acid numbers are indicated on both sides of each line. In-frame stop codons defining the 3′-ends of the reading frame are indicated by underlining and an asterisk. The dashed underlining indicates the putative transmembrane domain. Boxes indicate two C2 domains. (**B**) Hydropathy profile of mouse Syt XIII, obtained according to Kyte and Doolittle [53]. Note that the mouse Syt XIII has one hydrophobic region at the N-terminal domain, which may be responsible for the transmembrane domain.

After transfection (3 days), COS-7 cells (10 cm dish) expressing T7-Syt XIIIΔN or T7-Syt VIΔTM were homogenized in 50 mM Hepes/KOH, pH 7.2/0.1 mM PMSF/10 μM leupeptin/10 μM pepstatin A, and the proteins were then solubilized with 1% Triton X-100 at 4 °C for 1 h. After centrifugation at 17360 ***g*** for 10 min at 4 °C, each lysate (400 μl) was incubated with glutathione–Sepharose beads coupled with 5 μg of GST-NxIα-C (or GST alone; wet volume, 15 μl; Amersham Pharmacia Biotech) for 1 h at 4 °C. After washing the beads with 1 ml of the incubation buffer five times, GST-NxIα-C protein-bound fractions were examined by SDS/PAGE (10% gels) and immunoblotting with HRP-conjugated anti-(T7 tag) antibody as described previously [33].

RT-PCR analysis

Mouse first-strand cDNAs prepared from various tissues and developmental stages were obtained from Clontech Laboratories (Mouse MTC Panel I). PCR reactions were carried out in the presence of Perfect Match PCR Enhancer (Stratagene) for 30 or 35 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 C2B upper and C1 primers described above were used for amplification. The PCR products were analysed by 1% agarose gel and ethidium bromide staining. The authenticity of the products was verified by subcloning into the pGEM-T Easy vector and DNA sequencing.

Sequence analyses

Multiple sequence alignment and depiction of the phylogenetic tree of the mouse Syt family were performed using the CLUSTALW program (http://watson.genes.nig.ac.jp/homology/clustalw.shtml) and the appropriate default parameters (gapopen = 10, gapext = 0.05, gapdist = 8 and maxdiv = 40).

Miscellaneous procedures

Co-transfection of pEF-T7-Syts and pEF-FLAG-Syts into COS-7 cells (5×10^5 cells/10 cm dish, the day before transfection) was carried out using the LipofectAmine Plus reagent according to the manufacturer's instructions (Life Technologies, Rockville, MD, U.S.A.) [21,22,33]. Cells were harvested 3 days after transfection, and the proteins were solubilized with buffer containing 1% Triton X-100, 250 mM NaCl, 50 mM Hepes/KOH, pH 7.2, 0.1 mM PMSF, 10 μM leupeptin and 10 μM pepstatin A at 4 °C for 1 h. Immunoprecipitation of T7-Syts in the presence or absence of 500 μM Ca^{2+} by anti-(T7 tag) antibody-conjugated agarose, SDS/PAGE (10% gels) and immunoblotting analyses were also performed as described previously [33]. Preparation of liposomes consisting of PC and PS (1:1, w/w) and a phospholipid-binding assay were performed as described previously [38,39]. The protein concentrations were determined with a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, U.S.A.) using BSA as a reference.

RESULTS

Characterization of KIAA1427 protein as Syt XIII

In a previous study, we searched for novel tandem C2 proteins from the mouse EST and genome sequence databases using the mouse Syts I–XII protein sequences [33] as bait [32]. We have identified and cloned five novel genes, including Doc2γ [32], three related tandem C2 proteins without a transmembrane domain (M. Fukuda, unpublished work) and one putative Syt isoform (Figure 1A). The 1293 nucleotide sequence encoding a novel Syt isoform contains an open reading frame encoding 426 amino acids with a calculated molecular mass of 46867 Da (Figure 1A). Homology search analysis indicated that this protein is an orthologue of human KIAA1427 protein (90% identity at the amino acid level) [31]. Unexpectedly, however, the KIAA1427 protein is reportedly one of the single C2 domain-

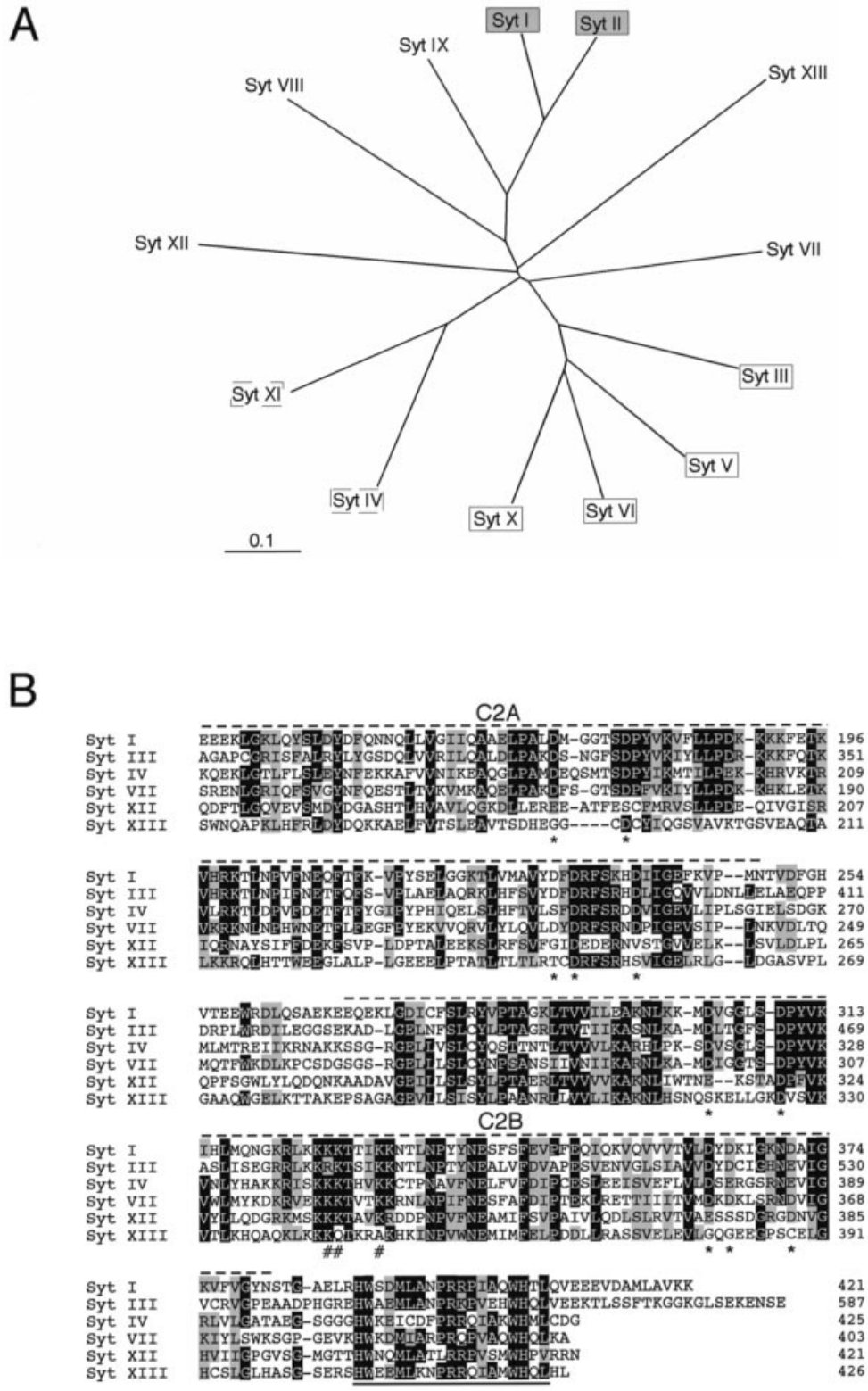

Figure 2 Phylogenetic tree and the sequence alignment of the mouse Syt family

(**A**) Phylogenetic tree of mouse Syts I–XIII (Syt XII is also called srg1 [54]). The phylogenetic tree is depicted as described in the Materials and methods section. Subfamilies of Syt isoforms are in shaded boxes (Syts I and II), white boxes (Syts III, V, VI and X) [33,55] or dashed boxes (Syts IV and XI). (**B**) Sequence alignment of mouse Syt family C2 domains. Residues for which half of the sequences are conserved or similar are shown on black or shaded backgrounds, respectively. Asterisks indicate the five conserved aspartate or glutamate residues, which may be crucial for Ca^{2+} binding by analogy with the Syt I C2A domain [49,50]. # Indicates the position of the Lys residues responsible for Ins(1,3,4,5)P_4 binding, AP-2 [clathrin assembly (adapter) protein] binding and self-oligomerization of Syt I (the so-called C2B effector domain) [47,51]. The dashed lines and solid line indicate the two C2 domains and the Nx-binding site [40], respectively. Amino acid numbers are indicated to the right.

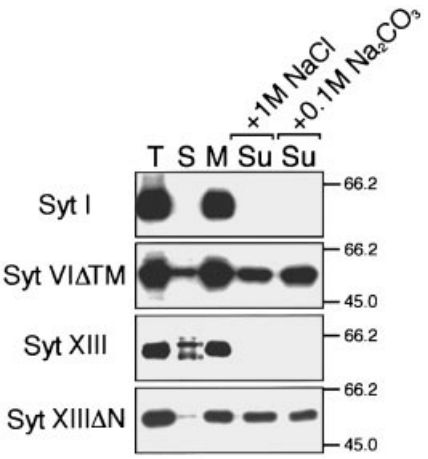

Figure 3 Subcellular fractionation of COS-7 cells expressing T7-Syt XIII

Subcellular fractionation of COS-7 cells expressing pEF-T7-Syt I (top panel), -Syt VIΔTM (second panel), -Syt XIII (third panel) or -Syt XIIIΔN (bottom panel). Membrane (M) and soluble (S) fractions were separated as described in the Materials and methods section. The membrane fraction was further resuspended in a buffer containing 1 M NaCl or 0.1 M Na_2CO_3, at pH 11, and incubated for 1 h at 4 °C. After centrifugation at 100 000 ***g*** for 1 h, the supernatants (Su) were recovered. Equal proportions (10 μl) of total (T), membrane, soluble and supernatant fractions treated with 1 M NaCl or 0.1 M Na_2CO_3, pH 11, were subjected to SDS/PAGE (10% gels) and transferred to a PVDF membrane (Millipore), followed by immunoblotting with HRP-conjugated anti-(T7 tag) antibody. Note that membrane association of Syt XIII is insensitive to both treatments, as is the case with Syt I. By contrast, deletion of the N-terminal hydrophobic region of Syt XIII (Syt XIIIΔN) resulted in dissociation from the membrane with the above treatments, as was observed with Syt VIΔTM. Asterisks indicate the non-specific interaction of HRP-conjugated anti-(T7 tag) antibody. The positions of the molecular-mass markers (kDa) are shown on the right. The results shown are representative of three independent experiments.

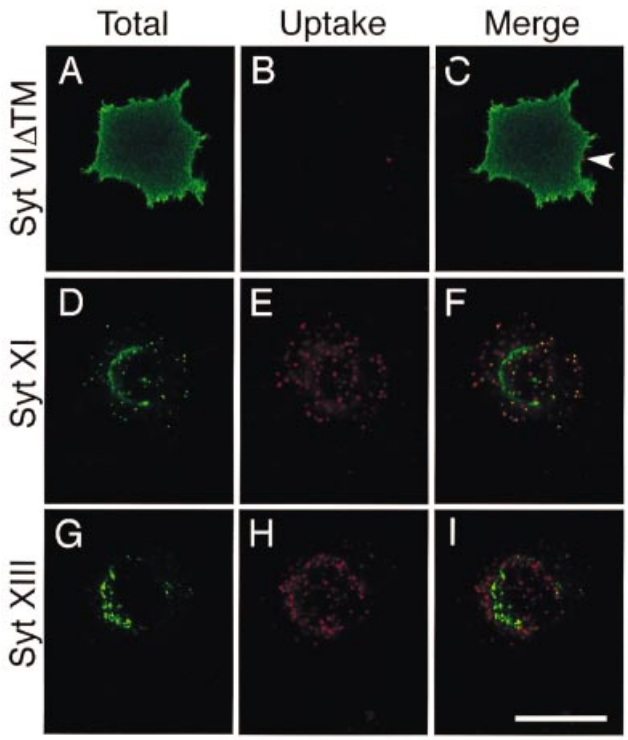

Figure 4 Characterization of Syt XIII as a type-I membrane protein

FLAG-Syt VIΔTM (**A**–**C**), -Syt XI (**D**–**F**) and -Syt XIII (**G**–**I**) were expressed in PC12 cells. PC12 cells were cultured in medium containing anti-FLAG (M2) (in **B**, **E** and **H**). After being washed with PBS to remove the unincorporated antibodies, PC12 cells were fixed and permeabilized, and the incorporated antibodies and total recombinant proteins were differently visualized by employing two different antibodies. (**A**, **D** and **G**) Total expressed proteins visualized by anti-FLAG (rabbit) antibody (green). (**B**, **E** and **H**) Incorporated anti-FLAG (M2) antibodies (red). (**C**, **F** and **I**) Superimpositions of **A** and **B**, **D** and **E**, and **G** and **H**, respectively. Note that FLAG-Syt XIII- and -Syt XI-expressing cells took up antibodies, as shown by numerous red dots, whereas Syt VIΔTM-expressing cells did not include antibodies (in **B**). The arrowhead probably indicates non-specific interaction of the antibodies with the plasma membrane. Scale bar, 20 μm.

containing proteins lacking a transmembrane domain [31]. Sequence alignment of the mouse orthologue of human KIAA1427 protein with mouse Syt isoforms shows clearly that it contains the C2A domain, C2B domain (broken lines in Figure 2B) and $HWX_{13}WHXL$ motif at the C-terminus (solid line in Figure 2B), which is a consensus sequence for Nx binding [40]. In addition, the hydrophobic profile of the mouse orthologue of human KIAA1427 protein shows the hydrophobic region to be present at the N-terminus (Figure 1B), although its hydrophobicity is weaker than that of Syt I (results not shown). Therefore, we tentatively identified KIAA1427 as the 13th isoform of Syt (Syt XIII).

To examine whether the N-terminal hydrophobic region is indeed essential for membrane association, the mouse Syt XIII protein was expressed in COS-7 cells and its membrane association was evaluated by subcellular fractionation (see the Materials and methods section for details). As shown in Figure 3 (top and third panels), the Syt XIII proteins were recovered only from the membrane fraction, and this association was resistant to treatments with 1 M NaCl and 0.1 M Na_2CO_3, at pH 11, as were Syt I proteins. Under these conditions, most peripheral membrane proteins were expected to be released from membranes. By contrast, membrane association of Syt VIΔTM, a major alternatively spliced variant lacking a transmembrane domain [22], was highly sensitive to treatments with both 1 M NaCl and 0.1 M Na_2CO_3 at pH 11 (Figure 3, second panel from the top). When the hydrophobic region of Syt XIII (amino acid residues 1–30) was deleted, Syt XIIIΔN proteins were also recovered from the membrane fraction, but were released from the membrane by the above treatments (Figure 3, bottom panel). These results indicate that the Syt XIII protein has at least two independent mechanisms for membrane association; the N-terminal hydrophobic region responsible for 1 M NaCl- and 0.1 M Na_2CO_3-resistant membrane associations, and the C-terminal domain containing two C2 domains responsible for 1 M NaCl- and 0.1 M Na_2CO_3-sensitive membrane associations (see also Figure 6, below).

Since Syt XIII has a putative palmitoylation site just after the hydrophobic region, which may also be responsible for membrane association [41,42], we further examined whether Syt XIII is an integral membrane protein (i.e. type-I membrane protein). To address this possibility, we performed anti-FLAG antibody-uptake experiments (Figure 4). When PC12 cells expressing FLAG-Syt XI, an integral membrane protein mainly associated with Golgi (Figure 4D, green), were cultured in the presence of anti-FLAG (M2) antibody for 3 h, the anti-FLAG antibodies were incorporated into the cells via endocytosis by recognizing the extracellular N-terminal domain of FLAG-Syt XI (Figure 4E, red dots; Figure 4F, superimpositions of D and E). The incorporation of antibody completely depended on the expression of FLAG-Syt XI proteins, because untransfected cells took up virtually no antibodies (results not shown). When FLAG-Syt

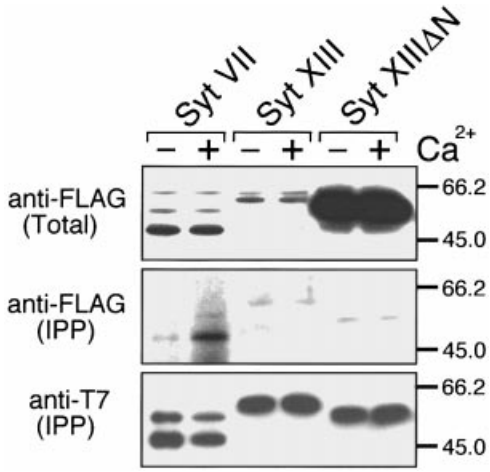

Figure 5 Self-oligomerization property of Syt XIII

Top panel: total homogenates of COS-7 cells expressing FLAG-Syt VII, -Syt XIII or -Syt XIIIΔN were subjected to SDS/PAGE (10%) and analysed by immunoblotting with anti-FLAG rabbit antibody (5 μg/ml). Middle panel: pEF-T7-Syt VII, XIII or XIIIΔN and pEF-FLAG-Syt VII, XIII or XIIIΔN were co-transfected into COS-7 cells. Expressed proteins were solubilized with 1% Triton X-100 and immunoprecipitated by anti-(T7 tag) antibody-conjugated agarose as described previously [22,33]. Co-immunoprecipitated FLAG-Syts (IPP) were first detected by anti-FLAG rabbit antibody (5 μg/ml). Bottom panel: the blot from the middle panel was stripped and reprobed with HRP-conjugated anti-(T7 tag) antibody to ensure loading of equal amounts of T7-Syt proteins. Note that Syt XIII and Syt XIIIΔN show no apparent Ca^{2+}-dependent self-oligomerization activities, as compared with Syt VII. The positions of the molecular-mass markers (kDa) are shown on the right. The results shown are representative of four independent experiments.

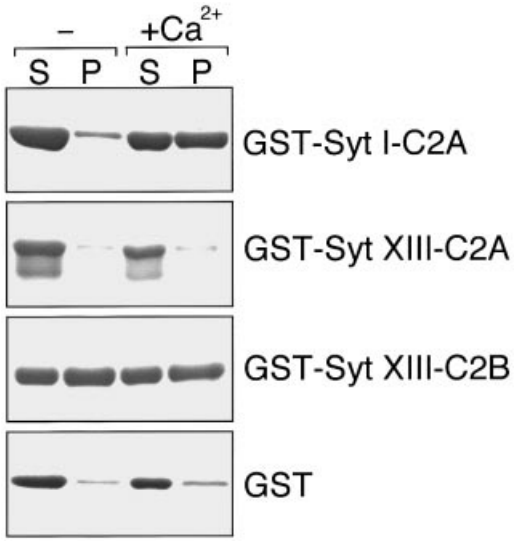

Figure 6 Phospholipid-binding properties of Syt XIII C2 domains

Liposomes and GST fusion proteins were incubated in 50 mM Hepes/KOH, at pH 7.2, in the presence of 2 mM EGTA (—) or 1 mM Ca^{2+} for 15 min at room temperature. After centrifugation at 12000 ***g*** for 10 min, the supernatants (S, non-binding fraction) and pellets (P, phospholipid-binding fraction) were separated as described previously [38,39]. Equal proportions of the supernatants and pellets were subjected to SDS/PAGE (10% gels) and then stained with Coomassie Brilliant Blue R-250. Note that the C2A domain of Syt XIII did not bind liposomes, whereas the C2B domain of Syt XIII did bind liposomes regardless of whether Ca^{2+} was present. By contrast, the C2A domain of Syt I bound liposomes only in a Ca^{2+}-dependent manner. The bottom panel indicates the negative control, GST. The results shown are representative of three independent experiments.

XIII proteins were expressed in PC12 cells, they were localized mainly in the peri-nuclear region (probably Golgi), but there were also several dots in the cytosol, apparently indicating transport from the Golgi to the plasma membrane (Figure 4G, green). The FLAG-Syt XIII-expressing cells also took up antibodies which appeared as numerous dots (Figure 4H, red dots; Figure 4I, superimpositions of G and H). By contrast, FLAG-Syt VIΔTM-expressing cells could not take up antibodies (Figure 4B, red), although FLAG-Syt VIΔTM protein itself showed enrichment around the plasma membrane (Figure 4A, green; Figure 4C, superimpositions of A and B) [22]. In some cases, a few red dots were associated with the plasma membrane in FLAG-Syt VIΔTM-expressing cells (Figure 4C, arrowhead), which may represent a non-specific interaction of antibody with the plasma membrane. In addition, T7-*N*-Gly-Syt XIII, which carries an artificial N-glycosylation site at the N-terminus [21], could not be N-glycosylated, probably due to the proximity of the recognition sequence to the transmembrane domain (results not shown). Therefore, we concluded that Syt XIII is indeed a type-I membrane protein lacking an extracellular N-terminal domain, although it lacks a signal peptide sequence.

Self-oligomerization properties of Syt XIII

Recently, we and others found several Syt isoforms from Ca^{2+}-dependent (via the C2 domains) and -independent (via the N-terminal domain) homo- or hetero-oligomers [21,33,43–48]. At least in Syt I, self-oligomerization activity is believed to be essential for efficient Ca^{2+}-dependent neurotransmitter release, based on analysis of *Drosophila syt I* mutants (reviewed in [10]). To examine whether Syt XIII also shows this oligomerization property, we performed T7- and FLAG-Syts co-expression assays [21,33,43]. As shown in Figure 5, both Syt XIII and Syt XIIIΔN showed only weak Ca^{2+}-independent self-oligomerization activity, whereas there was no Ca^{2+}-dependent oligomerization activity. Consistent with this, Syt XIII essentially lacks the C2B effector domain responsible for Ca^{2+}-dependent oligomerization, as demonstrated in Syt I [47] (Figure 2B, #). By contrast, Syt VII showed robust Ca^{2+}-dependent oligomerization activity [21,43].

Phospholipid-binding properties of Syt XIII C2 domains

The C2 domains of Syt family members were originally identified in Ca^{2+}-dependent protein kinase C, and some have been shown to possess Ca^{2+}-dependent phospholipid-binding activity (reviewed in [5]). In Syt I, the Ca^{2+}/phospholipid-binding site of the C2A domain has been shown to be important for secretory vesicle exocytosis [13,15–17]. Crystallographic and mutational analyses of the Syt I C2A domain indicated that five aspartate (or glutamate) residues are essential for Ca^{2+} binding [49,50] (see Figure 2B, *). Interestingly, Syt XIII lacks such aspartate or glutamate residues in both C2A and C2B domains, suggesting that Syt XIII should be classified as a Ca^{2+}-independent Syt isoform. To address this issue, a phospholipid (PS/PC liposomes)-binding assay was performed using GST fusion proteins (Figure 6). GST-Syt XIII-C2A did not bind PS/PC liposomes regardless of whether Ca^{2+} was present [only recovered in the supernatant (S) fraction; Figure 6, second panel from the top], whereas GST-Syt XIII-C2B bound PS/PC liposomes even in the absence of Ca^{2+} (third panel from the top). Due to this Ca^{2+}-independent phospholipid-binding activity of the Syt XIII C2B domain, Syt XIIIΔN proteins without a transmembrane domain can associate with the membrane fraction (Figure 3,

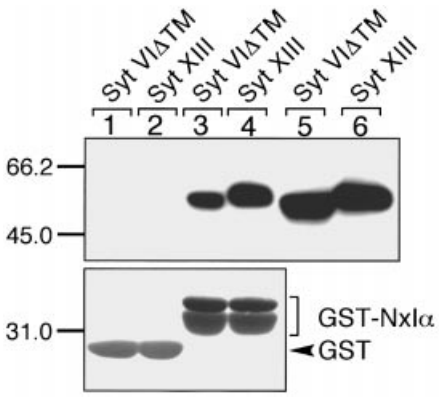

Figure 7 NxIα-binding property of Syt XIII

A GST pull-down assay was performed as described in the Materials and methods section. After immunoblotting with HRP-conjugated anti-(T7 tag) antibody (upper panel), blots were stained with Amido Black to ensure loading of equivalent amounts of GST fusion proteins (lower panel). T7-Syt VIΔTM or Syt XIIIΔN proteins were incubated with GST (lane 1 or 2) or GST-NxIα (lane 3 or 4), respectively. A 1/40 volume of total proteins used for the assay was loaded on lanes 5 and 6. Note that Syt XIIIΔN bound GST-NxIα, but not GST alone, as was the case with Syt VIΔTM in the absence of Ca^{2+}. The results shown are representative of three independent experiments.

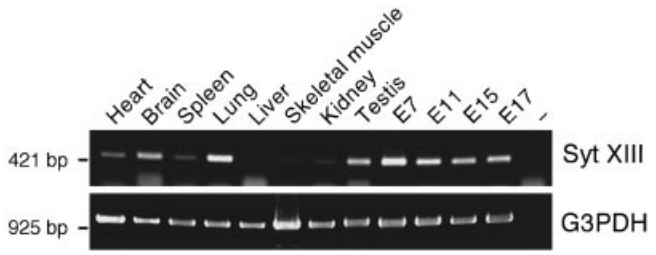

Figure 8 Tissue distribution of mouse Syt XIII

RT-PCR analysis of Syt XIII mRNAs in various tissues (heart, brain, spleen, lung, liver, skeletal muscle, kidney and testis) and on embryonic days (E) 7, 11, 15 and 17 (upper panel). RT-PCR analysis of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA was also performed (lower panel) to ensure the use of equivalent amounts of first-strand cDNA for RT-PCR analysis. The sizes of the molecular-mass markers (λ/StyI) are shown on the left. The results shown are representative of two independent experiments.

bottom panel). By contrast, GST-Syt I-C2A bound liposomes only in the presence of Ca^{2+} (Figure 5, top panel).

NxIα binding to Syt XIII

Although Syt XIII lacks the effector domain for Ca^{2+} in the C2A domain (Figure 2B, *), as well as that for self-oligomerization in the C2B domain (Figure 2B, #), the short C-terminus of Syt XIII is highly homologous to that of other isoforms (Figure 2B, underlined sequence). This domain is essential for NxIα binding *in vitro* [40], and is suggested to be involved in the docking of vesicles to plasma membranes. As expected, Syt XIII bound GST-NxIα-C, but not GST alone, regardless of whether Ca^{2+} was present (Figure 7, lanes 3 and 4).

Tissue distribution of Syt XIII

To examine the tissue distribution of mouse Syt XIII, RT-PCR was performed with specific primers (Figure 8). Syt XIII mRNA was found in all tissues tested, except for liver, and its expression levels were highest in the brain, lung and testis. In addition, expression of Syt XIII mRNA seemed to peak on embryonic day 7 and thereafter gradually decreased to the adult level.

DISCUSSION

In this study, we isolated and characterized KIAA1427 protein as a novel Syt isoform that has a single transmembrane domain, two C2 domains and a short C-terminus, and this protein was thus designated Syt XIII. Syt XIII is distinguishable from other Syt isoforms in that it lacks an extracellular N-terminal domain (summarized in Figure 9) and is classified as belonging to a new Syt subfamily, as demonstrated by phylogenetic analysis (Figure 2A). Sequence alignment of the mouse Syt family indicates that Syt XIII lacks aspartate (or glutamate) residues responsible for Ca^{2+} binding in both C2 domains [49,50] (G189, T245 and S253 in the C2A domain; S319, G380, G382 and C388 in the C2B domain; Figure 2B, asterisks). Because of this substitution, the Syt XIII C2A domain has no phospholipid-binding activity, whereas the Syt XIII C2B domain binds phospholipids regardless of whether Ca^{2+} is present (Figure 6). In addition, although the putative C2B effector domain is highly conserved among Syts I–XII (Lys clusters, # in Figure 2B), the corresponding region of Syt XIII is not always occupied by positively charged amino acids (Lys or Arg) (Figure 2B, #). Therefore, the Syt XIII C2B domain is unlikely to bind inositol polyphosphates or the C2B domain itself with high affinity [47,51]. Indeed, Syt XIII did not show Ca^{2+}-dependent self-oligomerization activity (Figure 5). In contrast with the different biochemical properties of Syt family C2 domains, the short C-terminal domain ($HWX_{13}WHXL$ motif) has been highly conserved during evolution as well as among Syt family members, and Syt XIII also bound NxIα *in vitro* (Figure 7). In addition, we recently showed that the conserved C-terminus of the Syt family is essential for plasma-membrane association [22,52] and that the Syt I C-terminus is indeed involved in synaptic vesicle docking to presynaptic plasma membranes in the squid giant synapse [52]. Therefore, it is likely

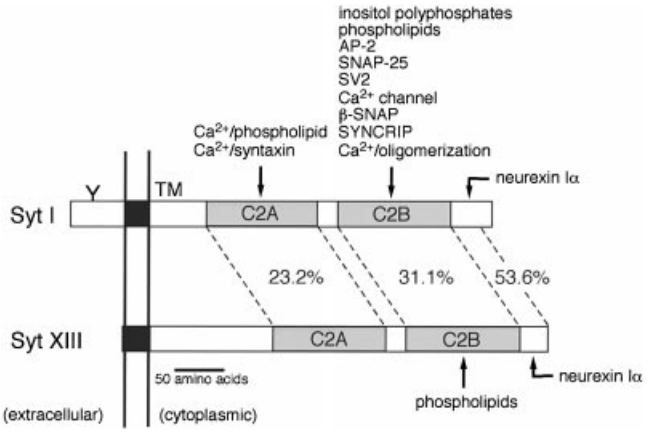

Figure 9 Schematic representation of Syt I and Syt XIII

The transmembrane domain (TM) and both C2 domains are shown as black boxes and shaded boxes, respectively. Y indicates N-glycosylation. Amino acid identities of each domain of Syt I and Syt XIII are indicated by percentages. Only the short C-termini of the two isoforms are highly conserved (53.6% identity). Arrows indicate the ligands of Syt I reported to date (reviewed in [5]), and those of Syt XIII identified in this study. SNAP-25, 25 kDa synaptosome-associated protein; AP-2, clathrin assembly (adapter) protein; SV2, synaptic vesicle protein 2; SNAP, soluble *N*-ethylmaleimide-sensitive-factor attachment protein; SYNCRIP, Syt-binding, cytoplasmic RNA-interacting protein.

that Syt XIII may be involved in the docking of vesicles to plasma membranes [40] rather than Ca^{2+}-regulated exocytosis, because the C2 domains of Syt XIII did not show Ca^{2+}-dependent properties. When Syt XIII is expressed in PC12 cells, Syt XIII proteins are apparently transported from the Golgi-like perinuclear compartment to the cell periphery (plasma membrane) constitutively via vesicle transport (Figure 4). Taking these observations together, and given that Syt XIII is expressed in various tissues outside the brain (Figure 8), we speculate that Syt XIII is involved in constitutive vesicle traffic, especially at docking step of transport vesicles to plasma membrane. Further work is necessary to determine whether Syt XIII is involved in transport vesicle docking to the plasma membrane.

In summary, we identified an atypical isoform of Syt, and designated it Syt XIII. Based on the biochemical properties of Syt XIII C2 domains and the C-terminus, we suggest that Syt XIII may be involved in the docking of vesicles to plasma membranes via a constitutive pathway.

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