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Synaptotagmin V: a novel synaptotagmin isoform expressed in rat brain

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Abstract Regulated Ca^{2+}-dependent release of transmitters from synaptic vesicles is an important characteristic of chemical neurotransmission. Synaptotagmins are abundant synaptic vesicle transmembrane proteins that probably function as Ca^{2+} sensors. Molecular cloning has identified four different synaptotagmin isoforms in mammals. We report here the cloning and sequencing of a novel isoform of 386 amino acids. Synaptotagmin V is 54% identical in sequence to synaptotagmin I and possesses all the domains that characterise this multigene family. It is expressed at high levels in rat brain, but not in spinal cord or a number of peripheral non-neuronal tissues.

Key words: Neurotransmission; Synaptotagmin V; C2 domain; Ca^{2+} sensor

1. Introduction

Neurotransmitters are stored in synaptic vesicles in presynaptic nerve terminals from which they are released by exocytosis following an influx of Ca^{2+} during the action potential [1]. Neurotransmitter release includes the docking and fusion of synaptic vesicles with the presynaptic membrane. The synaptic vesicle membrane is then retrieved by endocytosis and reused for the formation of new synaptic vesicles. Although the steps leading to neurotransmitter release share many processes with constitutive membrane trafficking the regulated Ca^{2+}-evoked release implies the existence of mechanisms that inactivate the fusion machinery until the signal for exocytosis is received [2].

Members of the synaptotagmin gene family are good candidates for fulfilling the role of such a Ca^{2+} sensor [3–7]. Synaptotagmins are abundant intrinsic membrane proteins of synaptic vesicles, with synaptotagmin I being the best characterised member of this gene family. It consists of a short amino-terminal intravesicular domain, a single transmembrane region and a larger cytoplasmic region [4]. The latter contains two internal repeats that are similar to the C2 domains found in several isoforms of protein kinase C, some phospholipases, rabphilin 3A, GTPase activating protein, brain protein Doc2 and the *C. elegans* protein unc-13 [8–14]. C2 domains confer Ca^{2+}-dependent phospholipid binding to at least some of these proteins. Similarly, purified synaptotagmin I binds Ca^{2+} and phospholipids and this binding is mediated through its first C2 domain [5,15,16]. A Ca^{2+}-dependent interaction between synaptotagmin and putative receptors for activated protein kinase C has also been reported [17]. In vitro experiments have shown that synaptotagmin I can bind to at least three additional proteins in a Ca^{2+}-independent manner [18–20]. These are neurexins which are synaptic cell surface receptors that include the receptor for alpha-latrotoxin, syntaxin which is involved in synaptic vesicle fusion and AP2, a protein complex that mediates clathrin assembly into coated pits. The suggestion that synaptotagmin I functions as a Ca^{2+} sensor has received direct support from the demonstration that mice carrying an inactivating mutation in the synaptotagmin I gene lack the fast component of Ca^{2+}-evoked neurotransmitter release in hippocampal neurons [21].

Molecular cloning has identified four separate synaptotagmin isoforms in mammals [4,22–24]. They differ to some extent in their distribution in central and peripheral nervous systems and are characterised by overlapping but distinct functional properties [25]. We report here the cloning and sequencing of the cDNA encoding a fifth rat synaptotagmin isoform which is expressed predominantly in brain.

2. Materials and methods

2.1. cDNA cloning and sequencing

A cDNA library designed to be screened by DNA sequencing was constructed as follows. Double-stranded cDNA was prepared from E17 rat forebrain poly(A)$^+$ RNA, as described [26]. The cDNA was treated with Klenow DNA polymerase and Mung bean nuclease, size-fractionated and ligated to *Hin*cII-digested, phosphatased pBS$^+$ or pUC18 plasmid vectors. Following transformation by electroporation 19,200 recombinant colonies were picked individually, grown and stored in Corning 25850 96-well round bottom plates. The insert of clone 1D4 from this library was used as a probe to screen a lambda gt10 cDNA library prepared from adult rat brain. Ten hybridisation-positive clones were isolated, subcloned into pBS$^+$ and partially sequenced.

Cycle sequencing reactions [27] with either forward or reverse fluorescent primers were run on an Applied Biosystems 373A DNA sequencer. Full-length sequence was compiled from both strands of the three overlapping cDNA clones 1D4, RB2 and RB8. Trace files from the sequencing machine were converted to Staden format, edited using ted and assembled into a bap database, as described [28]. The EMBL, Swissprot, GSDB and dBEST sequence databases were searched using the BLAST algorithm [29]. A multiple alignment of the five rat synaptotagmin amino acid sequences was built up by eye, based on the optimal alignments of each pair of sequences produced by the Align program [30].

2.2. RNA blot analysis

Central and peripheral tissues were dissected from adult Sprague–Dawley rats and immediately frozen on dry-ice. Total RNA was prepared using guanidine isothiocyanate and poly(A) selected. Poly(A)$^+$ RNA (2 μg) was fractionated on a 1.2% agarose-formaldehyde gel, blotted onto nitrocellulose, hybridised and washed as described [31]. Probes were labelled with [^{32}P]dCTP by random priming. The synaptotagmin V probe was prepared from the gel-purified insert of cDNA clone 1D4. The human glyceraldehyde phosphate dehydrogenase (GAPDH) probe was purchased from Clontech. The synaptotagmin I probe was prepared by polymerase chain reaction (PCR) amplification of rat brain cDNA using oligonucleotide primers RAT1F 5′-GGAGGAAAGAACGCCATTAAC-3′ and RAT1R 5′-GGTTCTTGGCTTCCAGAATG-3′. RNA size markers were purchased from GIBCO-

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The nucleotide sequence data reported in this paper will appear in the EMBL, Genbank and Nucleotide Sequence Databases under Accession Number X84884.

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BRL. Results of RNA blot hybridisations were collected on a Molecular Dynamics Phosphorimager.

3. Results

3.1. Cloning and sequencing of rat synaptotagmin V

Single pass sequencing of clones from an E17 rat forebrain cDNA library gave one clone (1D4) with significant similarities to synaptotagmins. Sequencing of 1D4 indicated that it encodes the carboxy-terminal half of the protein. Clone 1D4 was then used as a probe to screen an adult rat brain cDNA library at high-stringency. Screening of 200,000 plaques gave 10 positives. The nucleotide and deduced amino acid sequence of overlapping clones RB2 and RB8 is shown in Fig. 1. In-frame termination codons in the 5′ and 3′ regions of the DNA sequence indicate that it contains the entire coding region. A single open reading frame encodes a protein of 386 amino acids (Figs. 1 and 2). The predicted amino acid sequence and overall structure identify the protein as a novel member of the synaptotagmin family which we designate synaptotagmin V. The regions common to RB2 and RB8 were identical in sequence. However, 1D4 showed a proline to serine substitution at position 182 and lacked the glutamine residue at position 237. The latter change is similar to that observed in the synaptotagmin p65A isoform from the marine ray *D. ommata* [32].

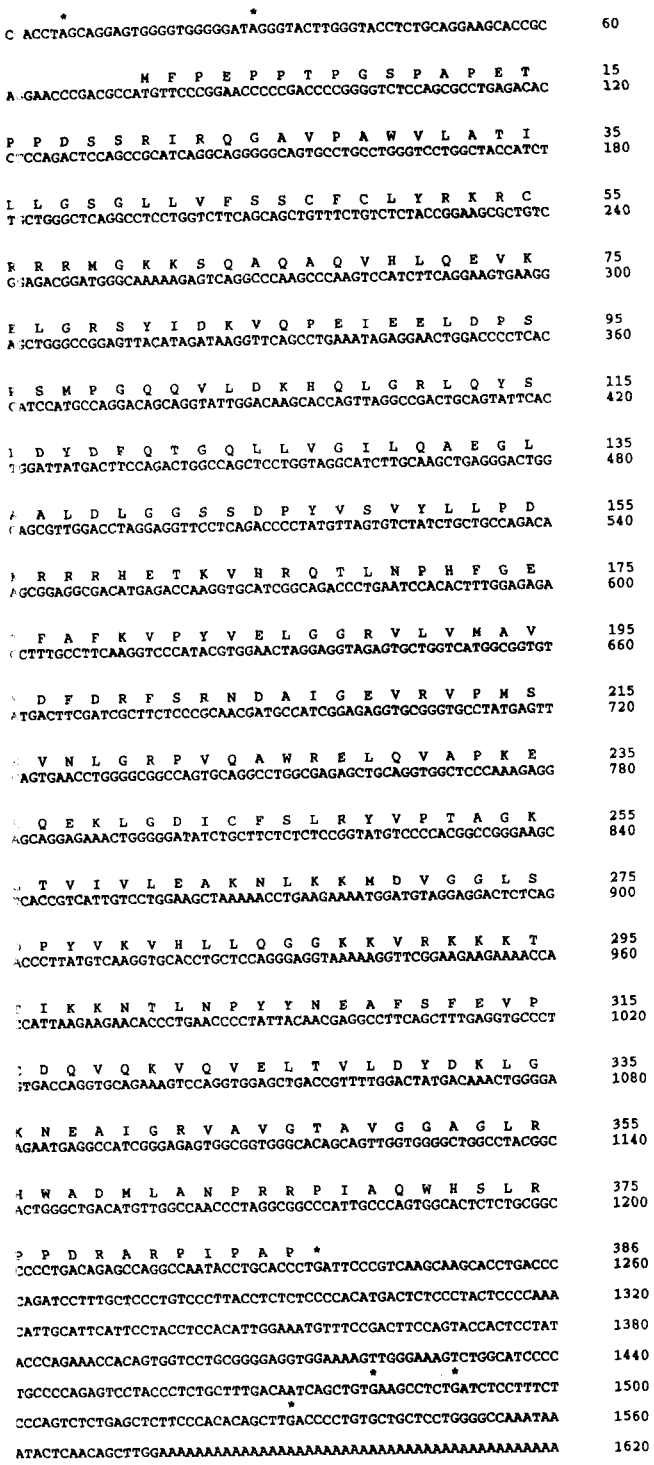


Fig. 1. Nucleotide and predicted amino acid sequence of rat synaptotagmin V. Nucleotides are numbered in the 5′ to 3′ direction and the amino acids are shown in single-letter code above the nucleotide sequence. In-frame termination codons are marked by an asterisk.

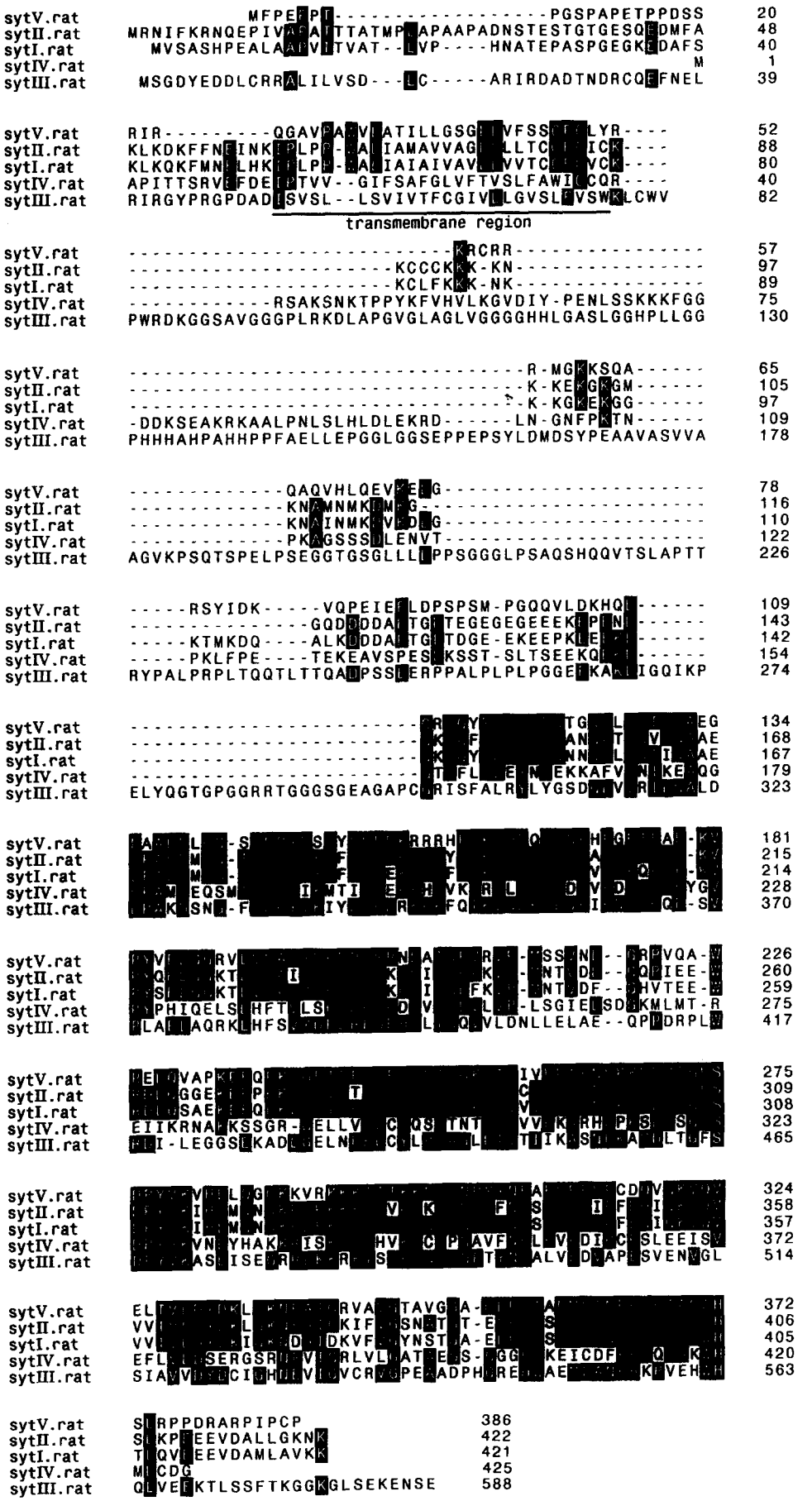
Synaptotagmins are defined by a characteristic sequence which comprises five distinct regions. These consist of an amino-terminal intravesicular sequence of variable length, a single hydrophobic stretch of 26–28 amino acids, a cytoplasmic spacer region of variable length, the highly conserved C2 domains and a short carboxy-terminal tail. Synaptotagmin V has a single hydrophobic stretch of 28 amino acids near the amino-terminus. This putative transmembrane region is preceded by a stretch of 23 amino acids with little similarity to amino-terminal sequences of rat synaptotagmins I-IV. The hydrophobic stretch is followed by a 54 amino acid sequence that precedes the two internal repeats that are very similar to the C2 domains found in other synaptotagmins. These are followed by a carboxy-terminal tail of 25 amino acids. The cytoplasmic domain of synaptotagmin V contains consensus phosphorylation sites for cyclic AMP-dependent protein kinase, Ca^{2+}/calmodulin-dependent protein kinase II, casein kinase II and proline-dependent protein kinases, consistent with evidence indicating that synaptotagmin is a phosphoprotein [33–36]. The overall identity of synaptotagmin V to synaptotagmin I is 54%. It is 53% identical to synaptotagmin II, 29% identical to synaptotagmin III and 32% identical to synaptotagmin IV. As with other synaptotagmins the similarities are strongest in the C2 domains (Fig. 2).

3.2. Expression of synaptotagmin V mRNA in rat tissues

The distribution of synaptotagmin V mRNA in various tissues from adult rat was analysed by RNA blotting (Fig. 3). A cDNA probe specific for synaptotagmin V recognised a single transcript band of approximately 1.7 kb in brain. This size is similar to that of the DNA sequence shown in Fig. 1, indicating that we have probably sequenced the full-length cDNA. No specific signal was detected in poly(A)$^+$ RNA from rat spinal cord, heart, thymus, kidney, adrenal gland, skeletal muscle and spleen. The brain levels of synaptotagmin V mRNA were similar to those of synaptotagmin I mRNA (data not shown).

4. Discussion

The present results show that rat synaptotagmins are encoded by a multigene family that consists of at least five members, with synaptotagmin V being the smallest isoform. Synaptotagmin V is 54% identical in sequence to synaptotagmin I and


sytV.rat
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sytI.rat
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sytIII.rat
transmembrane region

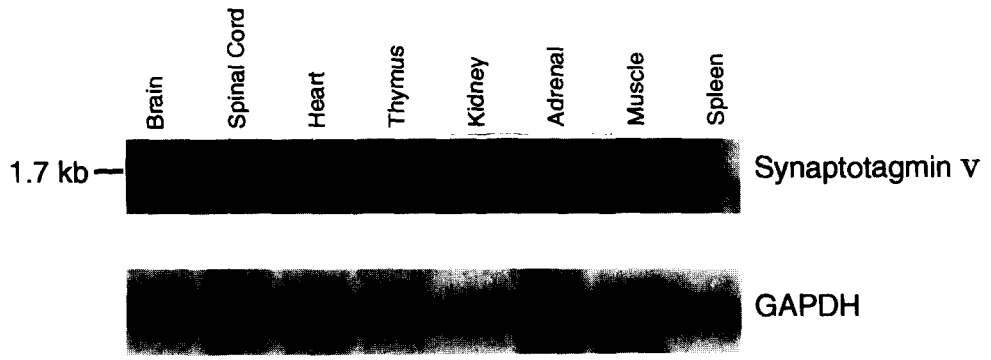

Fig. 3. RNA blot analysis of poly(A)$^+$ RNA from adult rat tissues, with ^{32}P-labelled rat synaptotagmin V DNA or human glyceraldehyde phosphate dehydrogenase (GAPDH) DNA used as the probe. Each lane contained approximately 2 μg poly(A)$^+$ RNA. The blot was re-hybridised with the GAPDH probe following elution of the synaptotagmin V signal.

has the features common to all known members of this gene family. Thus, it possesses a short intravesicular sequence which is followed by a single hydrophobic putative membrane spanning domain. The latter is separated by a 54 amino acid stretch from the two highly conserved C2 domains which are followed by a short carboxy-terminal tail.

The first C2 domain mediates Ca^{2+}-dependent phospholipid binding of synaptotagmins I-III, whereas the first C2 domain of synaptotagmin IV is devoid of Ca^{2+} and phospholipid binding activity, possibly because of an aspartate to serine substitution at position 244 of synaptotagmin IV [25]. Synaptotagmin V has an aspartate at an equivalent position and its sequence in the first C2 domain is very similar to that of synaptotagmins I-III, including the amino-terminal sequence in the first C2 domain that has been shown to be essential for Ca^{2+}-dependent phospholipid binding [16]. Synaptotagmin V is therefore likely to be a Ca^{2+} and phospholipid binding protein. Knowledge of its Ca^{2+} affinity must await experimental determination. The second C2 domain mediates high-affinity AP2 binding [20]; it is also highly conserved between synaptotagmins I-V, suggesting that synaptotagmin V may also be an AP2-binding protein. The binding of synaptotagmin I to neurexins is mediated through its carboxy-terminal 34 amino acids [37]. Synaptotagmin V shares a portion of this sequence with synaptotagmin I, suggesting that it may also bind to neurexins.

Synaptotagmins are synaptic vesicle proteins and are therefore predominantly expressed in nerve cells. A 1.7 kb band corresponding to synaptotagmin V mRNA was found to be expressed in rat brain, where its relative levels were similar to those of synaptotagmin I mRNA. It has been shown previously that synaptotagmin I and synaptotagmin III mRNAs are more abundant in brain than synaptotagmin IV mRNA, with synaptotagmin II mRNA being the least abundant [22–24]. No hybridisation-positive bands for synaptotagmin V were observed in poly(A)$^+$ RNA prepared rat from spinal cord, adrenal gland or a number of peripheral tissues, indicating that synaptotagmin V expression is largely brain-specific. However, by PCR, a synaptotagmin V-specific band could also be obtained from spinal cord cDNA, as well as from cDNAs prepared from a number of non-neuronal peripheral tissues. This suggests that synaptotagmin V may be expressed more widely, albeit at only very low levels. Similar results have been reported for synaptotagmin III mRNA [23].

By in situ hybridisation on adult rat brain it has been shown that rostral brain regions contain more synaptotagmin I mRNA and caudal regions more synaptotagmin II mRNA, with most nerve cells expressing predominantly one or the other isoform [25]. Synaptotagmin III and IV mRNAs are less abundant and are co-expressed with synaptotagmin I and II mRNAs in most nerve cells [25]. The cellular distribution of synaptotagmin V mRNA in brain remains to be determined.

Mutational studies in *C. elegans* and *D. melanogaster* have indicated a role for synaptotagmin in neurotransmission [38–40]. However, mutant animals still responded to sensory stimuli and exhibited coordinated motor movements, indicating at least some synaptic transmission. This may be explained by the existence of multiple synaptotagmin genes. Thus, two additional synaptotagmin genes have recently been identified in *C. elegans* through genomic sequencing of cosmid clones from chromosome III (cosmid F37A4, Accession Number U00032; cosmid F42G9, Accession Number U00051). Mice with an inactivating mutation in the synaptotagmin I gene lack the fast component of Ca^{2+}-evoked neurotransmitter release in hippocampal neurons, indicating that synaptotagmin I functions as the major low-affinity Ca^{2+} sensor in these nerve cells [21]. Other synaptotagmin isoforms may play a similar role in other nerve cell populations. Although the nature of the high-affinity Ca^{2+} sensor is unknown it could also be a synaptotagmin. An understanding of the function of synaptotagmin will require knowledge of all isoforms and of the specific roles played by each isoform in neurotransmission.

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Fig. 2. Sequence comparison of rat synaptotagmins I-V. Amino acids were aligned and gaps were introduced to maximise the homology. Amino acid identities between at least three of the five synaptotagmins are indicated by black bars.

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