

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 pro-

teins 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 *Hinc*II-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 gap database, as described [28]. The EMBL, Swissprot, GSDS 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 [³²P]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'-GGAG-GAAAGAACGCCATTAAAC-3' and RAT1R 5'-GGTTCCTGGCTT-CCAGAATG-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.

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	MFPEPTI-----PGSPAPETPPDSS	20
sytII.rat	MRNIFKRNQEPVAPATTATMPAPAAPADNSTESTGTGESQDMFA	48
sytI.rat	MVSASHPEALAAIVITVAT--LVP---HNATEPASPGEGKDAFS	40
sytIV.rat	MSGDYEDDLRRLILVSD--LC-----ARIRDADTNDRCQEFNEL	39
sytIII.rat		
sytV.rat	RIR-----QGAVPAVATILLGSGVVFSSFLYR----	52
sytII.rat	KLKDKFFNINKPLPP--AIAIAVAVGLLTCTICK----	88
sytI.rat	KLKQKFMNLHKPLPP--AIAIAVAVGLVVTCTVCK----	80
sytIV.rat	APITTSRVFDEITVV--GIFSAFGLVFTVSLFAWLCQR----	40
sytIII.rat	RIRGYPRGPDADISVSL--LSVIVTFCGIVLGVSLVSWLCLCWV	82
	transmembrane region	
sytV.rat	-----KRCRR-----	57
sytII.rat	-----KCCCKK-KN-----	97
sytI.rat	-----KCLFKK-KN-----	89
sytIV.rat	-----RSAKSNTTPPYKFVHVLKGVDIY-PENLSSKKKKFGG	75
sytIII.rat	PWRDKGGSVAVGGGGLRKLAPGVGLAGLVGGGGHHLGASLGGHPLLGG	130
sytV.rat	-----R-MGKKSQA-----	65
sytII.rat	-----K-KEGFGM-----	105
sytI.rat	-----K-KGEGGG-----	97
sytIV.rat	---DDKSEAKRKAALPNLSLHLDLEKRD-----LN-GNFPNTN-----	109
sytIII.rat	PHHHAHPAHPPFAELLEPGGLGGSEPPEPSYLDMDSYPEAAVASVVA	178
sytV.rat	-----QAQVHLQEVLEG-----	78
sytII.rat	-----KNMNMKMG-----	116
sytI.rat	-----KNMNMKMG-----	110
sytIV.rat	-----PKGSSSULENVT-----	122
sytIII.rat	AGVKPSQTSPELPSEGGTGSGLLLPPSGGGLPSAQSHQVTS LAPTT	226
sytV.rat	-----RSYIDK-----VQPEIEFLDPSPSM-PGQQVLDKHQ-----	109
sytII.rat	-----GQDDDAITGTEGEGEGEEEEKPNN-----	143
sytI.rat	-----KTMKDQ-----ALKDDAITGTDGE-EKEEPKIE-----	142
sytIV.rat	-----PKLFPE-----TEKEAVSPESKSSST-SLTSEEKQ-----	154
sytIII.rat	RYPALPRPLTQQTLTTQALPSSERPAPLPLPGGEKALIGQIKP	274
sytV.rat	-----RKY-----TG-----EG	134
sytII.rat	-----KFF-----AN-----AE	168
sytI.rat	-----KRY-----NN-----AE	167
sytIV.rat	-----ITFL-----EKEKAFV-----KEQG	179
sytIII.rat	ELYQGTGPGGRRRTGGGSGEAGAPCRISFALRLYGSDIVRIALD	323
sytV.rat	ALSL-SYRRRH-----HGA-----K	181
sytII.rat	ML-----F-----A-----K	215
sytI.rat	ML-----F-----A-----K	214
sytIV.rat	MEQSM-----MTI-----HVKRL-----D-----V-----D-----Y-----G-----S-----	228
sytIII.rat	AKSNF-----IY-----R-----FQ-----I-----Q-----S-----	370
sytV.rat	IVRV-----NAR-----SSNI-----RVVQA-----W	226
sytII.rat	QK-----K-----NTD-----QPIEE-----	260
sytI.rat	SK-----K-----NTD-----QVTEE-----	259
sytIV.rat	PHIQELS-----HFT-----LS-----D-----V-----L-----LSGIE-----SDKMLMT-----R	275
sytIII.rat	LALIAQRKHFS-----L-----QVLDNLLLEAE-----QPDRPL-----	417
sytV.rat	REIVAPKIQ-----IV-----	275
sytII.rat	REIGGE-----P-----T-----C-----	309
sytI.rat	REISAE-----Q-----V-----	308
sytIV.rat	EIKRNAKSSGR-----ELLV-----CQS-----TNT-----VV-----K-----RH-----P-----S-----S-----	323
sytIII.rat	RII-LEGGSKAD-----ELN-----C-----L-----L-----TIK-----S-----A-----ILT-----FS-----	465
sytV.rat	IVV-----L-----G-----KVR-----A-----CDV-----	324
sytII.rat	IMN-----V-----K-----F-----S-----I-----F-----I-----	358
sytI.rat	IMN-----V-----K-----F-----S-----I-----F-----I-----	357
sytIV.rat	VN-----YHAK-----IS-----HV-----CP-----AVF-----LV-----DI-----C-----SLEEIS-----	372
sytIII.rat	AS-----ISE-----R-----R-----S-----T-----ALV-----D-----AP-----SVEN-----VGL-----	514
sytV.rat	ELV-----K-----L-----R-----VA-----TAVG-----A-----A-----	372
sytII.rat	VV-----L-----K-----I-----F-----SN-----T-----E-----S-----	406
sytI.rat	VV-----L-----K-----I-----F-----SN-----T-----E-----S-----	405
sytIV.rat	EFL-----SERGSR-----V-----RLVL-----AT-----E-----S-----GG-----KEICDF-----Q-----K-----	420
sytIII.rat	SIADV-----ICI-----H-----V-----V-----CRV-----G-----PEADPH-----RE-----AE-----K-----VEH-----	563
sytV.rat	SRPPDRARPIPCP	386
sytII.rat	SKPTEEDVALLGKNK	422
sytI.rat	TQVTEEDVAMLAVKK	421
sytIV.rat	MCDCG	425
sytIII.rat	QVVEKTLSSFTKGGKGLSEKENSE	588

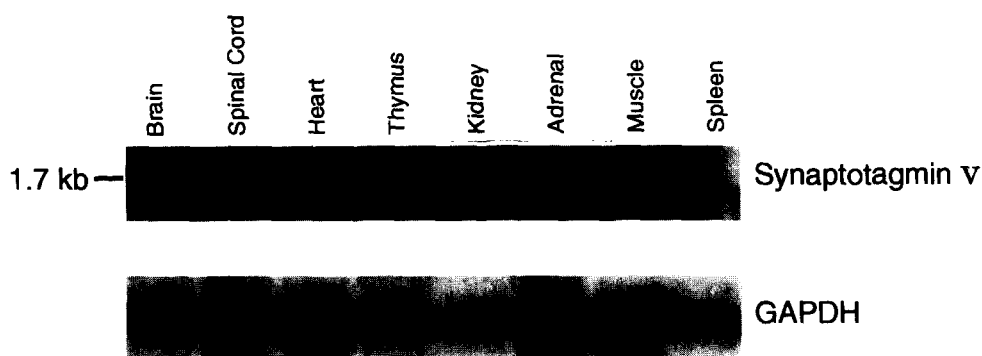


Fig. 3. RNA blot analysis of poly(A)⁺ RNA from adult rat tissues, with ³²P-labelled rat synaptotagmin V DNA or human glyceraldehyde phosphate dehydrogenase (GAPDH) DNA used as the probe. Each lane contained approximately 2 µg poly(A)⁺ RNA. The blot was re-hybridised with the GAPDH probe following elution 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 Ca²⁺-dependent phospholipid binding of synaptotagmins I–III, whereas the first C2 domain of synaptotagmin IV is devoid of Ca²⁺ 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 Ca²⁺-dependent phospholipid binding [16]. Synaptotagmin V is therefore likely to be a Ca²⁺ and phospholipid binding protein. Knowledge of its Ca²⁺ 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 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 Ca²⁺-evoked neurotransmitter release in hippocampal neurons, indicating that synaptotagmin I functions as the major low-affinity Ca²⁺ 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 Ca²⁺ 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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References

- [1] Jahn, R. and Südhof, T.C. (1994) *Annu. Rev. Neurosci.* 17, 219–246.
- [2] Rothman, J.E. (1994) *Nature* 372, 55–63.
- [3] Matthews, W.D., Tsavaler, L. and Reichardt, L.F. (1981) *J. Cell Biol.* 91, 257–269.
- [4] Perin, M.S., Fried, V.A., Mignery, G.A., Jahn, R. and Südhof, T.C. (1991) *Nature* 345, 260–263.

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.

- [5] Brose, N., Petrenko, A.G., Südhof, T.C. and Jahn, R. (1992) *Science* 256, 1021–1027.
- [6] Elferink, L.A., Peterson, M.R. and Scheller, R.H. (1993) *Cell* 72, 153–159.
- [7] Bommert, K., Charlton, M.P., DeBello, W.M., Chin, G.J., Betz, H. and DeAugustine, G.J. (1993) *Nature* 363, 163–165.
- [8] Nishizuka, Y. (1992) *Science* 258, 607–614.
- [9] Stahl, M.L., Ferenz, C.R., Kelleher, K.L., Kriz, R.W. and Knopf, J.L. (1988) *Nature* 332, 269–272.
- [10] Clark, J.D., Lin, L.L., Kriz, R.W., Ramesha, C.S., Sultaman, L.A., Lin, A., Milona, N. and Knopf, J.L. (1991) *Cell* 65, 1043–1051.
- [11] Shirataki, H., Kaibuchi, K., Sakoda, T., Kishida, S., Yamaguchi, T., Wada, K., Miyazaki, M. and Takai, Y. (1993) *Mol. Cell. Biol.* 13, 2061–2068.
- [12] Vogel, U.S., Dixon, R.A.F., Schaber, M.D., Diehl, R.E., Marshall, M.S., Scolnick, E.M., Sigal, I.S. and Gibbs, J.B. (1988) *Nature* 335, 90–93.
- [13] Orita, S., Sasaki, T., Naito, A., Komuro, R., Ohtsuka, T., Maeda, M., Suzuki, H., Igarashi, H. and Takai, Y. (1995) *Biochem. Biophys. Res. Commun.* 206, 439–448.
- [14] Maruyama, I.N. and Brenner, S. (1991) *Proc. Natl. Acad. Sci. USA* 88, 5729–5733.
- [15] Davletov, B.A. and Südhof, T.C. (1993) *J. Biol. Chem.* 268, 26386–26390.
- [16] Chapman, E.R. and Jahn, R. (1994) *J. Biol. Chem.* 269, 5735–5741.
- [17] Mochly-Rosen, D., Miller, K.G., Scheller, R.H., Khaner, H., Lopez, J. and Smith, B. (1992) *Biochemistry* 31, 8120–8124.
- [18] Hata, Y., Davletov, B., Petrenko, A.G., Jahn, R. and Südhof, T.C. (1993) *Neuron* 10, 307–315.
- [19] Bennett, M.K., Calakos, N. and Scheller, R.H. (1992) *Science* 257, 255–259.
- [20] Zhang, J.Z., Davletov, B.A., Südhof, T.C. and Anderson, R.G.W. (1994) *Cell* 78, 751–760.
- [21] Geppert, M., Goda, Y., Hammer, R.E., Li, C., Rosahl, T.W., Stevens, C.F. and Südhof, T.C. (1994) *Cell* 79, 717–727.
- [22] Geppert, M., Archer, B.T. and Südhof, T.C. (1991) *J. Biol. Chem.* 266, 13548–13552.
- [23] Mizuta, M., Inagaki, N., Nemoto, Y., Matsukara, S., Takahashi, M. and Seino, S. (1994) *J. Biol. Chem.* 269, 11675–11678.
- [24] Hilbush, B.S. and Morgan, J.I. (1994) *Proc. Natl. Acad. Sci. USA* 91, 8195–8199.
- [25] Ullrich, B., Li, C., Zhang, J.Z., McMahon, H., Anderson, R.G.W., Geppert, M. and Südhof, T.C. (1994) *Neuron* 13, 1281–1291.
- [26] Gubler, U. and Hoffman, B.J. (1983) *Gene* 25, 263–269.
- [27] Craxton, M. (1991) *Methods: A Companion to Methods in Enzymology* 3, 20–26.
- [28] Staden, R. (1994) in: *Methods in Molecular Biology, Computer Analysis of Sequence Data, Part II* (A.M. Griffin and H.G. Griffin, Eds.) pp. 27–67, Humana Press.
- [29] Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) *J. Mol. Biol.* 215, 403–410.
- [30] Pearson, W.R. (1994) in: *Methods in Molecular Biology, Computer Analysis of Sequence Data, Part II* (A.M. Griffin and H.G. Griffin, Eds.) pp. 365–389, Humana Press.
- [31] Yang, H., McLeese, J., Weishart, M., Dionne, J.L., Lemaire, I. and Aubin, R.A. (1993) *Nucleic Acids Res.* 21, 3337–3338.
- [32] Wendland, B., Miller, K.G., Schilling, J. and Scheller, R.H. (1991) *Neuron* 6, 993–1007.
- [33] Petrenko, A.G., Perin, M.S., Davletov, B.A., Ushkaryov, Y.A., Geppert, M. and Südhof, T.C. (1991) *Nature* 353, 65–68.
- [34] Popoli, M. (1993) *FEBS Lett.* 317, 85–88.
- [35] Davletov, B., Sontag, J.M., Hata, Y., Petrenko, A.G., Fykse, E.M., Jahn, R. and Südhof, T.C. (1993) *J. Biol. Chem.* 268, 6816–6822.
- [36] Bennett, M.K., Miller, K.G. and Scheller, R.H. (1993) *J. Neurosci.* 13, 1701–1707.
- [37] Perin, M.S. (1994) *J. Biol. Chem.* 269, 8576–8581.
- [38] DiAntonio, A., Parfitt, K.D. and Schwartz, T.L. (1993) *Cell* 73, 1281–1290.
- [39] Nonet, M.L., Grundahl, K., Meyer, B.J. and Rand, J.B. (1993) *Cell* 73, 1291–1305.
- [40] Littleton, J.T., Stern, M., Schulze, K., Perin, M. and Bellen, H.J. (1993) *Cell* 74, 1125–1134.