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Synaptotagmin I-ΔC2B. A novel synaptotagmin isoform with a single C2 domain in the bovine adrenal medulla

Eric W. Hewitt [a,1], Jing-Xian Tao [b], Jane E. Strasser [a,b,2], Daniel F. Cutler [a,*], Gary E. Dean [b]

[a] *MRC Laboratory for Molecular Cell Biology, Cell Biology Unit, and Department of Biochemistry and Molecular Biology, University College London, Gower Street, London WC1E 6BT, UK*

[b] *Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati College of Medicine, Cincinnati, OH 45267, USA*

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Abstract

Synaptotagmin I is a 65 kDa type 1 membrane glycoprotein found in secretory organelles that plays a key role in regulated exocytosis. We have characterised two forms (long and short) of synaptotagmin I that are present in the bovine adrenal medulla. The long form is a type I integral membrane protein which has two cytoplasmic C2 domains and corresponds to the previously characterised full-length synaptotagmin I isoform. The short-form synaptotagmin I-ΔC2B has the same structure in the lumenal and transmembrane sequences, but synaptotagmin I-ΔC2B is truncated such that it only has a single cytoplasmic C2 domain. Analysis of synaptotagmin I-ΔC2B expression indicates that synaptotagmin I-ΔC2B is preferentially expressed in the bovine adrenal medulla. However, it is absent from the dense core chromaffin granules. Furthermore, when expressed in the rat pheochromocytoma cell line PC12 bovine synaptotagmin I-ΔC2B is largely absent from dense core granules and synaptic-like microvesicles. Instead, indirect immunofluorescence microscopy reveals the intracellular location of synaptotagmin I-ΔC2B to be the plasma membrane.

Keywords: Synaptotagmin; C2 domain; Chromaffin cell; PC12 cell; Exocytosis

1. Introduction

Synaptotagmins are a family of proteins comprising at least 13 genes, all of which are type I integral membrane proteins with two cytoplasmic C2 domains [1–5]. The synaptic vesicle protein synaptotag-

Abbreviations: aa, amino acid(s); DCG, dense core granules; NSF, *N*-ethylmaleimide sensitive fusion factor; ORF open reading frame; PCR, polymerase chain reaction; RSO, regulatory secretory organelle; RT–PCR, reverse transcriptase–polymerase chain reaction; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; SNAP, soluble NSF attachment protein; SLMV, synaptic like microvesicles; t-SNARE, target-SNAP receptor

* Corresponding author. Fax: +44-20-7679-7805. *E-mail address:* d.cutler@ucl.ac.uk (D.F. Cutler).

[1] Present address: Cambridge Institute for Medical Research, Wellcome Trust/MRC Building, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2XY, UK.

[2] Present address: Department of Infectious Disease, Children's Hospital, 333 Burnet Avenue, Cincinnati, OH 45229-3039, USA.

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min I is the best characterised member of this family and is a key component of the molecular machinery which regulates the Ca^{2+}-dependent exocytosis of synaptic vesicles [6]. The two C2 domains of synaptotagmin I participate in a range of molecular interactions thought to be involved in both exocytosis and endocytic recycling. The membrane proximal C2 domain (C2A) of synaptotagmin I interacts with a subset of negatively charged phospholipids [7,8] and with the soluble NEM sensitive factor (NSF) attachment protein (SNAP) receptor (t-SNARE) syntaxin as a function of Ca^{2+} binding [9–11]. These interactions suggest that synaptotagmin I may participate in the fusion of vesicles with the plasma membrane, and indeed the C2A domain can interact directly with the surface of lipid bilayers after Ca^{2+} binding [12,13]. In addition to these binding studies, there are more direct demonstrations of the role of synaptotagmin I in synaptic vesicle exocytosis, as mutants in organisms as diverse as *Drosophila*, *Caenorhabditis elegans* and mice show defects in evoked neurotransmitter release [14–17].

The membrane distal (C2B) domain also displays Ca^{2+}-dependent interactions, namely an increased affinity for phosphatidylinositol-4,5-bisphosphate and homo- and hetero-oligomerisation with other synaptotagmin isoforms [18–22]. In addition the C2B domain also exhibits Ca^{2+} independent activities. The C2B domain binds to the β-isoform of SNAP (β-SNAP), thereby forming a triple complex which includes NSF [23] implying that the C2B domain may act to adapt the general membrane fusion machinery to Ca^{2+} regulation. The C2B domain may also be involved in the recycling of synaptic vesicles after fusion as it can bind to the AP2 clathrin adaptor complex [24,25], although the AP2 binding site is not an internalisation motif per se [26]. Instead the interaction between the C2B domain and AP2 is thought to promote the nucleation of clathrin coated vesicles at the plasma membrane after synaptic vesicle exocytosis [27,28]. Indeed, the interaction of synaptotagmin I with AP2 is promoted by peptides which contain a tyrosine-based endocytosis motif [27].

The exocytosis of dense core granules (DCG) from bovine adrenal medulla chromaffin cells may also involve synaptotagmin I. Synaptotagmin I is found on the DCG of the adrenal medulla [29,30] and antibodies specific to the C2A domain block DCG release [31]. Moreover, soluble fragments of the C2A domain or antibodies raised against that domain inhibit granule release in the rat pheochromocytoma cell line PC12 [32], which shares many characteristics with bovine adrenal medulla chromaffin cells. However, a mutant PC12 cell line which lacks synaptotagmin I can still undergo degranulation suggesting that synaptotagmin I is not obligatory for fusion [33]. Furthermore, the calcium requirement for DCG release by chromaffin cells is lower than that for synaptic vesicles and the rate of release much slower [34,35]. These observations prompt the question, does the exocytosis of DCG in chromaffin cells require other synaptotagmins in addition to synaptotagmin I?

In this study we screened bovine adrenal medulla cDNA libraries for novel synaptotagmin isoforms that might be components of the regulated exocytic apparatus in these cells. We describe two isoforms, the first corresponding to the previously reported bovine synaptotagmin I sequence [36]. The 5′ end of the second sequence, which we have designated synaptotagmin I-ΔC2B, is identical to the published bovine synaptotagmin I sequence, whereas its 3′ end is completely different (Fig. 1). Indeed, the synaptotagmin I-ΔC2B cDNA is predicted to encode a truncation of synaptotagmin I that lacks the C-terminal 152 amino acids (aa), which include the majority of the C2B domain. Analysis of the expression of synaptotagmin I-ΔC2B indicates that it is preferentially expressed in the adrenal medulla, implying a role in DCG exocytosis. However, one consequence of the truncated sequence of synaptotagmin I-ΔC2B is an altered intracellular localisation. Subcellular fractionation of adrenal medulla homogenates indicates that synaptotagmin I-ΔC2B is absent from DCG. Furthermore, when we analysed the expression of synaptotagmin I-ΔC2B transfected into PC12 cells, synaptotagmin I-ΔC2B is largely if not completely absent from both regulatory secretory organelles (RSOs) – the synaptic-like microvesicles (SLMV) – and DCG. Instead it is localised on the plasma membrane.

2. Materials and methods

2.1. Library screening and cloning of synaptotagmin isoforms

Uni-directional, double-stranded cDNA was prepared from bovine chromaffin cell mRNA, the 1.5–4.5 kb size fraction cloned into Lambda ZAP, and the cDNA packaged to produce six separate libraries containing between 5×10^5 and 2×10^6 independent clones each. A second size-fraction of 4.5 kb and bigger cDNA was similarly cloned and packaged to produce another library of about 10^6 independent clones. Oligonucleotides were synthesised corresponding to the human synaptotagmin I open reading frame (ORF) sequence [37] which spanned positions 523–540 (GCTGCTGAACTGCCCGCC) in the sense direction and positions 931–948 (GCCACCCACATCCATCTT) in the antisense direction. These primers amplified a 425 bp fragment in polymerase chain reactions (PCRs) with 10 ng of human brain poly(A)-selected RNA. The PCR reaction product was sub-cloned into Bluescript SK(−) at the *Eco*RV site and the DNA sequence matched the human synaptotagmin I sequence exactly [37]. 5×10^5 plaques from two of the mid-range cDNA and 2×10^5 from a lambda ZAP large-range cDNA bovine libraries [38] were screened using this DNA as a probe. Plaque lifts were hybridised under stringent conditions with the ^{32}P-labelled probe in 48% formamide, 4.8× SSC, 20 mM Tris–HCl (pH 7.6), 1× Denhardt's solution, 10% dextran sulphate, 0.1% SDS at 42°C. Filters were then washed with 0.2× SSC, 0.1% SDS at 68°C. Subsequent to cloning, super-infection with VCSM13 helper bacteriophage was used to excise the hybridisation positive Bluescript SK(−) plasmid containing inserts [39]. Preliminary analysis by restriction mapping established that the 13 final clones could be grouped into three different classes corresponding to long, intermediate and short forms. Only those clones corresponding to the long and short forms were further characterised by DNA sequencing. DNA sequencing demonstrated that all these clones had a poly(A) tract at their 3′ end, indicating that they are bona fide cDNAs. The long form was identical to the published bovine synaptotagmin I sequence [36], in contrast the short form represented a truncation of the synaptotagmin I ORF (synaptotagmin I-ΔC2B).

2.2. Reverse transcriptase–PCR (RT–PCR) analysis of the bovine synaptotagmin isoforms

Antisense primers designed to hybridise at the 3′ ends of specific synaptotagmin isoforms were used to produce single-stranded cDNA copies, using as a template whole RNA isolated from both bovine brain and adrenal medulla. The PCR primer for the full-length synaptotagmin I isoform was at position 1921 (CAGGCTGGATGCTGCT) and for the synaptotagmin I-ΔC2B isoform was at position 1660 (TGGCCCCAAAGTGAC). These cDNA transcripts were in turn used in PCR reactions with a sense oligonucleotide primer at position 9 (primer 5′-CATCTCAGCTGAACCCAC) specific for the common 5′ sequence of both synaptotagmin isoforms and antisense primers specific for either the 3′ sequences of the full-length synaptotagmin I or I-ΔC2B isoforms. The antisense PCR primer for synaptotagmin I was at position 940 (primer 3′FL-ATCAGACAAGCCACCCAC) and, with the sense primer I, produced a fragment 949 bp long. The antisense PCR primer for synaptotagmin I-ΔC2B was at position 1048 (primer 3′ΔC2B-GGGAATGATCCAAGACC) and in combination with primer I produced a fragment 1056 nucleotides long whose sequence, when cloned, exactly matched that of synaptotagmin I-ΔC2B.

2.3. Northern blot analysis

Total RNA was isolated from flash-frozen adrenal medullae as described [40]. RNA was denatured in 50.7% formamide and separated by electrophoresis in 1% agarose gels in 220 mM formaldehyde. Capillary transfer to GeneScreen Plus membrane was performed for 12 h in a buffer containing 25 mM $NaPO_4$ buffer, pH 6.5. The membrane was baked at 65°C for 2 h, pre-hybridised under standard conditions [40], and then probed by incubation in the same buffer containing DNA probe at approximately 10^6 cpm/ml, 10^8 cpm/μg. The synaptotagmin I-ΔC2B probe included nucleotides 2483–3417 of the I-ΔC2B sequence and was labelled with [^{32}P]dATP by ran-

dom primer labelling or PCR amplification [40]. Samples were normalised by blotting with an 18S rRNA-specific probe, 18S-20 [41].

2.4. Primary antibodies

An isoform-common cytoplasmic domain rabbit antiserum (729 Ab) was raised to a region held in common by both the full-length synaptotagmin I and the synaptotagmin I-ΔC2B isoforms. The antigen in this case was a T7 gene 9 fusion protein with aa 74–243 from the full-length synaptotagmin I which are identical to the same region in synaptotagmin I-ΔC2B. A full-length synaptotagmin I-specific rabbit polyclonal antiserum (I-FL Ab) was raised to the C-terminal 122 aa fused to the T7 gene 9 protein [42]. This region covers approximately half of the C2B domain and the extreme C-terminus of the protein. A synaptotagmin I-ΔC2B-specific rabbit polyclonal antiserum (I-ΔC2B Ab) was raised to a synthetic peptide (CEKEEVRKTLAY), corresponding to the C-terminal aa 267–276 of the synaptotagmin I-ΔC2B isoform. The peptide was coupled to keyhole limpet haemocyanin with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (Pierce Chemical Co.) via the N-terminal cysteine residue. This antiserum shows no immunoreactivity with the bacterially expressed C-terminal 122 aa of the full-length synaptotagmin I isoform. All synaptotagmin polyclonal antisera were immunoaffinity purified by sequential adsorption and desorption to agarose-coupled glutathione-*S*-transferase fusions with either the full-length synaptotagmin I or I-ΔC2B cytoplasmic domains. The common 729 and full-length specific antisera were used at 1/5000 dilution on immunoblots whereas the I-ΔC2B antiserum was used at a 1/500 dilution.

The M2 FLAG epitope tag specific monoclonal antibody was purchased from Sigma. Synaptophysin (p38) antiserum was raised against a GST-fusion protein containing the carboxy-terminal domain of the rat synaptophysin cDNA [43]. The chromogranin A antiserum was a gift from Dr. D. Apps and the synaptotagmin M48 monoclonal antibody [44] was a gift from Dr. G. Schiavo.

2.5. Constructs

Expression vectors for the bovine full-length synaptotagmin I and synaptotagmin I-ΔC2B isoforms were generated by cloning their ORFs as *Bam*HI/*Xho*I fragments from the original Bluescript SK(−) clones into the *Bam*HI/*Sal*I sites of pGW1 [45]. A FLAG (DYKDDDDK) epitope tag was fused to the C-terminus of synaptotagmin I-ΔC2B by PCR amplification of the synaptotagmin I-ΔC2B ORF cloned into pGW1 with the 5′ primer ACTGCACCTCGGTTCTATCGATTG and the 3′ primer TATAAAGCTTATTTATCATCATCATCTTTGTAATCTATTAGAAATGCTAATGTTTT. The resultant synaptotagmin I-ΔC2B-Flag PCR product was cut with the enzymes *Eco*RI and *Hin*dIII and cloned into the same sites of pRK34 [43].

2.6. Generation and sucrose gradient fractionation of adrenal medulla homogenates

Adrenal medullae were homogenised in 300 mM sucrose, 10 mM MOPS (pH 7.5), 1.0 mM phenylmethylsulfonyl fluoride, 0.1 mM *N*-tosyl-L-phenylalanine chloromethyl ketone, 0.1 mM *p*-aminobenzamidine, 2 μg/ml pepstatin. Debris and nuclei were removed by centrifugation in a JA20 rotor (Beckman) for 10 min at 3000 rpm to generate post nuclear supernatants used in either SDS–PAGE or subsequent sucrose gradient fractionation. For subsequent analysis by sucrose gradient fractionation, 3 ml of this supernatant was layered onto a sucrose step gradient consisting of 3 ml each of 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, and 2.0 M sucrose steps in 10 mM MOPS (pH 7.5) and centrifuged in an SW28 rotor (Beckman) overnight at 20 000 rpm. After centrifugation, 15 2-ml fractions were collected from the top of the tube using an Autodensi-Flow llC (Buchler Instruments).

2.7. Expression of synaptotagmin I-ΔC2B in PC12 cells

The day before transfection the PC12 cells were plated to about 75% confluency. On the day of transfection a 9-cm dish was trypsinised, washed once in DMEM plus sera, then twice in ice-cold HeBS buffer (20 mM HEPES (pH 7.05), 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM glucose) and the cells resuspended into 250 μl of HeBS in a 0.4-cm cuvette. One μg of DNA was added and the DNA/cell mix

was given two pulses in a BioRad genepulser (400 V, 125 μF and infinite Ω), cells were reseeded into a 9-cm dish in DMEM plus sera.

2.8. Ficoll gradient fractionation of PC12 cells

The procedure used was described by Cramer and Cutler [46]. After transfection cells were grown under normal conditions for 2 days. The cells were washed twice with ice-cold homogenisation buffer (320 mM sucrose, 10 mM HEPES (pH 7.4), 1 mM phenylmethylsulfonyl fluoride (HB)), washed into 1 ml HB and homogenised on ice by nine passages through a ball-bearing homogeniser, with a 0.012-mm clearance (EMBL workshop). The cell homogenate was spun in a microfuge at 11 000 rpm for 5 min and 1 ml of the supernatant was layered onto 11 ml of a 1–16% Ficoll linear gradient made in HB. The gradients were centrifuged for 45 min at 35 000 rpm in a SW40Ti rotor (Beckman), and fractionated into 0.5-ml fractions.

2.9. Glycerol gradient SLMV isolation

The procedure used was a modification of that described by Desnos et al. [43,47]. Briefly, the cells were washed twice with and then resuspended in 1 ml of buffer A (150 mM NaCl, 0.1 mM $MgCl_2$, 1 mM EGTA 1.0 mM phenylmethylsulfonyl fluoride and 10 mM HEPES, pH 7.4) and then homogenised (as above) with a ball-bearing homogeniser. The homogenate was then centrifuged in a microfuge at 14 000 rpm for 15 min. One ml of the supernatant was loaded onto the top of an 11-ml 5–25% glycerol (in buffer A) gradient and centrifuged in a SW40Ti (Beckman) rotor at 35 000 rpm for 2 h 50 min, and fractionated into 0.5-ml fractions.

2.10. SDS–PAGE and immunoblotting

Adrenal medulla homogenates were separated by SDS–PAGE on 7.5% gels, with 3-μl samples of sucrose gradient fractions loaded per lane. For Ficoll and glycerol gradients of PC12 homogenates, 20 μl of each fraction was separated on 12% acrylamide gels. All samples were loaded sequentially such that fraction 1 corresponds to the top of the gradient. Nitrocellulose replicas of the gels were made and probed with the appropriate immunoaffinity-purified rabbit antisera and HRP conjugated goat anti-rabbit secondary antibody. Reactions were visualised with the Amersham ECL detection system.

2.11. Immunofluorescence confocal microscopy

Cells were fixed in 4% paraformaldehyde in PBS and permeabilised in 0.2% saponin, 1% fish skin gelatin in PBS. Cells were then stained with either the anti-synaptotagmin M48 or the anti-FLAG epitope tag M2 monoclonal antibodies followed by an anti-mouse fluorescein-labelled secondary antibody (Jackson Laboratories). Antibody fluorescence was visualised by confocal microscopy using an MRC1024 microscope (BioRad).

3. Results

3.1. Isolation of synaptotagmin I-ΔC2B

To identify synaptotagmin isoforms present in bovine adrenal medulla chromaffin cells, we screened bovine adrenal medulla libraries [38] with a fragment of human synaptotagmin I cDNA, corresponding to nucleotides 523–948 [37]. This region encodes aa 167–307 incorporating part of the first C2 domain which is conserved throughout the synaptotagmin family [1–5]. This analysis revealed three different classes of synaptotagmin-like clones of which two were analysed in detail (Figs. 1 and 2). Both have a poly(A) tract at their 3′ end, indicating that they are bona fide cDNAs. Indeed, the first clone corresponds to the previously characterised bovine synaptotagmin I isoform [36]. However, whereas the 5′ end of the second clone cDNA designated synaptotagmin I-ΔC2B is identical to the published bovine synaptotagmin I sequence, its 3′ end is completely different (Fig. 1). The synaptotagmin I-ΔC2B isoform may arise from alternative splicing of the mRNA or be the result of a partially processed message in which an intron is retained. RT–PCR amplification of total adrenal medulla RNA with a 5′ primer specific to the sequence common to both isoforms in combination with 3′ primers specific for each cDNA clone confirmed that each message is present in the adrenal medulla (Fig. 2A and Section 2). Furthermore, the

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CAAAAGAACATCTCAGCTGAACCCACGATGGTGAGCGAGAGCCATCACGAGGCGCTGGCAGCCCCGCCGGTCACCACTGT      80
                              M  V  S  E  S  H  H  E  A  L  A  A  P  P  V  T  T  V      18
GGCGACGGTCCTGCCACACAACGCCACAGAGCCAGCCAGTCCCGGGGAAGGGAAGGAGGATGCCTTCTCCAAGCTGAAGG      160
  A  T  V  L  P  H  N  A  T  E  P  A  S  P  G  E  G  K  E  D  A  F  S  K  L  K       44
AGAAGTTTATGAATGAGTTGCATAAAATTCCATTGCCACCATGGGCCTTAATTGCCATAGCCATAGTTGCGGTCCTTTTA      240
E  K  F  M  N  E  L  H  K  I  P  L  P  P  W  A  L  I  A  I  A  I  V  A  V  L  L      71
GTCCTGACCTGCTGCTTTTGTATCTGTAAGAAATGCTTATTCAAAAAGAAAAACAAGAAGAAGGGAAAGGAAAAGGGAGG      320
 V  L  T  C  C  F  C  I  C  K  K  C  L  F  K  K  K  N  K  K  K  G  K  E  K  G  G      98
GAAGAATGCCATTAACATGAAGGATGTGAAAGACTTGGGGAAGACCATGAAAGATCAGGCCCTGAAGGATGATGATGCTG      400
  K  N  A  I  N  M  K  D  V  K  D  L  G  K  T  M  K  D  Q  A  L  K  D  D  D  A      124
AAACCGGATTGACTGATGGTGAAGAGAAAGAAGAACCCAAAGAAGAAGAGAAACTGGGAAAACTTCAGTACTCACTGGAT      480
E  T  G  L  T  D  G  E  E  K  E  E  P  K  E  E  E  K  L  G  K  L  Q  Y  S  L  D      151
TATGATTTCCAGAATAACCAGCTGCTGGTAGGAATCATCCAGGCTGCCGAGTTGCCCGCCTTGGACATGGGGGGCACCTC      560
 Y  D  F  Q  N  N  Q  L  L  V  G  I  I  Q  A  A  E  L  P  A  L  D  M  G  G  T  S      178
TGATCCCTATGTGAAGGTTTTTCTGCTGCCCGATAAAAAGAAGAAATTTGAGACAAAAGTCCACCGAAAGACCCTTAATC      640
  D  P  Y  V  K  V  F  L  L  P  D  K  K  K  K  F  E  T  K  V  H  R  K  T  L  N      204
CTGTCTTCAACGAGCAATTTACTTTCAAGGTGCCATATTCGGAATTGGGTGGCAAAACTCTGGTGATGGCCGTCTATGAT      720
P  V  F  N  E  Q  F  T  F  K  V  P  Y  S  E  L  G  G  K  T  L  V  M  A  V  Y  D      231
TTTGATCGCTTCTCAAAGCATGACATCATTGGGGAATTTAAAGTCCCCATGAACACGGTGGATTTCGGTCACGTAACTGA      800
 F  D  R  F  S  K  H  D  I  I  G  E  F  K  V  P  M  N  T  V  D  F  G  H  V  T  E      258
GGAGTGGCGTGATCTGCAAAGTGCAGAGAAGGAAGAGGTAAGAAAAACATTAGCATTTCTAATATGACAAGCTGGACTCG      880
  E  W  R  D  L  Q  S  A  E  K  E  E  V  R  K  T  L  A  F  L  I  .                  279
CCAGTTGCTACTCTCAAACTAACCTACTGAGAGATGGTGGGCTGGTGTGAGTAATGTGGGCCTCAGCTGCTGACACGTGG      960
CAAAATAGGGTCAGAGGATTGCATAACCTCGTTAAAAAGTATGCAGGTCCACTGAATCTTATTTGAAGGAAAATAGAGAT     1040
TTGTGCAGGTCTTGGATCATTCCCTGGAGCCAGACACACATACACACACATGTGCTTCTTTAAAAAGACAGTGAAAAAGT     1120
CATCTTATCCTTTGTATTTTTCTACAAAGATTTTGTTTTCTACACAGGTTATGTAGCTATTAAATAGAAATGAAATGGCA     1200
CTTTTTCGTTGATTATTTCTGCATATCACAGACAGGTCATTGTTTCTCCTAAAAATTTCAGAGCACAATAAAACATCAAA     1280
TAAAATGCACAAAGGTGGATAGATATTAAGTCCATTTTTTAAATGACTCATTTTTATAGTATCTTCACATTGTTTTTCCC     1360
CAGTTTTATAGGTTAGCCCAGGATTTTCCTGTGATTTAAAAACGGGAAATTCAATATCCTTAGTGCATTTAAACTTCTCT     1440
ATGATTATGCTGTGCCAGTCTAACACTAGCGTTTTTCTCTGCTAGTGTCTCAACAGGCCAATACCAGGAAAGCAAACAAC     1520
TCTTAGCTTCCCTTAATTGCTAAAGCAACTCCTTTCCCAACCATTTTTTTGTTCCTAATATAGTCTATAATTACCCAAGG     1600
GATTTCAACTGCCTTTGTCATTTAGGATCCTTAGACCCCCATGGATTTTGTAAGGTTTGGTCACTTTGGGGCCACCAGGG     1680
AAATCAGAAAGCAAGCCAAGCCTTCAGAGAAACTCAGCCAAGACCATAACCTGGGAATCCTTTCGATCTAAGGTTTCTGT     1760
GACCATTATGATTACTCAGTAAGGGTTTAGAACAGTTTGTTTACTACCGTTCTGAAATTGAATTTTATTAGTGCTCTAAA     1840
ATAATTTCTTGGTTGGACAAAAGAAGTCAAAATTAAGTTCTGCTTTTTCTTTGAAAGGAAATATACATAACTTACCCAAA     1920
GAAGTTAGTCAAAATTAGATTCCTGGATACTGAAATACATTGAGCCCCACTGTGGATAGAACCACCCAAGGATAATATTG     2000
AGTTTTTAAGTTTTATAACTTTAATAAGTTGTTCTTCCAGATGAGCTACAACCAATATCTATTTTAATATCCAATATATG     2080
TGACATTTACCCAATTGGACATAATGTTTCTAATCATTTAACAAAGTAGTGAGGCTGTTTCATTTAATTTTTATCTTTTA     2160
TTGGCATATTTGACTTTGTGTAAATTCCTTGTTCAGTTCAGTCGCTCAGTCGTGTCCAACTCTTTACAACCCCATGGACT     2240
GCAGCAGGCCAGTCCTTCCTGTCCATCACCAGCTCCCAGAGTTTACTAAAACTCATGTCTATCTAGTCGGTGACGCCATC     2320
CAACCTCTCATCCTCTGTCGTCCCCTTCTCCTCCTGCCTTCAATCTTTCCCAGCAGCAGAGTTTTTTCCAATGAGTCAGT     2400
TCTTTGCATCAAGTAGCTGAACTATTGGAGTTTTAGCTTTAGGATCAGTCGTTCCAATGAATATTCAGTACTGATTTCCT     2480
TTAGGATGGACTGGTTGGATCTCCTTGCAGTCCAAGGGACCCTCAAGAGTCTTCTCCAACACCACAGTTCAAAAGCATCA     2560
ATTCTTTGGCTCTCAGTTTTCTTTAGAGTCCAACTCTCACATCCATACATGACTACTGGAAAAACCATAGCTTTCACTAG     2640
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TGTGTCTTTTAATTCCATGGCTGCAGTCAACATCTGCAGTGATTTTGGAGCCCCCCAAAATAAAGTCTGTCACTGTTTCC     2800
CCAACAATTTGCCATGAAGTGAAGGAACCAGATGCCATGATCTTAAGTTTTCTGAATGCTGAGTTTTAAGCCAACTTTTT     2880
CACTCTCCTCTTTCACTTTCATCAAGAGGCTTTTTAGTTCCTCTTCACTTTCTGCCATAAGGGTGGTGTCATCTGCATAT     2960
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TTGAACATCTGGAAGTTCATGGTTCACATATGGTTCATGTAAATTCCTTGTGGCCAGGGCTAAAATGGGGCCATTAGTAT     3440
TCATTAACTGATAGGTTTTTGTTAATTGATTATTGGTCGGCTTTTTGGTGAGTCCCAGAGGTGACCCACTTGTGCTTCAC     3520
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CCCCACCAGGTCTCTGAAAAATTGGTAAATATTTTTGTGTGAATTAGGAAATGTAAGCCATTCAACCATTATCTTCTTTT     3680
TTAAATTCTTTGAATATTGGGCAGAGTTTGAGACTTGACACTAAGCTGACAGGTTTGACTAAAATTTCCAACTTTAGGTA     3760
GATTACCCAAAAAGAATCACATTTTTATTCTCACCCTTGACTTGCCTGTTCTCTGTTTCTTTCATAACTTTTTTCCTCCA     3840
TGGCTTCTCTCCCTGTAGAAGATGGTTTTGTCATTCAACGTATTCAAAATGACTCATTATGAAGATGTTTATTATGTACT     3920
GCTGTATTCCTGGGACTATGCCAAGTGTTTAAAAAAAAAAAAA                                          3963
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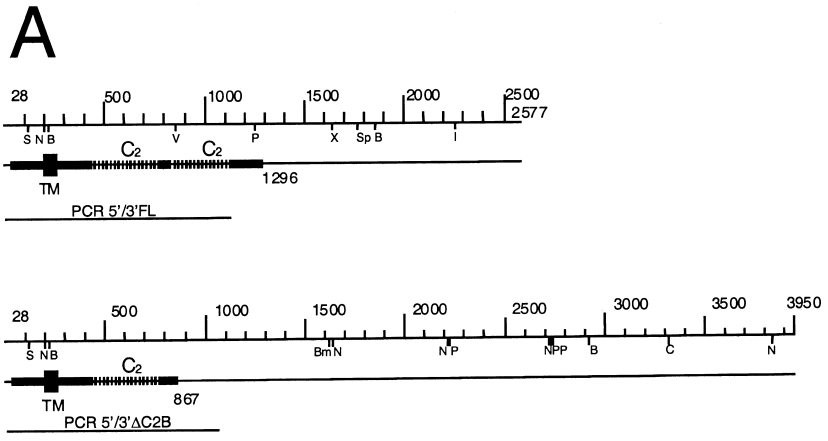
Fig. 1. Nucleotide sequence of synaptotagmin I-ΔC2B cDNA and predicted amino acid sequence. The amino acid sequence is shown in single-letter code beneath the cDNA sequence. The DNA and amino acids sequences are numbered on the right. The GenBank accession number for synaptotagmin I-ΔC2B is AF239741.

synaptotagmin I-ΔC2B-specific RT–PCR product was sequenced and matched the library cloned sequence exactly. Thus the synaptotagmin I-ΔC2B cDNA, which has the longer 3′-untranslated tail, encodes a protein of 279 aa, that is identical to the previously published synaptotagmin I sequence to aa 270, but which diverges thereafter (Fig. 2B). Like the full-length synaptotagmin I protein synap-



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Bt I FL   MVSESHHEALAAPPVTTVATVLPHNATEPASPGEGKEDAFSKLKEKFMNE    50
Bt I-ΔC2B ..................................................    50
Rn I      ...A..P.....-.......LV.....................Q.....    49

Bt I FL   LHKIPLPPWALIAIAIVAVLLVLTCCFCICKKCLFKKKNKKKGKEKGGKN   100
Bt I-ΔC2B ..................................................   100
Rn I      .....................V.....V.....................    99

Bt I FL   AINMKDVKDLGKTMKDQALKDDDAETGLTDGEEKEEPKEEEKLGKLQYSL   150
Bt I-ΔC2B ..................................................   150
Rn I      ..................................................   149

Bt I FL   DYDFQNNQLLVGIIQAAELPALDMGGTSDPYVKVFLLPDKKKKFETKVHR   200
Bt I-ΔC2B ..................................................   200
Rn I      .....................................E............   199

Bt I FL   KTLNPVFNEQFTFKVPYSELGGKTLVMAVYDFDRFSKHDIIGEFKVPMNT   250
Bt I-ΔC2B ..................................................   250
Rn I      ..................................................   249

Bt I FL   VDFGHVTEEWRDLQSAEKEEQEKLGDICFSLRYVPTAGKLTVVILEAKNL   300
Bt I-ΔC2B .................____VRKTLAFLI                      273
Rn I      ..................................................   299

Bt I FL   KKMDVGGLSDPYVKIHLMQHGKRLKKKKTTIKKNTLNPYYNESFSFEVPF   350
Rn I      ...................N..............................   349

Bt I FL   EQIQKVQVVVTVLDYDKIGKNDAIGKVFVGYNSTGAELRHWSDMLANPRR   400
Rn I      .....................D..............................   399

Bt I FL   PIAQWHTLQVEEEVDAMLAVKK                               422
Rn I      ......................                               421
```

Fig. 2. Comparison of full-length synaptotagmin I and synaptotagmin I-ΔC2B cDNAs. (A) Schematic diagram of the full-length synaptotagmin I and synaptotagmin I-ΔC2B genes. Restriction sites indicated: Bm, *Bam*HI; B, *Bst*XI; C, *Cla*I; I, *Eco*RI; V, *Eco*RV; N, *Nco*I; P, *Pst*I; S, *Sma*I; Sp, *Spe*I; X, *Xba*I. RT–PCR products from the full-length synaptotagmins I and I-ΔC2B are shown as PCR 5′/3′FL and PCR 5′/3′ΔC2B, respectively. 5′, 3′FL and 3′ΔC2B represent the primers used as described in Section 2. The dark bar below each restriction map is the predicted protein, with the single transmembrane domain indicated as a box labelled 'TM'. Numbering in the figure refers to the nucleotide numbers. (B) Alignment of the full-length (FL) and synaptotagmin I-ΔC2B predicted protein sequences. Periods indicate aa of identity with the top-most sequence. Bt refers to the *Bos taurus* synaptotagmin isoforms and Rn refers to the *Rattus norvegicus* synaptotagmin I [52]. The two predicted C2 domains are italicised, while the transmembrane domain is indicated by single underlining. Regions used for the production of isoform-specific antisera are indicated by double underlining.

totagmin I-ΔC2B therefore possesses the membrane-proximal C2A domain responsible for Ca^{2+}/phospholipid/syntaxin binding, but the synaptotagmin I-ΔC2B isoform lacks the distal C2B domain thought to be involved in β-SNAP and, AP2 binding and the Ca^{2+}-dependent association with phosphatidylinositol-4,5-bisphosphate and homo- and hetero-oligomerisation [18–22].

3.2. Tissue distribution of the synaptotagmin I-ΔC2B mRNA

The tissue distribution of synaptotagmin I-ΔC2B isoform expression was analysed by probing Northern blots of flash-frozen tissue samples with a DNA probe specific for the unique 3′ sequence of this isoform (Fig. 3A). The synaptotagmin I-ΔC2B mRNA (4.5 and 9 kb) was more abundant in the adrenal medulla than in either brain cortex or stem (Fig. 3A, compare lane 1 with lanes 2 and 3), but was absent from liver (Fig. 3A, lane 4) and lung (data not shown). Synaptotagmin I-ΔC2B therefore represents a novel isoform that is preferentially expressed in the adrenal medulla. This is in contrast to the distribution of the full-length synaptotagmin I isoform, which is instead preferentially expressed in the brain versus the adrenal medulla [48,49].

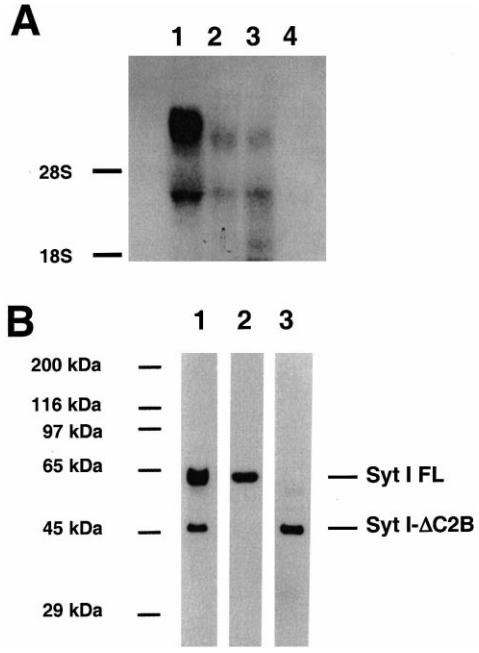

Fig. 3. Analysis of synaptotagmin I-ΔC2B expression in the bovine adrenal medulla. (A) Northern blots of bovine tissues for synaptotagmin I-ΔC2B. RNA was isolated, separated by electrophoresis and probed as described in Section 2 using a specific probe to synaptotagmin I-ΔC2B. Lane 1, adrenal medulla; lane 2, brain cortex; lane 3, brain stem; lane 4, liver. Samples were normalised by blotting with an 18S rRNA-specific probe, 18S-20 as described by [41], data not shown. (B) Immunoblots of adrenal medulla homogenates probed with synaptotagmin antibodies. Mono-specific rabbit immunoaffinity-purified polyclonal antibodies were raised against regions unique to full-length synaptotagmin I (Syt I FL) and to synaptotagmin I-ΔC2B (Syt I-ΔC2B), as described in Section 2. In addition an antiserum (729) was raised against a region common to both synaptotagmin isoforms. These were used to probe Western blots of total tissue homogenates derived from bovine adrenal medullae as described in Section 2. Lane 1, 729 antiserum; lane 2, full-length synaptotagmin I-specific antiserum; lane 3, synaptotagmin I-ΔC2B-specific antiserum.

3.3. Analysis of synaptotagmin I-ΔC2B in adrenal medullae homogenates

In order to examine the expression of the synaptotagmin I-ΔC2B protein in the adrenal medulla, we raised an antiserum specific for synaptotagmin I-ΔC2B, as well as an antiserum specific for the full-length synaptotagmin I isoform and a common antiserum that recognises both isoforms. The common 729 antiserum was raised against a fusion protein containing aa 74–243 from the common N-terminal sequences of the full-length synaptotagmin I and synaptotagmin I-ΔC2B isoforms. In Western blots of homogenised flash-frozen adrenal medulla the 729 antiserum recognised two different species at 65 and 45 kDa (Fig. 3B, lane 1). The 65 kDa species corresponds to the previously characterised full-length synaptotagmin I isoform that is expressed in the bovine adrenal medulla [29,30]. The presence of a 45 kDa species is consistent with the expression of synaptotagmin I-ΔC2B with its predicted shorter cytoplasmic sequence. However, it has been reported that in rat brain homogenates the full-length 65 kDa synaptotagmin I molecule can be cleaved by proteases C-terminal to the transmembrane domain to generate a 40 kDa breakdown product comprising the cytoplasmic region [50]. In order to demonstrate that the 45 kDa band was indeed synaptotagmin I-ΔC2B and not simply a breakdown product of the full-length synaptotagmin I isoform, we used antisera specific for each isoform. Antiserum raised against a fusion protein containing the C-terminal 122 aa of the full-length synaptotagmin I isoform recognised a single species of 65 kDa (Fig. 3B, lane 2) consistent with the size of the mature glycosylated full-length synaptotagmin I [36]. The full-length synaptotagmin I-specific antiserum did not recognise the 45 kDa species. This demonstrates that the 45 kDa band is not simply a breakdown product of the full-length synaptotagmin I as this would be predicted to be recognised by the full-length specific sera. A spe-

cific synaptotagmin I-ΔC2B antiserum was raised against a synthetic peptide comprising the C-terminal 9 aa unique to this molecule. Blots probed with this antiserum labelled a 45 kDa protein (Fig. 3B, lane 3), the same size as the smaller protein labelled with the common 729 antiserum. Together, these data are consistent with the expression of two different synaptotagmin isoforms that have identical N-termini, but diverge thereafter. We therefore conclude that the full-length synaptotagmin I and synaptotagmin I-ΔC2B isoforms are both expressed in the adrenal medulla.

To determine the intracellular location of the two synaptotagmin I variants, subcellular fractionation was used. Bovine adrenal medulla homogenates were centrifuged on sucrose gradients and fractions analysed with the isoform-specific antisera (Fig. 4). The main immunoreactivity seen with the antisera to the full-length synaptotagmin I isoform shows a peak in fractions 10–12, paralleling the peak of chromogranin A within DCG. The additional chromogranin A immunoreactivity at the top of the gradient is soluble material within the load fractions. In addition, there is immunoreactivity for the full-length synaptotagmin I isoform in light membrane fractions which co-sediments with both the SLMV marker

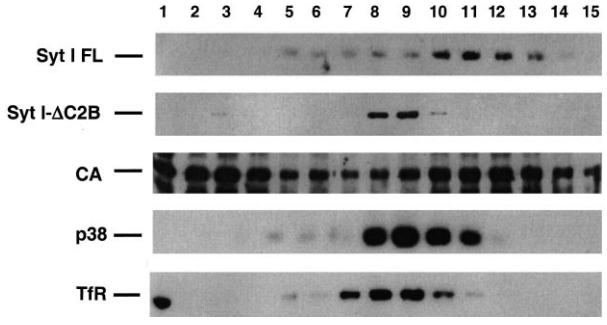

Fig. 4. Sucrose gradient fractionation of adrenal medullae homogenates. Bovine adrenal medullae post-nuclear supernatants were prepared and fractionated on sucrose gradients as described in Section 2. Fractions were collected and Western blots probed with immunoaffinity-purified antisera specific to full-length synaptotagmin I (indicated by Syt I FL) and synaptotagmin I-ΔC2B (indicated by Syt I-ΔC2B). In addition fractions were probed with antisera specific to other marker proteins; chromogranin A (indicated by CA), synaptophysin (indicated by p38) and transferrin receptor (indicated by TfR). Chromogranin A in fractions 1–4 was released from granules during homogenisation; immunoreactivity found deeper in the gradient is in granules.

synaptophysin and the transferrin receptor (fractions 8–9), marking the distribution of the plasma membrane and of early endosomes. This broad distribution reflects the presence of the full-length synaptotagmin I isoform in both DCGs and SLMV [50]. In contrast, synaptotagmin I-ΔC2B immunoreactivity was mainly found within only 2 fractions (8–9), in the region in which membranes enriched in both synaptophysin and transferrin receptor were located. Unfortunately, an analysis of the intracellular localisation of synaptotagmin I-ΔC2B in bovine chromaffin cells by immunofluorescence microscopy using the I-ΔC2B-specific anti-peptide antisera proved to be unsuccessful. However, the fractionation data alone indicate that the full-length synaptotagmin I isoform largely co-fractionates with DCG whereas synaptotagmin I-ΔC2B is in a light membrane fraction distinct from the chromaffin granules.

3.4. Expression and analysis of synaptotagmin I-ΔC2B in PC12 cells

The above data demonstrate that chromaffin cells express the novel synaptotagmin I-ΔC2B isoform in a light membrane fraction, but the identity of this fraction was unclear. To pursue its identity, we turned to another system; PC12 cells. This was for three reasons. We anticipated an association of synaptotagmin I-ΔC2B with regulated secretory organelles, and so we wished to use a system in which the targeting to both SLMV and DCG membranes has been extensively characterised. Indeed, we have recently mapped a dileucine-like motif in the bovine full-length synaptotagmin I isoform that promotes SLMV targeting in PC12 cells [51]. Additionally, expression of synaptotagmin I-ΔC2B in PC12 cells avoids any complications arising from the large number of different synaptotagmin genes and any of their potential splicing products in adrenal medulla chromaffin cells. In the absence of a complete catalogue of synaptotagmin products in any given cell type, we could never be absolutely sure that our antibodies were only recognising a single gene product. By expressing a single cDNA and demonstrating the presence or absence of a single product, we could be sure that our serum was only revealing the location of the protein encoded by that particular mRNA. Finally, by examining the location of an over-expressed pro-

tein, we hoped to be able to use antibodies that recognise both forms to localise synaptotagmin I-ΔC2B by immunofluorescence, since our domain-specific antibodies had not provided useful immunofluorescence data.

The cDNAs encoding the bovine full-length synaptotagmin I and synaptotagmin I-ΔC2B isoforms were cloned into an expression vector, PC12 cells were transiently transfected with these two constructs and their expression analysed 2 days after transfection. Previously we have described a Ficoll gradient fractionation protocol used to analyse targeting to exocytic compartments [46]. This procedure was used to generally characterise the subcellular localisation of the two bovine synaptotagmin isoforms in PC12 cells. We were unable to differentiate between the distribution of the endogenous rat synaptotagmin I and the bovine full-length synaptotagmin I when this was expressed in PC12 cells. The common 729 antiserum recognised a 65 kDa species in untransfected PC12 cells, consistent with the endogenously expressed rat synaptotagmin I isoform (Fig. 5A, lane 1). When the bovine full-length synaptotagmin I was transfected into PC12 cells it was indistinguishable from the endogenous rat synaptotagmin I as both proteins migrated at 65 kDa (data not shown). However, due to the very high degree of sequence identity (97%) between bovine and rat synaptotagmin I [36,52], the two proteins should behave identically. In contrast, we found no evidence for the endogenous expression of a rat synaptotagmin I-ΔC2B in PC12 cells. The synaptotagmin I-ΔC2B antibody did not recognise a band of the predicted size and synaptotagmin I-ΔC2B-specific primers did not amplify a PC12 synaptotagmin I-ΔC2B cDNA (data not shown). However, when bovine synaptotagmin I-ΔC2B was expressed a single band with the same molecular mass as that seen for synaptotagmin, I-ΔC2B in chromaffin cells was seen in addition to the endogenous rat synaptotagmin I (Fig. 5A, lane 2). A faint 45 kDa band was also present in untransfected cells (Fig. 5A, lane 1), which might be a breakdown product of rat synaptotagmin I. However, the amount of 45 kDa immunoreactive material was much more intense in cells transfected with the synaptotagmin I-ΔC2B cDNA. Since this band was dependent on the expression of the cDNA encoding synaptotagmin I-ΔC2B and the antibody recognises both 65 and 45 kDa forms, we therefore conducted a parallel comparison by subcellular fractionation of the endogenous rat synaptotagmin I and the expressed bovine synaptotagmin I-ΔC2B in PC12 cells.

The highest concentration of rat synaptotagmin I immunoreactivity in Ficoll gradients was found in fractions 11–12 which correspond to the DCG peak identified by labelling with [^{3}H]dopamine prior to homogenisation (Fig. 5B,C). In addition, a second population of synaptotagmin I was found in lighter fractions including those enriched in the SLMV marker protein synaptophysin which peaks in fractions 3–5 (Fig. 5C). This corresponds to synaptotagmin I in the SLMV fraction, the analysis of which is described below. These data conform with the distribution of synaptotagmin I within PC12 cells found by others [53]. In contrast, synaptotagmin I-ΔC2B in PC12 cell homogenates was found in a light membrane fraction, with a broad peak in fractions 4–8 (Fig. 5C). There was only a very limited proportion of synaptotagmin I-ΔC2B immunoreactivity deeper in the gradient, where [^{3}H]dopamine-labelled DCGs were found. Although synaptotagmin I-ΔC2B was mainly in light fractions toward the top of the gradient, the distribution was not identical with that of the SLMV marker synaptophysin, the majority of synaptotagmin I-ΔC2B instead being found slightly deeper in the gradient. However, these data correlate with the sucrose gradient fractionation of adrenal medulla homogenates, in which synaptotagmin I-ΔC2B is present in a light membrane fraction distinct from the chromaffin granules (Fig. 4).

The identity of the membrane fraction to which synaptotagmin I-ΔC2B was targeted was unclear from Ficoll gradients. To determine if synaptotagmin I-ΔC2B was present in SLMVs, we used the glycerol gradient procedure [43,47] to isolate SLMV from cells transfected with this cDNA. We found that the rat synaptotagmin I co-fractionated with the SLMV marker protein synaptophysin, peaking in fractions 10–11 (Fig. 5D). In contrast to endogenous synaptotagmin I, much less immunoreactivity at 45 kDa was observed in these fractions. Furthermore the level of the 45 kDa band relative to rat synaptotagmin I is significantly less than the level of synaptotagmin I-ΔC2B relative to rat synaptotagmin I in homogenates of transfected cells (Fig. 5A). Due to the low level of immunoreactivity we cannot distin-

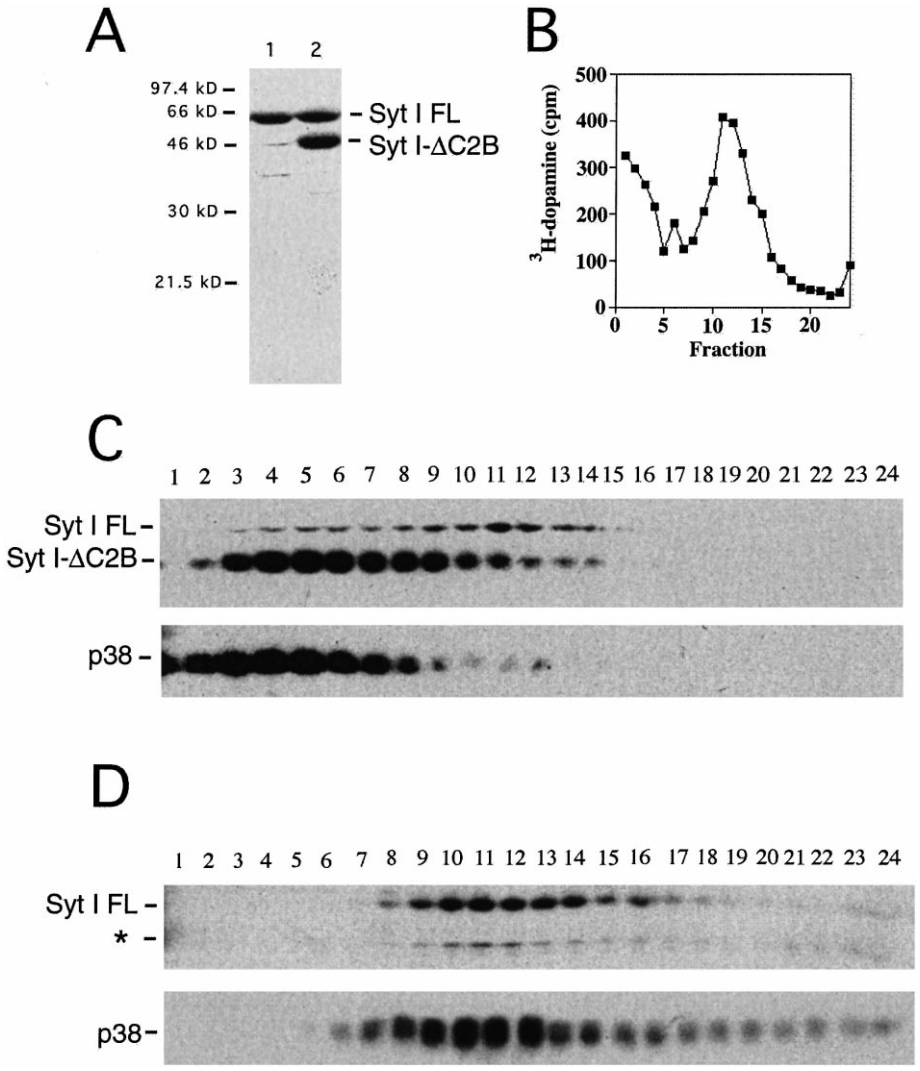

Fig. 5. Subcellular fractionation of synaptotagmin I-ΔC2B in PC12 cells. (A) Expression of synaptotagmin I-ΔC2B in PC12 cells. Homogenates of either mock (lane 1) or synaptotagmin I-ΔC2B (lane 2) transfected cells were probed with the common 729 antiserum. (B,C) Ficoll gradient fractionation of synaptotagmin I-ΔC2B transfected PC12 cells. PC12 cell homogenates were fractionated on Ficoll gradients as described in Section 2. Fractions were collected and counted for [^{3}H]dopamine labelling (B) and probed with either the isoform common 729 immunoaffinity purified antiserum or a specific synaptophysin antiserum. Synaptophysin is indicated by p38; rat synaptotagmin I and bovine synaptotagmin I-ΔC2B are indicated by Syt I FL and Syt I-ΔC2B, respectively (C). Soluble material is expected to be present in fraction 1, immunoreactivity and counts deeper in the gradient are associated with vesicular material. (D) Glycerol gradient isolation of SLMV from of synaptotagmin I-ΔC2B transfected PC12 cells. SLMV were isolated on glycerol gradients as described in Section 2. Fractions were collected and probed with either the isoform common 729 immunoaffinity-purified antiserum or the synaptophysin-specific antiserum (indicated by p38). Soluble material is expected to be present in fraction 1, while immunoreactivity found deeper in the gradient is associated with vesicular material. Rat synaptotagmin I is indicated by Syt I FL and * indicates immunoreactivity which may either correspond to bovine synaptotagmin I-ΔC2B or a breakdown product of rat synaptotagmin I.

guish between synaptotagmin I-ΔC2B and the breakdown product of rat synaptotagmin I on these gradients, as we see no difference between transfected and untransfected cells (data not shown). The majority of synaptotagmin I-ΔC2B was instead removed by centrifugation of the homogenate prior to the glycerol gradient (see Section 2). Therefore, even though synaptotagmin I-ΔC2B is in a light membrane fraction only a very limited proportion, if any, of synaptotagmin I-ΔC2B is targeted to SLMV. Thus we

conclude that unlike rat synaptotagmin I, synaptotagmin I-ΔC2B is enriched in neither SLMV nor DCG membranes.

To determine where synaptotagmin I-ΔC2B might be, PC12 cells transfected with either bovine full-length synaptotagmin I or synaptotagmin I-ΔC2B were analysed by confocal immunofluorescence microscopy. One advantage of analysing expression in transiently transfected cells is that not all cells express the DNA. This allowed an easy comparison of cells expressing the bovine synaptotagmins with those that only express the endogenous rat synaptotagmin I. Staining with antibody M48 (which recognises an epitope in the C2A domain) revealed that in untransfected PC12 cells endogenous synaptotagmin I was found in a punctate distribution characteristic

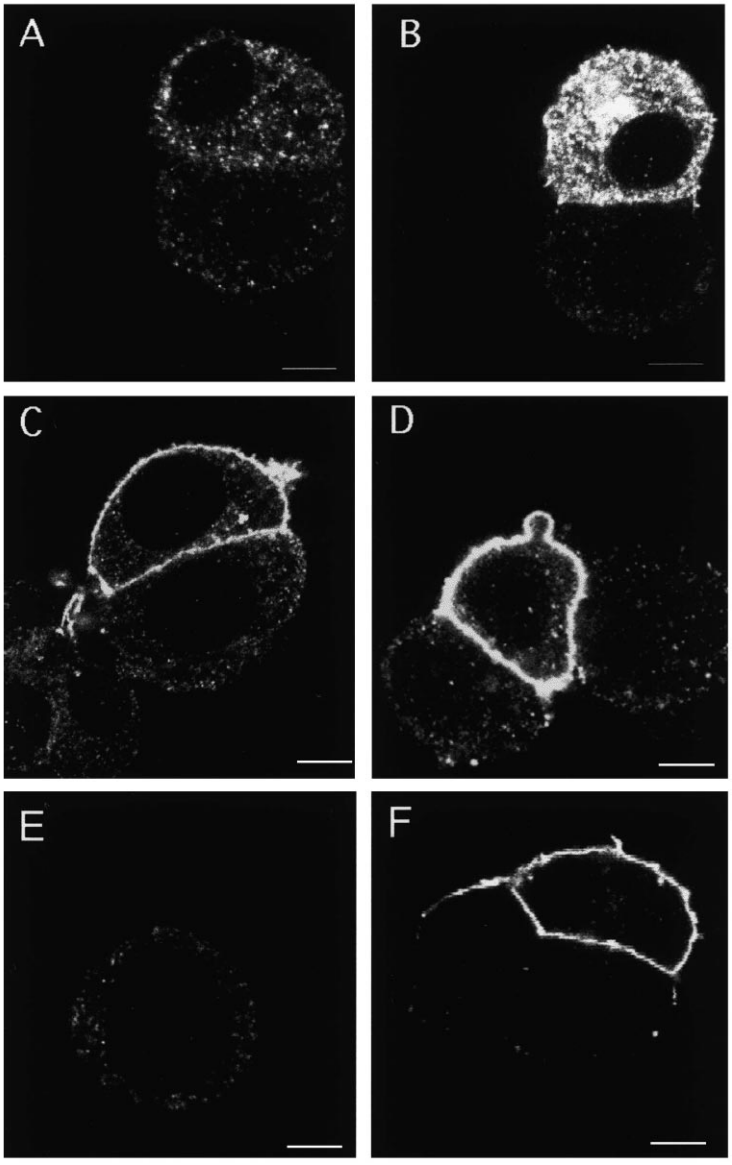

Fig. 6. Confocal immunofluorescence microscopy of PC12 cells transfected with bovine full-length synaptotagmin I and synaptotagmin I-ΔC2B. (A,B) PC12 cells transfected with bovine full-length synaptotagmin I. (C,D) PC12 cells transfected with bovine synaptotagmin I-ΔC2B. (E) an untransfected PC12 cell. (F) PC12 cells transfected with synaptotagmin I-ΔC2B-FLAG. Cells were fixed, permeabilised and stained with either the synaptotagmin I antibody M48 (A–E) or the M2 FLAG epitope tag-specific antibody (F) in conjunction with a fluorescein-conjugated secondary antibody as described in Section 2. Cells transfected with the expression vectors are uppermost in each panel (A–D,F). Scale bar = 5 μm.

of DCG staining (Fig. 6E). In cells transfected with full-length bovine synaptotagmin I, the bovine form was superimposed onto the endogenous rat synaptotagmin I, accentuating the staining of these granular structures in cells expressing either barely detectable levels (Fig. 6A) or very high levels of the protein (Fig. 6B). In marked contrast, when bovine synaptotagmin I-ΔC2B was expressed in PC12 cells it was found on the plasma membrane (Fig. 6C,D). This was not simply a consequence of over-expression, as there was no increase in staining of DCGs and a similar pattern was observed both in cells where synaptotagmin I-ΔC2B was barely detectable (Fig. 6C) and where expression was very high (Fig. 6D). Finally, to discriminate between the endogenous rat synaptotagmin I and the bovine synaptotagmin I-ΔC2B and therefore to confirm the plasma membrane localisation, a FLAG (DYKDDDDK) epitope tag was added to the C-terminus of synaptotagmin I-ΔC2B. PC12 cells transfected with this construct were stained with the FLAG epitope antibody M2 and analysed by immunofluorescence microscopy. The FLAG-tagged synaptotagmin I-ΔC2B was also localised to the plasma membrane (Fig. 6F), confirming the plasma membrane localisation of synaptotagmin I-ΔC2B. We therefore conclude that synaptotagmin I-ΔC2B is found on the plasma membrane in PC12 cells. Furthermore, due to the similarity between the RSOs in chromaffin and PC12 cells, we predict that synaptotagmin I-ΔC2B will also be present on the plasma membrane in bovine chromaffin cells.

4. Discussion

To date 13 members of the synaptotagmin family have been described, all of which have two C2 domains [1–5]. Here we describe two sequences, the first of which corresponds to the previously characterised bovine synaptotagmin I isoform [36], whereas the second is a novel sequence that encodes synaptotagmin I-ΔC2B. Synaptotagmin I-ΔC2B may be formed by alternative splicing of the synaptotagmin I gene, as the 5′ end of the cloned synaptotagmin I-ΔC2B cDNA is identical to that of the full-length synaptotagmin I, whereas the 3′ ends are completely different. Alternatively, synaptotagmin I-ΔC2B may result from the retention of a normally spliced intron. However, alternative splicing has been reported in synaptotagmins I, IV, V and VII, which have splice variants in which the transmembrane domain is removed or altered [54,55]. Although synaptotagmin I-ΔC2B is the only synaptotagmin isoform with a single C2 domain, a protein expressed in pancreatic β cells – granuphilin – which normally has two C2 domains also has a truncated isoform with a single C2 domain [56].

The consequence of the different 3′ sequences is to generate proteins that have identical N-termini, but different C-terminal cytoplasmic sequences. The I-ΔC2B isoform has a unique cytoplasmic structure in that it lacks the C-terminal 152 aa which include the majority of the C2B domain. The functional consequences of the different cytoplasmic sequences of the two synaptotagmin isoforms are unclear, but one obvious consequence is their distinct intracellular distributions. In bovine adrenal medulla chromaffin cells the full-length synaptotagmin I is enriched in DCG whereas synaptotagmin I-ΔC2B is localised to a light membrane fraction. Analysis of the two bovine isoforms in PC12 cells suggested that whereas the full-length synaptotagmin I is present in both SLMV and DCG, synaptotagmin I-ΔC2B is localised to the plasma membrane. Given the similarities between chromaffin and PC12 cells it is likely that synaptotagmin I-ΔC2B is also present on the plasma membrane in chromaffin cells.

The different subcellular localisation of the bovine synaptotagmin I isoforms indicates that the targeting information in the cytoplasmic regions differs between the two molecules. In PC12 cells SLMV are formed directly from the plasma membrane and or via an endocytic intermediate. The former route is dependent upon the AP2 adaptor complex and clathrin [57] whereas the latter is sensitive to brefeldin A (BFA) and dependent upon the AP3 adaptor complex [58,59]. Full-length synaptotagmin I can interact with AP2 via the C2B domain, which requires two lysines, corresponding to Lys 326/Lys 327 in the bovine sequence [24,25,27] and would be predicted to utilise this pathway of SLMV targeting. Additionally, we have described a dileucine-like motif (Met 417/Leu 418) located within the extreme C-terminus of the full-length synaptotagmin I isoform which is capable of promoting SLMV targeting in PC12 cells

[51]. Targeting via this motif is BFA sensitive suggesting that the full-length synaptotagmin I can also utilise the AP3-dependent pathway of SLMV biogenesis [51]. Synaptotagmin I-ΔC2B has neither of the motifs described for SLMV targeting, which is consistent with our observation that little if any synaptotagmin I-ΔC2B is present in SLMV. In addition, the C-terminus of full-length synaptotagmin I contains an endocytosis signal [26]. It is not known if this signal is required for SLMV targeting, but it is absent in synaptotagmin I-ΔC2B and may then cause the accumulation of synaptotagmin I-ΔC2B at the plasma membrane. In contrast to SLMV, DCG are thought to bud directly from the trans-Golgi [60,61], and no role has been proposed for either AP2 or AP3 in this process. The sequence requirements for DCG targeting of the full-length synaptotagmin I are unknown, yet the absence of synaptotagmin I-ΔC2B from DCG suggests that the C-terminal 152 aa of the full-length synaptotagmin I also contain sequence information necessary for this process.

In addition to affecting the subcellular localisation of the two bovine synaptotagmin I isoforms, the different cytoplasmic sequences would be predicted to affect the functional properties of the two isoforms. The absence of the C2B domain would reduce the repertoire of molecular interactions in which synaptotagmin I-ΔC2B could participate, with functional consequences. Indeed, the *Drosophila* AD1 mutant which lacks the C2B domain has a null phenotype [15], presumably because interactions mediated by the C2B domain are essential for synaptotagmin function. Synaptotagmin I-ΔC2B would be unable to bind to β-SNAP, or phosphatidylinositol-4,5-bisphosphate which bind to the C2B domain or participate in Ca^{2+}-dependent and homo- and hetero-oloigomerisation [18–22]. The absence of the C2B domain would not preclude interactions via the C2A domain, as the C2A domain alone can associate with both negatively charged phospholipids and syntaxin as a function of Ca^{2+} binding [8–11]. What then is the function of synaptotagmin I-ΔC2B? The expression of synaptotagmin I-ΔC2B by the chromaffin cells of the adrenal medulla suggests an involvement in some aspect of their physiology. A recent study may provide a clue to a potential function of synaptotagmin I-ΔC2B. Expression of truncated forms of synaptotagmin I lacking the C2B domain inhibits clathrin mediated endocytosis [62]. Synaptotagmin I-ΔC2B would be predicted based on these observations to similarly act as a negative regulator of endocytosis. However, further work is required to determine the function(s) of synaptotagmin I-ΔC2B in chromaffin cells and whether it is indeed an inhibitor of endocytosis.

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