Identification of a novel protein complex containing annexin A4, rabphilin and synaptotagmin

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Abstract Rabphilin is a synaptic vesicle-associated protein proposed to play a role in regulating neurotransmitter release. Here we report the isolation and identification of a novel protein complex containing rabphilin, annexin A4 and synaptotagmin 1. We show that the rabphilin C2B domain interacts directly with the N-terminus of annexin A4 and mediates the co-complexing of these two proteins in PC12 cells. Analyzing the cellular localisation of these co-complexing proteins we find that annexin A4 is located on synaptic membranes and co-localises with rabphilin at the plasma membrane in PC12 cells. Given that rabphilin and synaptotagmin are synaptic vesicle proteins involved in neurotransmitter release, the identification of this complex suggests that annexin A4 may play a role in synaptic exocytosis.

Key words: Annexin; Rabphilin; C2 domain; Ca^{2+}-regulated secretion

1. Introduction

Regulated exocytosis is triggered by intracellular Ca^{2+} elevation and because Ca^{2+} elevation is critical in steps preceding vesicle fusion, it is important to identify components acting as and through Ca^{2+} sensors. One possible Ca^{2+} sensor involved in the regulation of exocytosis is rabphilin. This protein, like other proposed Ca^{2+} sensors, contains C2 (conserved region 2) domains. C2 domains were originally identified in protein kinase C isoforms displaying Ca^{2+}-dependent membrane association properties [1]. C2 domains are structural modular units containing approximately 120 amino acids [2] that were initially proposed to be Ca^{2+}-dependent phospholipid binding units [3]. However, since then, evidence has accumulated to suggest that C2 domains may also play an important role in mediating protein–protein interactions [4–6]. These protein–protein interactions in turn may be involved in a wide range of intracellular signal transduction events.

In order to identify molecular mechanisms involved in exocytosis, we have looked for protein–protein interactions mediated by the C2 domains of rabphilin. Structurally, the full-length rabphilin protein contains an N-terminal rab3 interaction domain [7,8], a central phosphorylation region [9,10] and two C2 domains (denoted C2A and C2B) located in the C-terminal tail which bind phospholipids in a Ca^{2+}-dependent manner [11]. It is expressed in neuronal and neuroendocrine cells and has been shown to localise to synaptic vesicles and dense-core secretory granules [12,13]. On the basis of its interaction with GTP-bound (activated) rab3a it has been proposed to be an effector for this G protein [14]. However, increasing evidence suggests that rabphilin can vesicle-associate and function independently of rab3 proteins [13,15–19] and it has been shown to be a rab27a interaction protein [20]. Furthermore, previous work suggests that the C2B domain of rabphilin may be important for the protein's ability to regulate exocytosis [12,21]. However, the molecular mechanism for this activity has yet to be determined. Unlike proteins such as synaptotagmin, potential binding partners for rabphilin C2 domains have been little studied. However, β-adducin has been shown to bind to a rabphilin fragment containing amino acids 281–704. This region encompasses both C2 domains, but this interaction has only been demonstrated in vitro by overlay assay and requires the presence of lipids and Ca^{2+} [22]. In addition, neurexin has been shown to bind to a synaptotagmin-like motif located in the amino acid region 674–696 of rabphilin. However, it is unlikely that neurexin would bind to the C2B region alone since it does not contain amino acids 691–696 which appear to be critical for stable interaction to occur [23].

Therefore, in order to elucidate a signalling role for this C2 domain, we have looked for protein complex formations. In this study we report the isolation of a novel protein complex formed by the C2B domain of rabphilin and the identification of key protein components in this complex.

2. Materials and methods

2.1. *Glutathione* S-*transferase (GST) fusion proteins and isolation of protein complexes*

cDNA fragments encoding the C2A (amino acids 371–510) and C2B domains (528–684) of rat and the 14 amino acid N-terminal sequence of annexin A4 were subcloned into the pGEX GST fusion protein system and proteins expressed and purified as previously de-

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Abbreviations: PBS, phosphate-buffered saline; GST, glutathione *S*-transferase; ECL, enhanced chemiluminescence; TFA, trifluoroacetic acid; GFAP, glial fibrillary acidic protein

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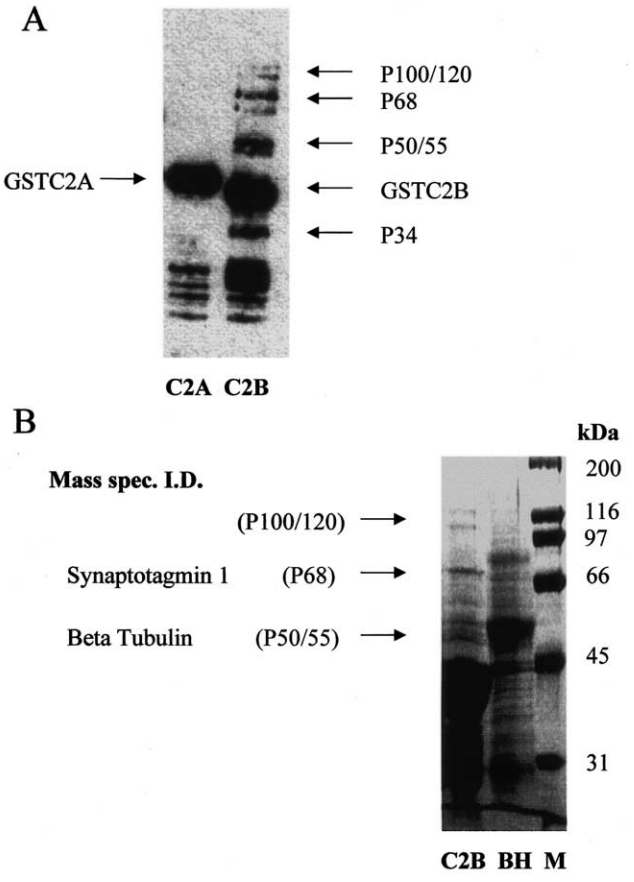

Fig. 1. Isolation of proteins co-complexing with the C2 domains of rabphilin. A: Visualisation of proteins co-complexing with rabphilin C2 domains in rat brain synaptosomes. Co-complexed proteins were labelled with NHS-biotin and detected using avidin-horseradish peroxidase and ECL. B: Scaled-up preparation of proteins co-complexing with the rabphilin C2B domain in rat brain homogenates. Proteins were detected by Coomassie blue staining. BH is a brain homogenate sample prior to purification and M are molecular weight markers.

scribed by us [5,24]. Cleavage of the rabphilin C2B domain from the GSTC2B protein was performed using thrombin digestion [24]. Rat brain synaptosomes were prepared as previously described [25]. Synaptosomal proteins were solubilised by homogenisation in phosphate-buffered saline (PBS) containing 1% (v/v) Triton X-100 and 100 μM Ca^{2+} followed by centrifugation at 20 000×*g*. GST fusion proteins (100 nM) bound to glutathione agarose beads were incubated for 1 h with synaptosome supernatants. Protein complexes were collected by centrifugation and washed three times with PBS. Co-complexed proteins were then labelled with normal horse serum (NHS)-biotin and blots analysed as previously described by us [5]. For scaled-up procedures, rat brains were homogenised and centrifuged as before and supernatants collected and incubated overnight with 100 nM GSTC2B. Complexes were then washed with RIPA buffer [5], proteins separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and detected by Coomassie blue staining.

2.2. Annexin A4 N-terminal tail Vp22 fusion protein (Vp22NT) construction

Oligonucleotides corresponding to the sense and antisense strands of DNA encoding the first 14 amino acids of the rat annexin A4 sequence were ligated into the pCRT7/VP22/NES-2 TOPO vector (Invitrogen), expressed and purified as per the instruction manual (Invitrogen).

2.3. Peptide preparation and mass spectrometric analysis

Protein bands from scaled-up purifications were extracted from stained gels using 50% acetonitrile in 50 mM ammonium bicarbonate. After alternative washing steps with 50 mM ammonium bicarbonate or 50% acetonitrile, the gel pieces were digested overnight at room temperature with trypsin, 50 mM ammonium bicarbonate (pH 8.0). Peptides were then extracted using sequential steps of 0.2% trifluoroacetic acid (TFA), followed by 50% acetonitrile in 0.1% TFA. The peptide extracts were desalted using Zip Tip C18 columns according to the manufacturer's protocol (Millipore) and eluted in 5 μl of 50% acetonitrile containing 0.1% TFA. MALDI mass spectra were recorded for peptide extracts using Ettan MALDI-TOF (Amersham Biosciences), operated in an automatic acquisition method under positive ion reflectron mode at 20 kV accelerating voltage with pulsed ion extraction enabled. α-Cyano-4-hydroxycinnamic acid (10 mg/ml in 50% acetonitrile and 0.1% TFA) was used as the matrix. The peptide masses obtained were searched against the National Center for Biotechnology Information (non-redundant all taxonomies) database using the built-in program ProFound. One missed cleavage per peptide was allowed and an initial mass tolerance of 50 ppm was used in all searches.

2.4. Annexin A4 and rabphilin C2B pulldowns

GST fusion proteins (400 nM) were immobilised on glutathione agarose beads and incubated in PBS in the absence or presence of Ca^{2+} (100 μM) as indicated with recombinant human annexin A4 protein (1 μM) [24]. Samples were washed three times with PBS containing 1% (v/v) Triton X-100 and Western blots probed with anti-annexin A4 antibodies, followed by enhanced chemiluminescence (ECL) detection. For rabphilin C2B pulldowns Vp22NT protein (1 μM) was incubated with GST or GSTC2B proteins (400 nM) on beads in PBS containing 10 μM Ca^{2+}. Samples were collected by

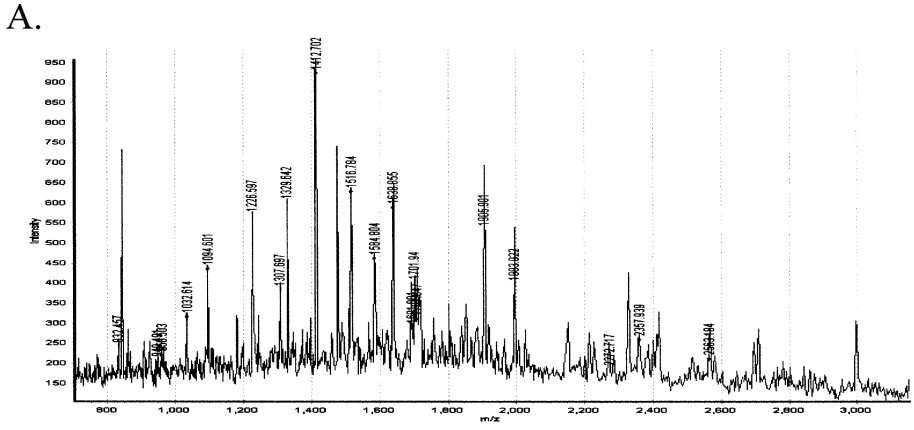

Fig. 2. MALDI-TOF analysis of isolated proteins. A: Mass spectral analysis for p68 protein gel bands. B: Peptides recovered from p68 (showed in red). C: Mass spectral analysis for p50/55 protein gel bands. D: Peptides recovered from p50/55 samples (shown in red).

centrifugation, washed and prepared as above. Blots were probed with anti-myc epitope antibodies to detect for Vp22 proteins.

2.5. Overlay binding assay and in vitro peptide competition studies

For overlay binding assay, recombinant annexin A4 protein (20 μg) samples were loaded onto polyacrylamide gels and Western transferred. Filters were cut into individual strips and incubated overnight with GST fusion proteins as indicated. Blots were probed with anti-GST antibodies followed by ECL detection. For peptide competition studies, recombinant GST annexin A4 (4 μg) and rabphilin C2B domain protein (2 μg cleaved from the GSTC2B fusion protein) were incubated at 4°C for 2 h in PBS containing 10 μM Ca^{2+} in the presence or absence of scrambled or wild type sequence N-terminal annexin A4 peptides (10 μg). After washing three times with PBS containing 1% Triton X-100, samples were analysed by SDS–PAGE and Coomassie staining.

2.6. Cell culture and loading of PC12 cells

Mixed astrocyte–neurone cultures were prepared from 6–8 day old Sprague–Dawley rats as previously described [41]. Cells were used for immunofluorescence studies after 7 days in culture. PC12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) contain-

B.

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1          11         21         31         41         51         61         71
MVSASHPEAL AAPVTTVATL VPHNATEPAS PGEGKEDAFS KLKQKFMNEL HKIPLPPWAL IAIAIVAVLL VVTCCFCVCK
81         91         101        111        121        131        141        151
KCLFKKKNKK KGKEKGGKNA INMKDVKDLG KTMKDQALKD DDAETGLTDG EEKEEPKEEE KLGKLQYSLD YDFQNNQLLV
161        171        181        191        201        211        221        231
GIIQAAELPA LDMGGTSDPY VKVFLLPEKK KKFETKVHRK TLNPVFNEQF TFKVPYSELG GKTLVMAVYD FDRFSKHDII
241        251        261        271        281        291        301        311
GEFKVPMNTV DFGHVTEEWR DLQSAEKEEQ EKLGDICFSL RYVPTAGKLT VVILEAKNLK KMDVGGLSDP YVKIHLMQNG
321        331        341        351        361        371        381        391
KRLKKKKTTI KKNTLNPYYN ESFSFEVPFE QIQKVQVVVT VLDYDKIGKN DAIDKVFVGY NSTGAELRHW SDMLANPRRP
401        411        421
IAQWHTLQVE EEVDAMLAVK K
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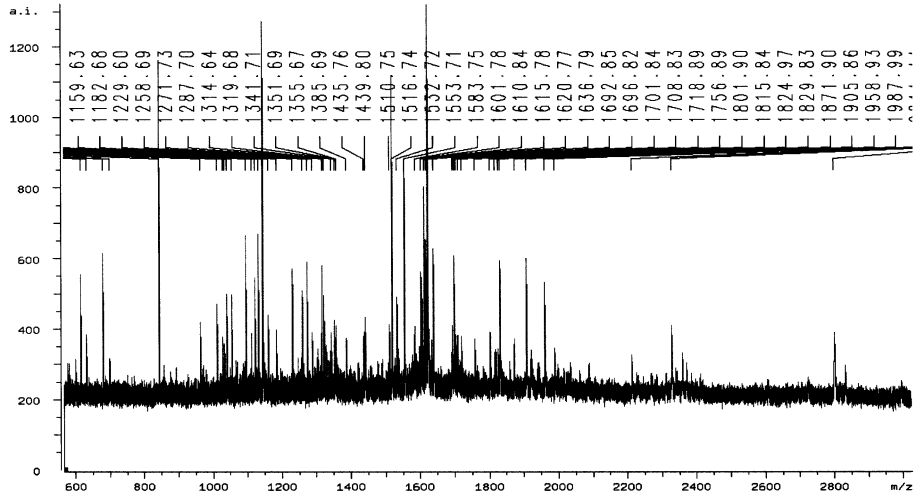
C.



D.

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1          11         21         31         41         51         61         71
MREIVHIQAG QCGNQIGAKF WEVISDEHGI DPTGSYHGDS DLQLERINVY YNEAAGNKYV PRAILVDLEP GTMDSVRSGP
81         91         101        111        121        131        141        151
FGQIFRPDNF VFGQSGAGNN WAKGHYTEGA ELVDSVLDVV RKESESCDCL QGFQLTHSLG GGTGSGMGTL LISKIREEYP
161        171        181        191        201        211        221        231
DRIMNTFSVM PSPKVSDTVV EPYNATLSVH QLVENTDETY CIDNEALYDI CFRTLKLTTP TYGDLNHLVS ATMSGVTTCL
241        251        261        271        281        291        301        311
RFPGQLNADL RKLAVNMVPF PRLHFFMPGF APLTSRGSQQ YRALTVPELT QQMFDSKNMM AACDPRHGRY LTVAAIFRGR
321        331        341        351        361        371        381        391
MSMKEVDEQM LNVQNKNSSY FVEWIPNNVK TAVCDIPPRG LKMSATFIGN STAIQELFKR ISEQFTAMFR RKAFLHWYTG
401        411        421        431        441
EGMDEMEFTE AESNMNELVS EYQQYQDATA DEQGEFEEEE GEDEA
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Fig. 2 (*Continued*).

ing 10% horse serum and 5% foetal bovine serum until use. PC12 cells that were 50–70% confluent were loaded in suspension by electroporation in serum-free DMEM medium containing 100 nM fusion proteins or N-terminal annexin A4 (METKGGTVKAASGF) or scrambled (MFEGTSKAGAGKVT) peptide as previously described by us [24].

2.7. Immunofluorescent labelling of cells

Immunostaining was performed as previously described [26]. Specifically, cells were grown on chamber slides and fixed in 4% formaldehyde. Cells were permeabilised with 0.1% Triton X-100 and blocked in 5% donkey serum. Cells were mounted in Vectashield medium prior to microscopic analysis.

2.8. Analysis of complex formation in synaptosomes

Synaptosome fractions were prepared as described previously [25]. Membrane and vesicle fractions were solubilised in PBS containing 1% (v/v) Triton X-100 and after collection by centrifugation at $20\,000 \times g$ supernatants were subjected to immunoprecipitation with appropriate antibodies. Immunoprecipitations and Western blotting procedures were as previously described by us [5].

3. Results and discussion

We began our studies attempting to isolate proteins which co-complex with either the C2A or C2B domain of rabphilin. Using biotin labelling, we detected six protein bands that co-purified specifically with the C2B domain: a doublet around 120/100 kDa ('p120/p100'), a strongly labelled protein at around 68 kDa ('p68'), a doublet around 55/50 kDa ('p55/50') and another strongly labelled protein of around 34 kDa ('p34'; Fig. 1A). Unfortunately, no proteins specifically interacting with the C2A domain were obtained under these conditions (Fig. 1A). However, since biotin labelling primarily labels lysine residues in proteins it is still possible that proteins were co-complexing with this domain but remained undetected using this procedure.

In order to obtain sufficient material for mass spectral identification of these proteins, the purification procedure was then scaled up and proteins were isolated from SDS–polyacrylamide gels stained with Coomassie blue (Fig. 1B). MALDI-TOF analysis allowed the successful identification of p68 and p50/55 proteins (Fig. 2). p68 was found to be synaptotagmin 1 and samples of the purified complex were subjected to Western blot probing with an antibody raised to the first 16 amino acids of synaptotagmin I, a sequence that is unique to this synaptotagmin isoform, and this further confirmed the identity of p68 (data not shown). The p50 and p55 bands were both identified as β-tubulin. Initially we were sceptical of this find because β-tubulin is a very abundant protein in brain homogenates. However, it was not found to purify with fusion proteins containing the C2A domain (Fig. 1A) or GST (data not shown) and a number of protein gel samples were analysed by MALDI-TOF. Therefore based on these criteria β-tubulin appears to be a specific interacting protein.

Unfortunately, identification of proteins below the 35 kDa size or the p100 and p120 proteins was not possible by MALDI-TOF analysis as the amount of material obtained was insufficient and probably contained a number of proteins/peptides. Similarly, due to the large amounts of fusion protein used in the scaled-up procedure, it was not possible to obtain a discrete p34 band for analysis (Fig. 1B).

However, we rationalised that since we [5] and others [4,6] have previously shown annexin-like proteins and annexin family members directly interacting with proteins via their C2 domains, there was a strong possibility that an annexin could be present in this protein complex. In addition, since

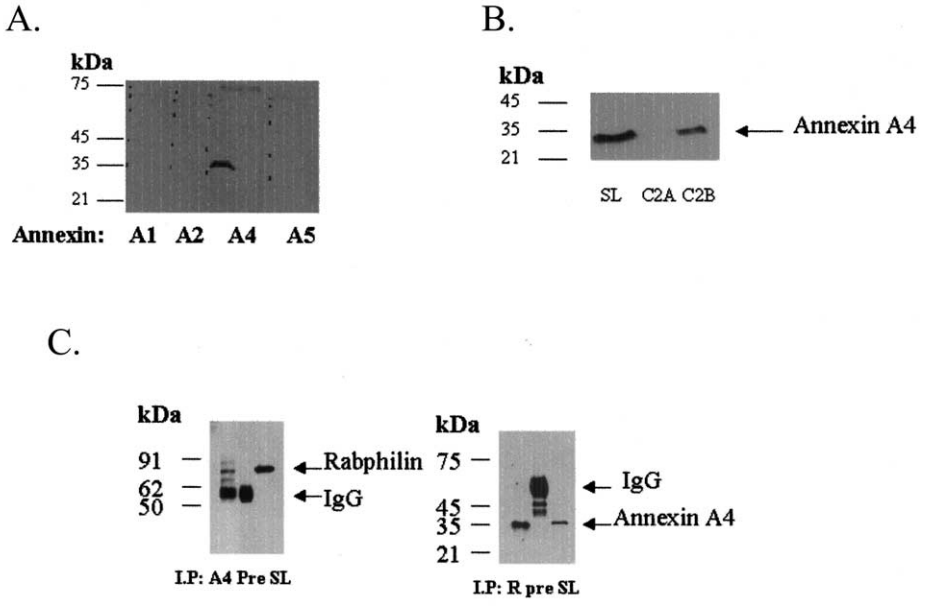


Fig. 3. Identification of p34 as annexin A4 and verification of annexin A4–rabphilin interaction. A: Western blot probing with a range of antibodies raised against annexin family members indicated that only annexin A4 was expressed in rat synaptosomes. B: Protein complexes were isolated as described in Fig. 1A. Western-blotted samples were then probed with anti-annexin A4 antibodies to verify that p34 was annexin A4. SL is a control lane containing synaptosome lysates to show the specificity of the antibody used. C: Annexin A4 and rabphilin co-immunoprecipitate from synaptosome lysates. Synaptosome lysates were immunoprecipitated (IP) with antibodies as indicated: anti-annexin A4 (A4), anti-rabphilin (R), pre-immune rabbit sera (Pre). Blots were probed with either anti-rabphilin (left panel) or anti-annexin A4 antibodies (right panel). Control lanes containing synaptosome lysates (SL) show specificity of antibodies used during probing.

most annexin family members have a molecular weight in the range of 32–36 kDa [27], we looked to see if p34 could be an annexin. To do this, we initially probed synaptosome lysates to see which annexins were expressed. Our studies indicated that only one annexin (annexin A4) was significantly expressed in synaptosomes (Fig. 3A). We next employed Western blotting and immunoprecipitation studies to see if annexin A4 was able to co-complex with rabphilin. We found that the 34 kDa protein isolated in our small-scale purifications was detected by anti-annexin A4 antibodies (Fig. 3B) and that annexin A4 was indeed able to co-immunoprecipitate with rabphilin from synaptosomes (Fig. 3C). Furthermore, using pulldown (Fig. 4A) and overlay assays (Fig. 4B), we were able to show that the rabphilin C2B domain was able to directly bind to annexin A4 in a Ca^{2+}-dependent manner. Moreover, because the C2B domain was able to bind to a denatured form of annexin A4 in the overlay assay, we reasoned that the conserved highly structured C-terminal protein core [27,28] characteristic of this and all annexin members may not be involved in this binding. Instead, the unique short N-terminal tail of annexin A4 looked like a prime candidate for binding.

Therefore, in order to test whether the rabphilin C2B domain interacts directly with the N-terminal tail of annexin A4 we employed another pulldown assay. This time, we incubated GST or GSTC2B fusion proteins (immobilised on beads) with a myc epitope-tagged recombinant annexin A4 N-terminal tail fusion protein (termed Vp22NT). We found that this annexin A4 N-terminal sequence was indeed able to bind to the rabphilin C2B domain (Fig. 4C). Next, we reasoned that if this N-terminal annexin A4 sequence is important for the rabphilin–annexin A4 interaction to occur in cells, then we should be able to disrupt the interaction of native proteins by loading a peptide corresponding to this N-terminal tail sequence. Using PC12 cells (since these endogenously express both rabphilin and annexin A4 proteins) we loaded either the wild type 14 amino acid sequence peptide or a control scrambled sequence peptide by electroporation. After cell recovery, co-immunoprecipitation studies and Western blotting were undertaken to detect rabphilin and annexin A4 co-complexing (Fig. 4D). We found that the loading of annexin A4 peptide (compared to scrambled control peptide) resulted in a significant reduction of the amount of annexin A4 co-complexing with rabphilin (by approximately 50%). In addition, we have undertaken an in vitro peptide competition assay, where we incubated recombinant GST annexin A4 and the rabphilin C2B domain in the presence or absence of excess scrambled and wild type sequence peptides and again blocked the annexin A4–rabphilin interaction with the wild type sequence peptide (Fig. 4E). Hence, we conclude that this N-terminal annexin A4 sequence does have the ability to interact directly with the C2B domain, but our results do not preclude the

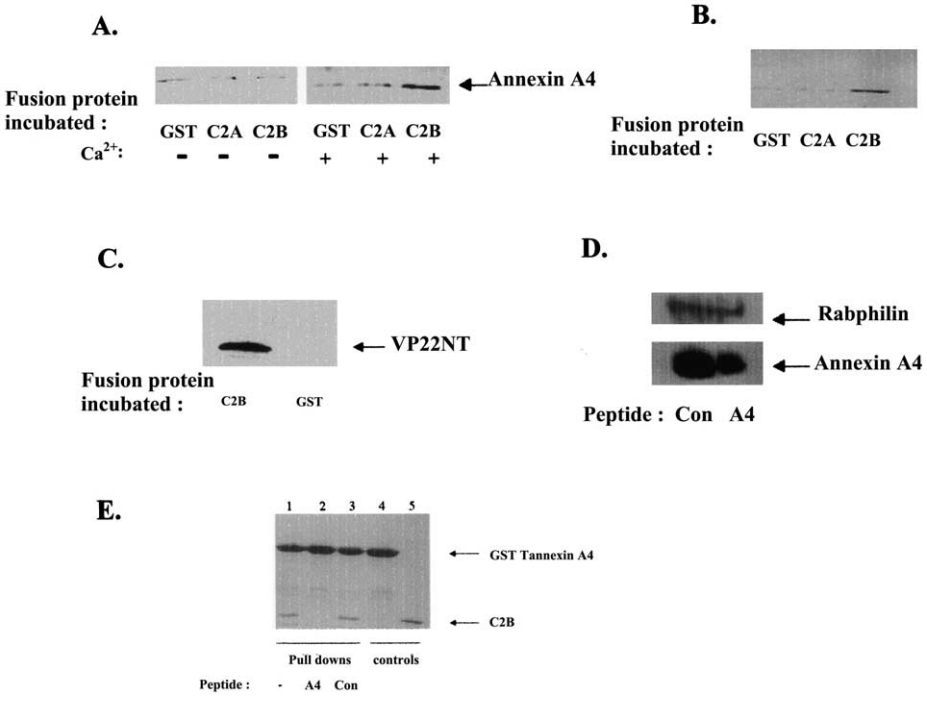
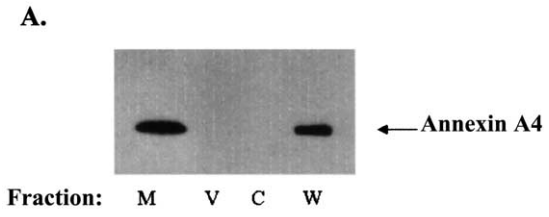
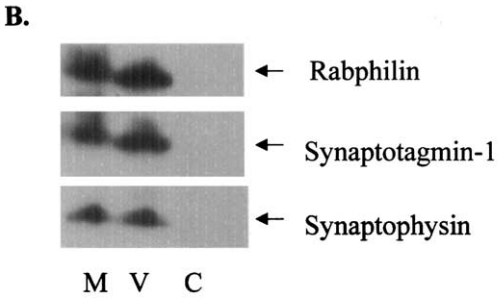


Fig. 4. The N-terminal tail of annexin A4 binds directly to the C2B domain of rabphilin. A: Annexin A4 pulldowns. GST fusion proteins were incubated as indicated with recombinant human annexin A4 protein (1 μM) in the absence or presence of Ca^{2+} (100 μM). Samples were Western-blotted and probed for the presence of annexin A4. B: Overlay (far Western) binding of fusion proteins to recombinant annexin A4. Annexin A4 protein (20 μg) was loaded onto gels and Western-transferred. Blots were incubated with GST fusion proteins as indicated and probed for fusion protein binding with anti-GST antibodies. C: Rabphilin C2B pulldowns using Vp22NT fusion protein. A Vp22 fusion protein containing the N-terminal tail sequence from annexin A4 was incubated with either GST or GSTrabphilinC2B (C2B) proteins. Samples were Western-blotted and blots probed with anti-myc antibodies to detect Vp22 fusion protein bound. D: Loading PC12 cells with wild type annexin A4 N-terminal tail peptide (A4) but not the scrambled sequence peptide (Con) inhibits the ability of rabphilin to co-complex with annexin A4 as assessed by anti-rabphilin immunoprecipitations. Cell lysates were immunoprecipitated with anti-rabphilin antibodies and samples were then Western-blotted and the blots probed with either anti-rabphilin (upper panel) or anti-annexin A4 (lower panel) antibodies. E: In vitro blocking of annexin A4–rabphilin C2B interaction with wild type (A4) or scrambled (Con) annexin A4 N-terminal sequence peptides. Lanes 1–3: GSTannexin A4 and rabphilin C2B pulldowns in the absence (lane 1) or presence (lanes 2 and 3) of peptides as indicated. Lanes 4 and 5 are control lanes showing recombinant proteins used: GSTannexin A4 (lane 4) and rabphilin C2B (lane 5).


A.
Annexin A4
Fraction: M V C W


B.
Rabphilin
Synaptotagmin-1
Synaptophysin
M V C

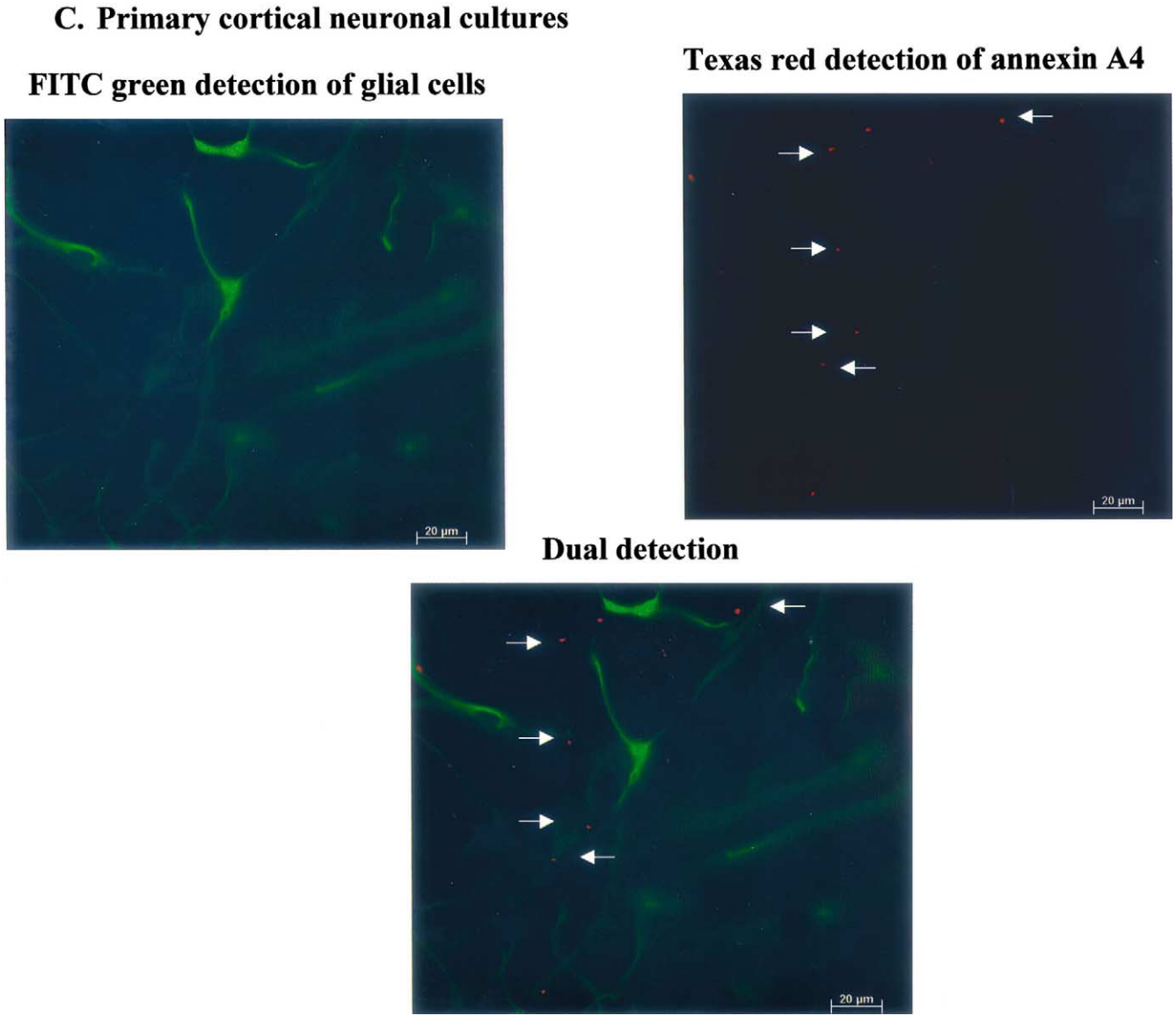

C. Primary cortical neuronal cultures
FITC green detection of glial cells
Texas red detection of annexin A4
Dual detection
20 µm
20 µm
20 µm

Fig. 5.

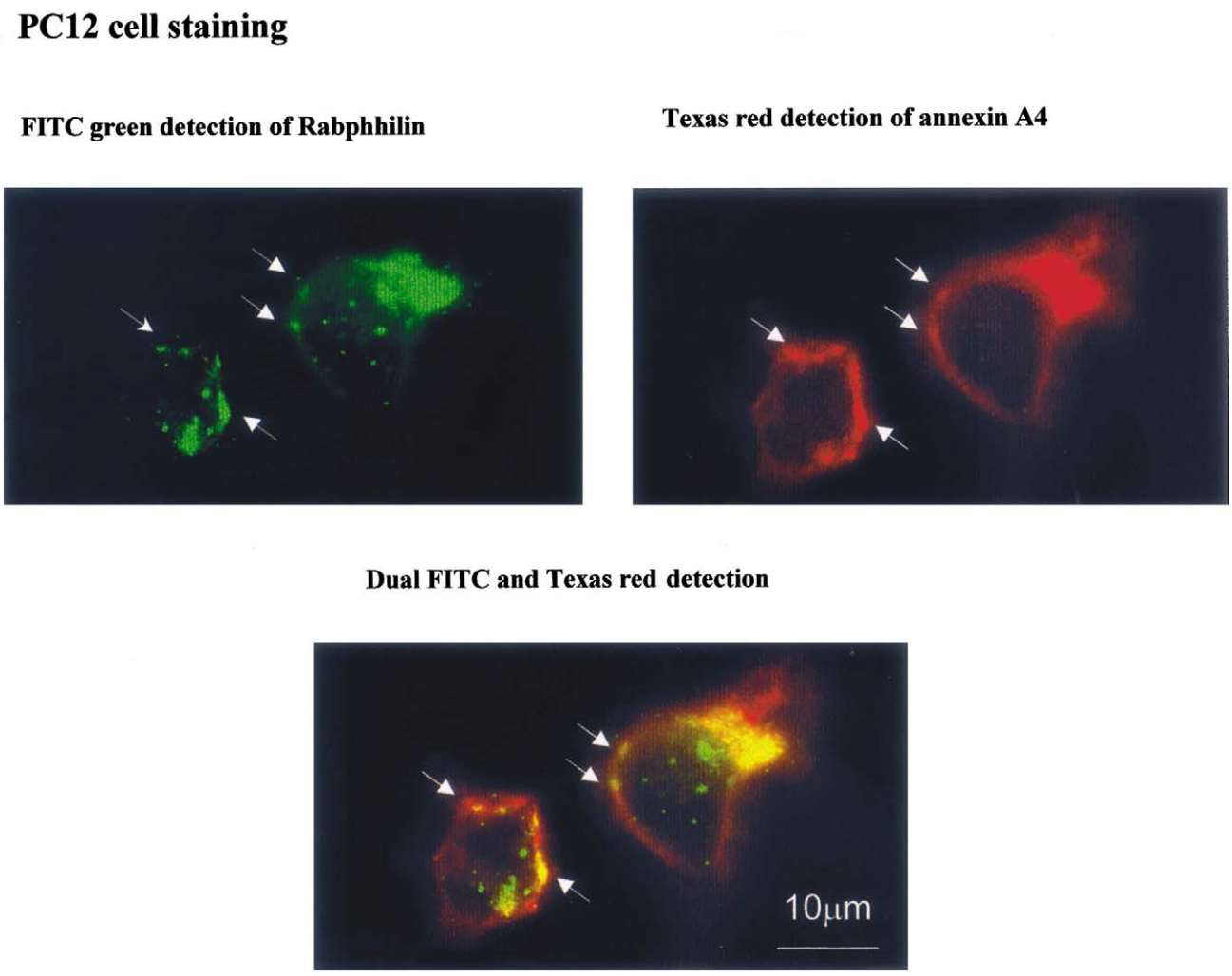


Fig. 5. Localisation of complex formation in synaptosomes, neuronal cultures and PC12 cells. A: Fractionation of synaptosomes. Equal proportions of membranes (M), vesicle (V) and cytosolic (C) fractions or whole synaptosomes (W) were loaded and probed by Western blotting with anti-annexin A4 antibodies. B: Synaptosome fractions (as defined in A) were probed for the presence of rabphilin (top panel), synaptotagmin-1 (middle panel) and synaptophysin (bottom panel). C: Rat primary cortical neuronal cultures were stained for either the glial cell-specific protein GFAP (FITC green), or annexin A4 (Texas red). D: Annexin A4 and rabphilin co-localise at the membrane periphery in PC12 cells. Rabphilin is labelled with FITC green, annexin A4 with Texas red and co-localised proteins appear yellow (dual green and red detection).

possibility that other regions of the annexin protein may also interact with the rabphilin protein in vivo.

This protein sequence within the N-terminal region is unique for this annexin family member and is believed to form an extension that lies across the concave (cytosol-facing) surface of the protein [29]. It is therefore conceivable that when annexin A4 is bound to the inner surface of the plasma membrane this tail region would still be available for highly specific protein–protein interactions to occur at the cytosolic surface. This tail region has also been proposed to play a role in regulating annexin A4 multimerisation [30] and in common with annexins A1 and A2 could play an important role in the Ca^{2+} sensitivity and cellular localisation of the protein [27,31–34].

Since, to our knowledge, annexin A4 has not previously been studied in synaptosomes, we probed for the localisation of this protein in synaptosomes. To do this we fractionated synaptosomes by centrifugation as previously described [25] and probed for the presence of annexin A4 in each fraction. We found that annexin A4 was only detected in synaptosome membranes and not in purified vesicle fractions (Fig. 5A). Furthermore, by probing synaptosome fractions with the presynaptic marker proteins rabphilin, synaptotagmin-I and synaptophysin, we have characterised our synaptosomal preparations (Fig. 5B). However, since presynaptic proteins are also found in astrocytes and annexin A4 has previously been reported to be found in glial cells in human hippocampus [34] it was conceivable that glial cell membranes could be present in our cortical synaptosome preparations. So, we probed synaptosomes with an antibody raised against a glial cell-specific protein glial fibrillary acidic protein (GFAP) and indeed we did detect some glial cell contamination (data not shown). Therefore, in order to determine whether annexin A4 was expressed and co-complexed with rabphilin and synaptotagmin in glial cells we undertook immunofluorescence studies using primary cortical neuronal cultures (Fig. 5C). Dual staining for GFAP and annexin A4 showed that annexin A4 gave a discrete punctate staining pattern and did not co-localise with glial cells and therefore annexin A4 is most probably expressed in nerve terminals (synaptosomes). In addition, we

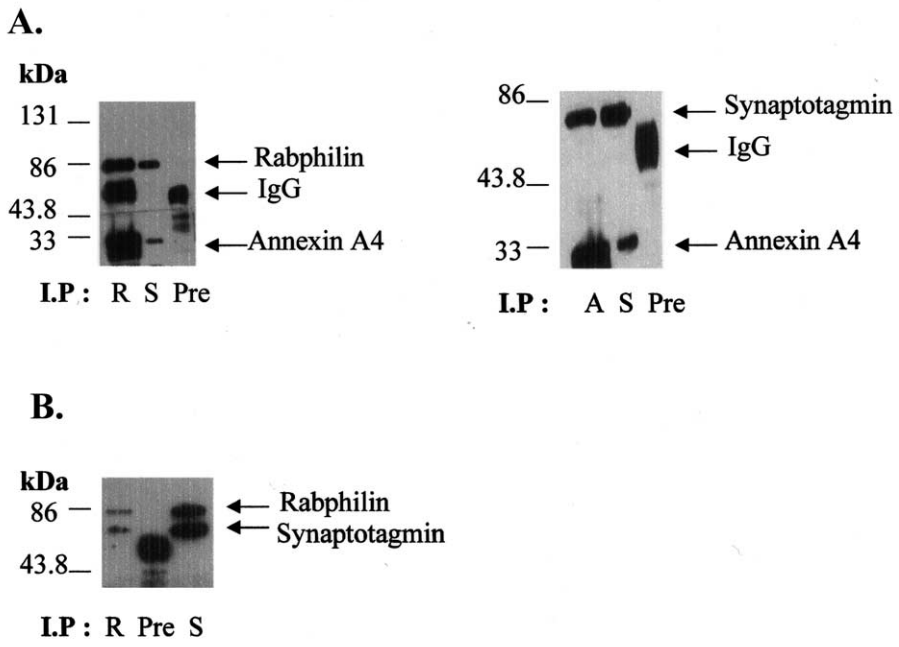


Fig. 6. Rabphilin and synaptotagmin co-complex independently of annexin A4 on synaptic vesicles. A: Triton-solubilised synaptosome membrane fractions were immunoprecipitated with either anti-rabphilin antibodies (R), anti-annexin A4 antibodies (A) or pre-immune rabbit sera (Pre). Protein complexes isolated were Western-blotted and blots probed for the presence of rabphilin and annexin A4 (left panel) or synaptotagmin and annexin A4 (right panel). Synaptosome membrane samples (S) verify the specificity of antibodies used during probing. B: Triton-solubilised vesicle fractions were immunoprecipitated with anti-rabphilin (R) antibodies or pre-immune sera (Pre) and isolated protein complexes were Western-blotted and blots probed for the presence of rabphilin and synaptotagmin.

also probed synaptosomal preparations for β-tubulin, and we were not able to detect its presence.

Furthermore, we have used the PC12 cell system in order to visualise the pattern of co-localisation of rabphilin and annexin A4 in a whole cell system that endogenously expresses annexin A4 and presynaptic proteins. These cells allow the visualisation of discrete plasma membrane and (punctate) vesicle structures (Fig. 5D). Although this cell system is non-neuronal, it is a recognised model for regulated exocytosis, and the molecular mechanisms of release are believed to be analogous to those occurring in nerve terminals. Our immunofluorescence studies confirmed that annexin A4 only displayed a plasma membrane distribution, whilst rabphilin was found to display a predominantly punctate distribution both at the plasma membrane and internally within cells (Fig. 5D). The co-localisation of annexin A4 and rabphilin was only observed at plasma membrane peripheral regions and the pattern of co-staining was clearly punctate, most probably indicative of vesicular staining.

Finally, to complete these studies, we went back into the synaptosome system and probed for co-complexing of rabphilin, annexin A4 and synaptotagmin in synaptic vesicle and membrane fractions (Fig. 6). We found that rabphilin and synaptotagmin co-complexed together with annexin A4 in the membrane fraction. In addition, in the synaptic vesicle fraction, rabphilin and synaptotagmin were still found to co-complex. These data together indicate that rabphilin and synaptotagmin must co-complex on vesicles independently of rabphilin's interaction with annexin A4 at the membrane (and indeed independently of β-tubulin since this protein is absent from synaptosomes). Therefore, we would speculate that the potential function of these proteins co-complexing with annexin A4 could be to facilitate membrane binding of rabphilin/rabphilin-associated vesicles. Such a process, in turn, could then play a role in the regulation of neurotransmitter release. Work is now under way to see if annexin A4 plays a functional role in regulated exocytosis. Although annexin A4 has previously been postulated to function as a modulator of chloride channels [35], a role for annexin A4 in exocytosis would not be unprecedented. Indeed, annexin A4, in common with a number of other annexin family members, has previously been proposed to play a role in phospholipid vesicle association and fusion events in mammalian and yeast cell systems [36–40]. Whether such a functional role can be attributed to this protein complex has yet to be established.

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