Identification and characterization of Snapin as a ubiquitously expressed SNARE-binding protein that interacts with SNAP23 in non-neuronal cells

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Members of the SNARE (soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor) superfamily [syntaxins, VAMPs (vesicle-associated membrane proteins) and SNAP25 (synaptosome-associated protein-25)-related proteins] are required for intracellular membrane-fusion events in eukaryotes. In neurons, assembly of SNARE core complexes comprising the presynaptic membrane-associated SNAREs syntaxin 1 and SNAP25, and the vesicle-associated SNARE VAMP2, is necessary for synaptic vesicle exocytosis. Several accessory factors have been described that associate with the synaptic SNAREs and modulate core complex assembly or mediate Ca^{2+} regulation. One such factor, Snapin, has been reported to be a brain-specific protein that interacts with SNAP25, and regulates association of the putative Ca^{2+}-sensor synaptotagmin with the synaptic SNARE complex [Ilardi, Mochida and Sheng (1999) Nat. Neurosci. **2**, 119–124]. Here we demonstrate that Snapin is expressed ubiquitously in neuronal and non-neuronal cells. Furthermore, using protein–protein-interaction assays we show that Snapin interacts with SNAP23, the widely expressed homologue of SNAP25, and that the predicted C-terminal helical domain of Snapin contains the SNAP23-binding site. Subcellular localization experiments revealed that Snapin is a soluble protein that exists in both cytosolic and peripheral membrane-bound pools in adipocytes. Moreover, association of Snapin with the plasma membrane was detected in cells overexpressing a Snapin–green fluorescent protein fusion protein. Finally, we show that Snapin is able to form a ternary complex with SNAP23 and syntaxin 4, suggesting that it is a component of non-neuronal SNARE complexes. An important implication of our results is that Snapin is likely to perform a general role in SNARE-mediated vesicle fusion events in non-neuronal cells in addition to its participation in Ca^{2+}-regulated neurosecretion.

Key words: exocytosis, soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE), synaptosome-associated protein-23 (SNAP23), syntaxin, trafficking, vesicle.

INTRODUCTION

The trafficking of proteins and lipids between organelles is mediated by transport vesicles that bud from a donor membrane and subsequently fuse with an appropriate acceptor compartment. Vesicle fusion is dependent on SNAREs (soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptors) [1,2]. SNAREs encompass three distinct protein families: the Syns (syntaxins), VAMPs (vesicle-associated membrane proteins; synaptobrevins) and SNAP25 (synaptosome-associated protein-25)-related proteins. Individual family members function in distinct vesicular trafficking pathways [2,3]. SNARE-mediated vesicle fusion has been studied extensively in neurons where Syn1, VAMP2 and SNAP25 are required for synaptic vesicle exocytosis. The interaction of VAMP2 on synaptic vesicles with Syn1 and SNAP25 on the presynaptic membrane to form a stable helical bundle is believed to be an obligatory step in the fusion event [4]. Importantly, SNARE complexes isolated from yeast [5] and non-neuronal mammalian cells [6] exhibit properties that are similar to the synaptic complex, suggesting that the features of SNARE-mediated vesicle fusion in the neuron are likely to be conserved.

Vesicle fusion is regulated by several accessory factors that bind to isolated SNAREs and/or assembled core complexes. In the synapse, Syn1-binding proteins include synaptotagmin I, munc18a, munc13–1, tomosyn, complexins and syntaphilin; SNAP25-binding proteins include Hrs-2 and Snapin, and VAMP2-binding proteins include synaptophysins. The best-characterized accessory factors are the synaptic Syn1-binding proteins, Munc18a and synaptotagmin I. Munc18a inhibits the interactions between the synaptic SNAREs, and may also mediate the structural transition in Syn1 that is required for fusion [7]. Synaptotagmin I is a component of synaptic vesicles that contains two cytosolic C2 domains that bind Ca^{2+} and phospholipid and mediate the interaction with Syn1 [8]. It exhibits Ca^{2+}-dependent binding to Syn1 as well as the core fusion complex, and has been proposed to function as a Ca^{2+} sensor for Ca^{2+}-regulated exocytosis. The association of synaptotagmin I with the SNARE complex has been shown to be modulated by an additional accessory protein termed Snapin [9,10]. Snapin has been reported to be a neuron-specific component of synaptic vesicles that interacts with isolated SNAP25 as well as the assembled synaptic SNARE complex [10].

Homologues of the synaptic SNAREs are involved in membrane-fusion events occurring in non-neuronal cells. SNAP23

Abbreviations used: GST, glutathione S-transferase; SNAP25, synaptosome-associated protein-25; SNAP23, synaptosome-associated protein-23; SNARE, soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor; Syn, syntaxin; VAMP, vesicle-associated membrane protein; PNS, post-nuclear supernatant; GFP, green fluorescent protein.

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(synaptosome-associated protein-23) is a ubiquitously expressed SNAP25-related protein that functions in vesicle fusion with the plasma membrane in these cell types. SNAP23 binds to Syns2, 3 and 4 [11], which are involved in exocytic trafficking pathways outside the brain [2]. Here, we identify Snapin as a SNAP23-binding protein in non-neuronal cells, and demonstrate that it is ubiquitously expressed. We show that Snapin is a soluble protein that exists in both cytosolic and peripheral membrane-associated pools. Finally, we have mapped the SNAP23-binding site on Snapin, and shown that SNAP23 can bind simultaneously to Snapin and Syn4. Our results suggest that Snapin may not function exclusively in Ca^{2+}-regulated exocytosis in neurons as previously suggested [10,12]. Rather, it is likely to function as a general component of the SNARE machinery that participates in the regulation of multiple SNAP23- as well as SNAP25-dependent vesicle trafficking pathways.

EXPERIMENTAL

Construction of cDNA clones

Murine cDNAs encoding SNAP23 and the cytoplasmic domain of Syn4 (amino acids 2–273) were amplified from a 3T3-L1 adipocyte cDNA library by PCR. The Snapin cDNA was isolated in a yeast two-hybrid screen in which the bait construct was generated by ligating the SNAP23 cDNA into the pEG202 vector [13]. An oligo-dT-primed 3T3-L1 adipocyte cDNA library in the pJG4–5 vector [13] was provided by J. Alleman-Sposeto (Metabolex, Hayward, CA, U.S.A.). The two-hybrid screen was performed as described [13], and positive clones were identified by DNA sequencing and BLAST searches. The wild type Snapin cDNA (amino acids 1–136) was generated by PCR using a full-length cDNA clone in pJG4–5 as template.

Recombinant proteins and antibodies

For protein synthesis in *Escherichia coli*, cDNAs encoding Snapin, SNAP23 or the cytoplasmic domain of Syn4 were ligated into the following vectors: pET28a (Novagen), pMAL-c2X (New England Biolabs) or pGEX-5X-1 (Amersham Biosciences). Fusion proteins were purified according to the manufacturer's instructions. SNAP23 and Syn4 antisera were prepared by immunizing rabbits with GST (glutathione S-transferase) fusion proteins. Rabbit anti-Snapin antibody was raised against maltose-binding-protein-tagged Snapin, and subsequently affinity purified from the antiserum using His_6-Snapin (histidine-tagged Snapin) immobilized on Amino Link Plus Coupling Gel (Pierce) according to the manufacturer's instructions. Anti-Xpress and -V5 antibodies were purchased from Invitrogen. For immunoblotting, primary antibodies were used at a 1:1000 dilution, followed by horseradish peroxidase-conjugated secondary antibodies (Silenus; 1:5000) and detection using ECL reagent (Pierce).

Analysis of tissue distribution

For Northern blotting, the full-length Snapin cDNA was labelled with [^{32}P]dCTP using the Multiprime DNA labelling kit (Amersham Biosciences). The radiolabelled probe was hybridized to a commercially available mouse RNA blot that was normalized for RNA loading by probing for β-actin (MTN blot; Clontech). Hybridization and washing were performed using the ULTRAhyb kit (Ambion) according to the manufacturer's instructions. The blot was exposed to X-ray film for 72 h at -80 °C prior to development. For immunoblotting, 3T3-L1 adipocytes or tissues dissected from freshly killed rats were homogenized using Polytron in lysis buffer (2 % Triton X-100, 0.15 M KCl, 2 mM EDTA, 1 mM dithiothreitol and 50 mM Hepes, pH 7.2) supplemented with a protease-inhibitor cocktail {10 μM leupeptin, 7 μM pepstatin A, 20 μM AEBSF [4-(2-aminoethyl)benzenesulphonyl fluoride] and 0.7 μM aprotinin}. The extracts were centrifuged at 20 000 ***g*** for 10 min and the supernatants diluted into SDS sample buffer, resolved by SDS/PAGE (15 % gels) and subjected to immunoblotting.

In vitro binding assays

Purified GST–SNAP23 or GST control (0.04 μM) were incubated with glutathione beads for 1 h at 4 °C in binding buffer (0.15 M KCl, 0.1 % Triton X-100 and 20 mM Hepes, pH 7.4). Following immobilization, the beads were washed and subsequently incubated for 30 min at room temperature with His_6-Snapin in 0.5 ml of binding buffer. For analysis of Syn4 binding the immobilized GST–SNAP23 was incubated with a saturating concentration (0.25 μM) of His_6-Snapin or buffer-only control. The beads were then washed extensively and incubated for 30 min with His_6-Syn4. Following incubation the beads were washed six times with 1 ml of binding buffer, and bound proteins eluted in SDS sample buffer. The samples were analysed by SDS/PAGE followed by immunoblotting using anti-His and anti-GST primary antibodies.

In vivo binding assays

For expression of GST-tagged proteins in COS-7 cells the Snapin or SNAP23 cDNAs were ligated into the pEBG vector [14]. The mammalian expression vectors encoding Snapin–V5, Snapin tagged with the V5 epitope, and Xpress–SNAP23, SNAP23 tagged with the Xpress epitope, were generated by ligating the wild-type cDNAs into the pcDNA4-V5.His and pcDNA4-His.Max vectors (Invitrogen), respectively. The Snapin deletion mutants were generated by PCR, and subcloned into the pcDNA4-V5.His vector. Transient transfection of COS-7 cells in suspension was achieved by electroporation at 200 V and 975 μF in the presence of 10 μg of each plasmid. Following transfection, cells were allowed to recover for 2 days, and lysed (one confluent 10-cm dish of cells/transfection) in 1 ml of PBS supplemented with 5 mM EDTA, 2 % Triton X-100 and protease-inhibitor cocktail. The cell lysates were centrifuged at 20 000 ***g*** for 10 min, and GST-fusion proteins recovered from the supernatants by incubation with glutathione beads for 1 h at 4 °C. The beads were washed three times with PBS supplemented with 0.1 % Triton X-100, and bound proteins eluted in SDS sample buffer. The samples were analysed by immunoblotting as indicated in the figure legends.

Subcellular localization experiments

Confluent cultures of 3T3-L1 preadipocytes were induced to differentiate in the presence of 0.6 μM insulin, 0.5 mM 3-isobutyl-1-methylxanthine and 0.25 μM dexamethasone for 7–14 days. For the fractionation experiment, one 15-cm dish of confluent adipocytes was homogenized in 1 ml of HES buffer (0.255 M sucrose, 1 mM EDTA and 20 mM Hepes, pH 7.4) supplemented with protease-inhibitor cocktail. The homogenate was centrifuged at 1000 ***g*** for 10 min, and the PNS (post-nuclear supernatant) divided into 100-μl aliquots. The PNS fractions were incubated for 30 min on ice, and centrifuged at 100 000 ***g*** for 10 min. Pellet and supernatant fractions were analysed by immunoblotting using affinity-purified Snapin antibody.

To examine the localization of endogenous Snapin by immunofluorescence microscopy, adipocytes on coverslips were serum-starved for 2 h, and exposed to 3 % paraformaldehyde for 10 min on ice, followed by 100 % methanol at − 20 °C for 2 min. The fixed cells were blocked with 5 % goat serum in PBS, and incubated with affinity-purified Snapin antibody (1:100) followed by Alexa488-conjugated secondary antibody (Molecular Probes). To examine the localization of Snapin–GFP (where GFP is green fluorescent protein) an expression construct was created by subcloning the Snapin cDNA into the pEGFP-N3 vector (Clontech). Differentiated adipocytes were co-transfected with 50 μg of the Snapin–GFP expression vector as described previously [15]. Optical sections of the cells were examined using a Leica TCS-NT confocal microscope.

RESULTS

Isolation of Snapin

In order to isolate factors that interact with SNAP23 in non-neuronal cells we utilized the yeast two-hybrid system [13] to screen a mouse 3T3-L1 adipocyte cDNA library using SNAP23 as bait. Eighteen independent positive clones were obtained, of which three encoded Snapin. A clone encoding full-length Snapin as well as two fragments were isolated (the most truncated of which lacked the N-terminal 12 amino acids of Snapin). To confirm the interaction, we transformed fresh yeast cells with the plasmids encoding SNAP23 bait and Snapin prey. Induction of the β-galactosidase reporter was observed with Snapin, but not with empty vector or various control prey constructs (results not shown). In addition, we were unable to detect an interaction between Snapin and VAMP2, indicating that Snapin does not associate non-specifically with the helical domains that are common to SNAREs. Snapin is a recently identified protein that was reported to be expressed exclusively in brain and to interact with SNAP25, but not SNAP23 [10]. The fact that we isolated Snapin from a non-neuronal cell line, and detected a strong interaction with SNAP23 in the two-hybrid system, prompted us to re-examine its tissue distribution and association with the SNARE.

Snapin is expressed ubiquitously

To investigate the tissue distribution of Snapin we performed Northern blotting using radiolabelled full-length Snapin cDNA to probe RNA samples extracted from multiple mouse tissues. This revealed the presence of a single $\approx$ 1.5-kb mRNA transcript that was detected in multiple tissues with the highest expression levels in heart, testis and liver (Figure 1). To confirm the ubiquitous expression of Snapin at the protein level, we generated an affinity-purified antibody against recombinant full-length Snapin. To confirm the specificity of the antibody we performed immunoblots where it was used to probe detergent extracts of COS-7 cells transiently transfected with an expression vector encoding a Snapin fusion protein (Snapin–V5), or empty vector control (Figure 2A). A robust immunoreactive band of the expected molecular mass was detected in cells expressing Snapin–V5 (Figure 2A, lane 2), but not the empty vector control (Figure 2A, lane 1). Furthermore, the immunoreactive Snapin band was not detected when the antibody was neutralized with purified His_6-Snapin fusion protein prior to immunoblotting (Figure 2A, lane 3). Having confirmed the specificity of the affinity-purified antibody, we used it to probe detergent extracts isolated from multiple rat tissues for expression of the endogenous protein. In these experiments we detected a $\approx$15-kDa band corresponding to the predicted molecular mass of Snapin in all tissues examined (Figure 2B). These results are consistent with the fact that we isolated the Snapin cDNA from a non-neuronal cell line and indicate that it is expressed ubiquitously.

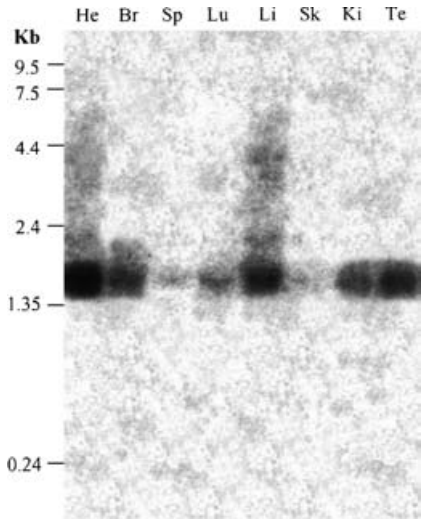

Figure 1 Tissue distribution of Snapin mRNA

Northern blotting was performed by probing a poly(A)$^+$ RNA blot isolated from mouse tissues with ^{32}P-labelled Snapin cDNA. A commercially available mouse RNA blot was used that was normalized for β-actin loading. Blots were washed and autoradiographed. Tissues: He, heart; Br, brain; Sp, spleen; Lu, lung; Li, liver; Sk, skeletal muscle; Ki, kidney; Te, testis.

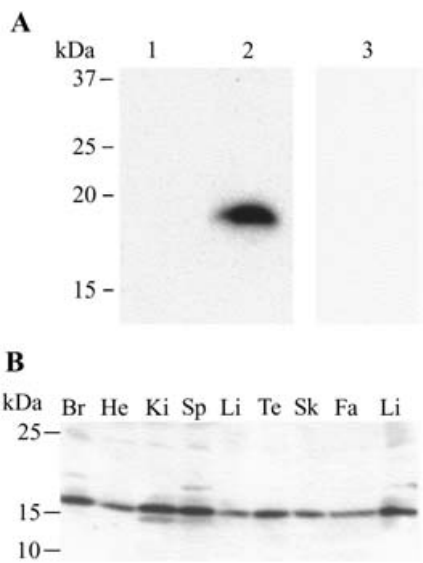

Figure 2 Snapin is expressed ubiquitously

(**A**) Specificity of affinity-purified Snapin antibody investigated by immunoblotting. COS-7 cells were transfected in parallel with a Snapin expression vector encoding an 18.5-kDa Snapin fusion protein (Snapin–V5; lanes 2 and 3), or with empty vector control (lane 1). Detergent-solubilized total cell lysates were prepared, and equal amounts of protein analysed by immunoblotting. In lane 3 the blot was probed with an equivalent concentration of Snapin antibody following pre-neutralization with purified His_6-Snapin protein. (**B**) Tissue distribution of Snapin analysed by immunoblotting. Detergent extracts of total cell protein (equal loading of 100 μg of protein/lane) from rat tissues were probed using affinity-purified Snapin antibody. The band detected across all tissues corresponds to the predicted molecular mass of Snapin ($\approx$ 15 kDa). Tissues: Br, brain; He, heart; Ki, kidney; Sp, spleen; Li, liver; Te, testis; Sk, skeletal muscle; Fa, fat; L1, 3T3-L1 adipocytes.

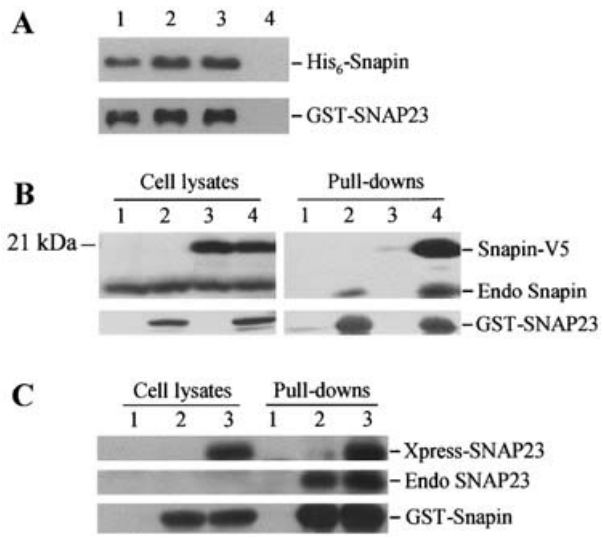

Figure 3 Snapin associates with SNAP23

(**A**) *In vitro* binding assay. Purified GST–SNAP23 (lanes 1–3) or control GST protein (lane 4) were immobilized on glutathione beads, and subsequently incubated with His_6-Snapin at the indicated concentrations. Binding was analysed by immunoblotting using anti-His and anti-GST antibodies. Under these conditions we reproducibly observed saturable binding at roughly 0.2 μM His_6-Snapin. (**B**) *In vivo* pull-down assay. COS-7 cells were transfected with expression vectors encoding GST–SNAP23 (lanes 2 and 4) or GST control vector (lanes 1 and 3) in combination with epitope-tagged Snapin (Snapin–V5) (lanes 3 and 4) or empty vector control (lanes 1 and 2). The transfected cells were solubilized in 1 ml of buffer containing 2 % Triton X-100 and GST proteins isolated from the lysates on glutathione beads. Cell lysate (40 μl) and the entire sample eluted from the glutathione beads were subjected to SDS/PAGE, and immunoblotted using anti-GST and affinity-purified Snapin antibodies. (**C**) COS-7 cells were co-transfected with expression vectors encoding GST–Snapin (lanes 2 and 3), or GST control (lane 1) in combination with epitope-tagged SNAP23 (Xpress–SNAP23; lane 3) or empty vector control (lanes 1 and 2). GST proteins were recovered from cell lysates on glutathione beads, and immunoblots probed using anti-GST and anti-SNAP23 antibodies as described above. These experiments were repeated several (>five) times and representative blots are shown. Endo, endogenous.

Snapin interacts with SNAP23

To investigate further the interaction between Snapin and SNAP23 observed in the two-hybrid system we performed an *in vitro* binding assay using recombinant fusion proteins (Figure 3A). In this experiment, SNAP23 fused to GST was immobilized on glutathione beads, and incubated with increasing concentrations of His_6-Snapin. Subsequently, the beads were washed extensively and bound proteins analysed by immunoblotting. A saturable association between His_6-Snapin and GST–SNAP23 was observed (Figure 3A, lanes 1–3), and no background Snapin binding was detected in controls where GST–SNAP23 was substituted by GST (Figure 3A, lane 4). Although the Snapin and SNAP23 antibodies were able to immunoprecipitate complexes formed from the purified bacterially expressed proteins, we were unable to detect an interaction between endogenous Snapin and SNAP23 in co-immunoprecipitation experiments (results not shown). The most likely explanation is that in cells the interaction is a transient intermediate in the SNARE functional cycle and/or inhibited by additional endogenous proteins. In light of this we hypothesized that by overexpressing Snapin or SNAP23 in cells we might be able to detect an interaction with the respective endogenous binding partner.

To test this hypothesis we performed a 'pull-down' assay by transfecting COS-7 cells with an expression vector encoding GST–SNAP23 (Figure 3B). Protein complexes containing the GST fusion protein were subsequently isolated from detergent-solubilized cell lysates on glutathione beads and analysed by immunoblotting. When GST–SNAP23 was co-expressed with Snapin tagged with the V5 epitope (Snapin–V5), a strong band corresponding to the recombinant Snapin protein was detected in the bead eluate (Figure 3B, lanes 4). This band was not detected when the GST–SNAP23 construct was substituted by a control vector encoding GST (Figure 3B, lanes 3). Importantly, an association between GST–SNAP23 and endogenous Snapin protein was detected by immunblotting using affinity-purified Snapin antibody (Figure 3B, lanes 2). Endogenous Snapin was not recovered on the beads in a control where the cells expressed GST (Figure 3B, lanes 1), confirming the specificity of the interaction.

In addition, we performed the converse experiment by transfecting cells with GST-tagged Snapin (GST–Snapin). An interaction was detected between the recombinant proteins when cells were co-transfected with GST–Snapin and SNAP23 tagged with the Xpress epitope (Figure 3C, lanes 3), indicating that the ability of Snapin to bind to SNAP23 was retained in the GST fusion construct. In cells expressing GST–Snapin alone, we detected an interaction with endogenous SNAP23 (Figure 3C, lanes 2). The endogenous SNAP23 band in the bead eluate was not observed when the expression vector encoding GST–Snapin was substituted by GST control (Figure 3C, lanes 1). Endogenous SNAP23 was detected in the GST–Snapin bead eluate, but not in the total cell lysate due to the fact that it is effectively concentrated on the beads during the pull-down procedure. In addition, we observed that a higher proportion of the total endogenous SNAP23 was isolated on the GST–Snapin beads compared with recombinant Xpress–SNAP23. This may be explained by the possibility that GST–Snapin has a higher affinity for endogenous SNAP23 than Xpress–SNAP23, presumably as a result of misfolding of the overexpressed fusion protein, or a steric effect of the epitope tag on binding.

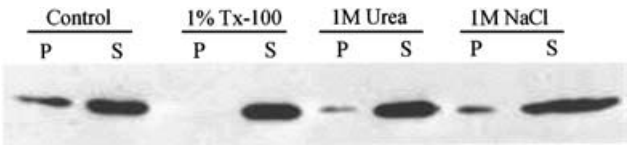

Figure 4 Soluble and peripheral membrane-associated pools of Snapin exist in cells

A PNS was prepared following homogenization of 3T3-L1 adipocytes. The PNS was divided into equal aliquots, and incubated in the presence of homogenization buffer (control), 1 % Triton X-100 (Tx-100), 1 M urea or 1 M NaCl as indicated. The samples were centrifuged at 100 000 ***g*** to sediment membranes, and equal proportions of the pellet (P) and supernatant (S) fractions were loaded on to gels. Snapin protein was detected by immunoblotting using affinity-purified antibody. These experiments were repeated four times and a representative blot is shown.

Intracellular localization of Snapin

Previously, it was suggested that the N-terminal hydrophobic region of Snapin (amino acids 1–20) constitutes a transmembrane domain [10]. However, we found that sequence analysis using the TMHMM transmembrane prediction program [16] did not identify a potential membrane-spanning segment. To investigate this issue further we performed a biochemical fractionation experiment. A PNS fraction was prepared from 3T3-L1 adipocytes, and centrifuged at 100 000 ***g*** to sediment total cell membranes (Figure 4). Immunoblotting of the soluble and particulate fractions using affinity-purified Snapin antibody revealed that roughly 70 % of total cellular Snapin is present in the cytosol (Figure 4, Control). This result is consistent with the sequence

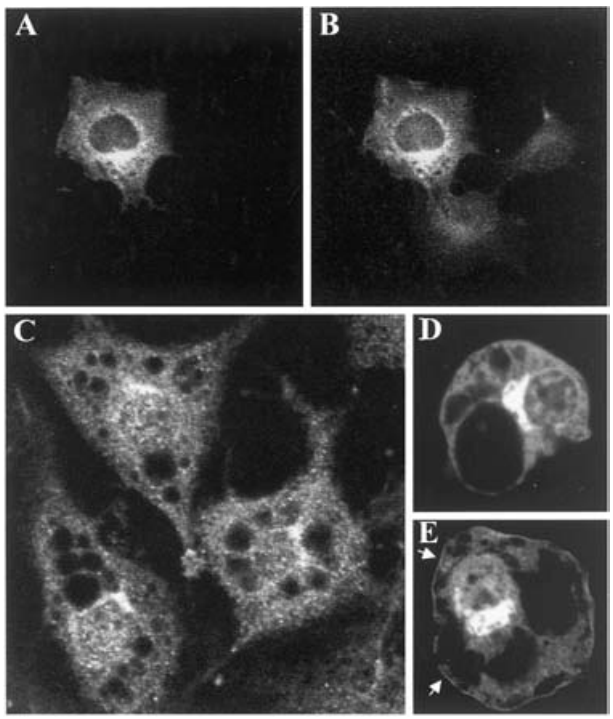

Figure 5 Subcellular localization of Snapin investigated by confocal fluorescence microscopy

(**A**, **B**) COS-7 cells were transfected with an expression vector encoding Snapin–V5. Then 2 days after transfection the cells were fixed and permeabilized, and double-stained using a mouse monoclonal antibody recognizing the V5 epitope tag (**A**) or the affinity-purified Snapin antibody (**B**). The cells were subsequently incubated with Alexa488-conjugated anti-mouse IgG and Alexa568-conjugated anti-rabbit IgG secondary antibodies and examined by confocal microscopy. (**C**) Differentiated 3T3-L1 adipocytes on glass coverslips were incubated in serum-free medium for 2 h, then fixed and permeabilized prior to confocal microscopy. The localization of endogenous Snapin was visualized by incubation with affinity-purified Snapin antibody, followed by Alexa488-conjugated secondary antibody. Fluorescent staining of the adipocytes was not observed in controls where the anti-Snapin antibody was omitted. (**D**, **E**) Adipocytes were transiently transfected with an expression vector encoding Snapin–GFP prior to incubation in serum-free medium and fixation. Two different transfected cells are shown. Plasma-membrane localization was detected in roughly half of the transfected cells examined (indicated with arrows in **E**). The large black holes in the cytoplasm are lipid droplets.

analysis, confirming that Snapin is not a transmembrane protein. The pool of Snapin present in the particulate fraction was completely solubilized by incubation of the PNS fraction with non-ionic detergent, and partially solubilized in the presence of high concentrations of salt or urea (Figure 4). These observations provide strong evidence that the sedimentable pool of Snapin is associated with membranes. Therefore, Snapin is present in cytosolic and peripheral membrane-associated pools in 3T3-L1 adipocytes.

In addition, we performed indirect immunofluorescence microscopy using affinity-purified Snapin antibody. To confirm that the antibody recognized Snapin under the experimental conditions employed we transfected COS-7 cells with an expression vector encoding Snapin–V5, then double-stained the cells with mouse anti-V5 and rabbit anti-Snapin antibodies. In transfected cells strong cytoplasmic staining was detected using the anti-V5 antibody (Figure 5A). The fluorescent staining observed using anti-Snapin was dramatically brighter in transfected cells than non-transfected cells, and overlapped precisely with that detected using anti-V5 antibody (Figure 5B). Furthermore, we found that pre-neutralization of the Snapin antibody under conditions identical to those employed for the immunoblotting experiments (Figure 2A) abolished the anti-Snapin fluorescence detected in transfected cells (results not shown). These observations confirmed the suitability of the Snapin antibody for analysis of the subcellular localization of the endogenous protein in cells.

In view of the faint fluorescence detected using anti-Snapin in non-transfected COS-7 cells (Figure 5B), we examined the localization of endogenous Snapin in a different cell line, 3T3-L1 adipocytes (Figure 5C). In these cells, we observed diffuse cytosolic staining as well as enrichment in the perinuclear region, which typically reflects association with Golgi and/or endosomal membranes. To confirm these data using an alternative approach, we constructed an expression vector encoding wild-type Snapin fused to GFP (Snapin–GFP), and then examined the localization of the fusion protein in adipocytes (Figures 5D and 5E). We observed that Snapin–GFP exhibited localization to the cytosol and perinuclear region (Figure 5D), consistent with the results obtained for the endogenous protein (Figure 5C). In roughly 50% of the transfected cells, we detected a rim of Snapin–GFP fluorescence around the surface of the cell (Figure 5E), indicative of localization to the plasma membrane. The plasma membrane and perinuclear fluorescence observed for Snapin–GFP was absent from control cells expressing GFP (results not shown). The fact that we detected Snapin–GFP, but not the endogenous protein, at the plasma membrane may simply reflect the higher levels of Snapin expression in the transfected cells. The Snapin-binding sites at the plasma membrane (possibly SNAP23) may not be fully occupied in the non-transfected cells, whereas in transfected cells these sites may be saturated with Snapin–GFP, leading to a higher signal that is more readily detectable by fluorescence microscopy. Our ability to detect localization of Snapin–GFP at the plasma membrane is consistent with the biochemical evidence supporting an interaction with SNAP23, a protein that is associated predominantly with this membrane in non-neuronal cells [11].

The predicted helical domains of Snapin are involved in SNAP23 binding

Snapin has been reported to contain a single predicted helical domain extending from amino acids 83 to 119 [10]. In contrast, we found that analysis of murine Snapin using the Coils program [17] identified two regions (H1, amino acids 37–65, and H2, amino acids 81–126) with a high probability of forming helical domains (Figure 6A). The C-terminus of Snapin (residues 79–136) has been reported to be sufficient for interaction with SNAP25 [10]. The most truncated Snapin cDNA isolated in the two-hybrid screen encoded a fragment lacking the N-terminal 12 amino acids, indicating that this region of Snapin is not critical for interaction with SNAP23. To investigate further the SNAP23-binding site on Snapin we constructed deletion mutants based on the domain map shown in Figure 6(A). Three mutants were constructed: Snapin(36–136), comprising both predicted helices (H1 and H2); Snapin(1–79), comprising the N-terminal hydrophobic region and H1, and Snapin(79–136), comprising H2 only. Expression vectors encoding the deletion mutants with a V5 epitope tag were co-transfected along with GST–SNAP23 into COS-7 cells, and complex formation assessed using a pull-down assay (Figure 6B).

As expected, a robust interaction was observed in cells co-expressing wild-type Snapin–V5 and GST–SNAP23 (Figure 6B, lanes 3). The Snapin–V5 band in the bead eluate was not detected in controls where cells were co-transfected with GST–SNAP23 and empty vector (Figure 6B, lanes 1), or empty GST vector and

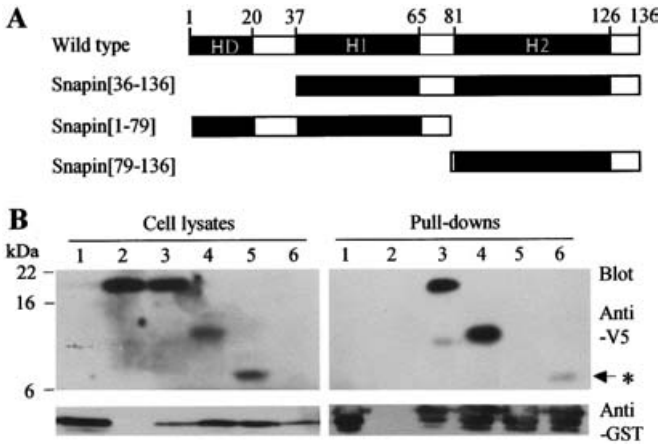

Figure 6 Analysis of the SNAP23-binding site on Snapin by deletion mutagenesis

(**A**) Domain map of Snapin indicating the positions of the N-terminal hydrophobic domain (HD; amino acids 1–20) and two predicted helical regions (H1 and H2; amino acids 37–65 and 81–126, respectively). The truncated mutants were constructed by deleting specific domains of Snapin as indicated. (**B**) Pull-down assay where cells were co-transfected with expression vectors encoding GST–SNAP23 and empty vector (lanes 1), wild-type Snapin (lanes 3), Snapin(36–136) (lanes 4), Snapin(1–79) (lanes 5) or Snapin(79–136) (lanes 6). As a control for non-specific binding, wild-type Snapin–V5 was co-transfected with an expression vector encoding GST alone (lanes 2). Transfected cells were solubilized in 1 ml of a 2 % Triton X-100-containing buffer, and protein complexes isolated on glutathione beads. Cell lysate (40 μl) and the entire bead eluate were subjected to SDS/PAGE, and immunoblotted using anti-V5 antibody to detect Snapin (upper panels) and anti-GST antibodies (lower panels). The Snapin(79–136) band in the bead eluate is indicated with an asterisk. The multiple bands detected using the anti-GST antibody in the bead eluates are likely to result from proteolysis of the GST–SNAP23 fusion protein during the procedure. This experiment was repeated three times and a single representative blot is shown.

Snapin–V5 (Figure 6B, lanes 2). Immunoblotting of the cell lysates revealed that the expression levels of Snapin(36–136) (Figure 6B, cell lysates, lane 4) and Snapin(1–79) (Figure 6B, cell lysates, lane 5) were reduced significantly compared with the wild-type protein, while Snapin(79–136) was not detected (Figure 6B, cell lysates, lane 6). This suggests that the amino acid deletions alter the structural integrity of Snapin, leading to the production of mutants with compromised stability. The amount of Snapin(36–136) in the bead eluate was roughly equivalent to the wild-type protein, indicating that the predicted helical domains are sufficient for binding to SNAP23. Snapin(1–79) did not exhibit detectable binding to GST–SNAP23, suggesting that H2 is critical for the interaction. Consistent with this we found that although Snapin(79–136) expression was below the limit of detection in the cell lysate, it was apparent in the bead eluate. This is presumably due to concentration of the mutant during isolation on the beads. Taken together the data suggest that the C-terminal predicted helical domain, H2, represents the primary SNAP23-binding site.

Binding of Snapin and Syn4 to SNAP23 is not competitive

In non-neuronal cells the association between SNAP23 and Syns2, 3 or 4 is critical for SNARE complex assembly and vesicle fusion in a variety of different exocytic pathways [2,11]. To begin to address whether Snapin might play a role in the regulation of this interaction an *in vitro* binding assay was performed in which we compared the binding of Syn4 with isolated SNAP23 and Snapin–SNAP23 complexes (Figure 7). In this experiment, purified GST–SNAP23 (Figure 7, lanes 2–11) or GST control (Figure 7, lane 1) were immobilized on glutathione beads, and subsequently incubated in the absence (Figure 7, lanes 1–6) or

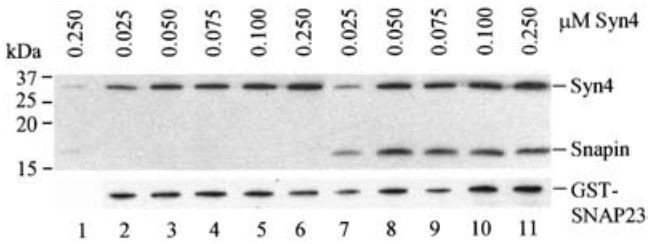

Figure 7 Snapin does not affect Syn4-SNAP23 complex formation

Binding of His_6-Syn4 to isolated GST–SNAP23 or His_6-Snapin–GST–SNAP23 complexes was measured using an *in vitro* binding assay. Purified GST–SNAP23 (lanes 2–11) or control GST protein (lane 1) were immobilized on glutathione beads, and subsequently incubated in the absence (lanes 1–6), or presence (lanes 7–11) of a saturating concentration of His_6-Snapin. The glutathione beads were washed, and incubated with increasing concentrations of His_6-Syn4 as indicated. Complex formation was analysed by immunoblotting using anti-His and anti-GST antibodies. The levels of GST–SNAP23 and His_6-Snapin detected on the beads provide internal controls for variations in sample recovery in the assay. This experiment was repeated several (> five) times and typical data are shown.

presence (Figure 7, lanes 7–11) of a saturating concentration of His_6-Snapin. Following washing to remove unbound protein, the immobilized GST–SNAP23 or preformed Snapin–SNAP23 complexes were incubated with increasing concentrations of purified His_6-Syn4. The amount of Syn4 associated with SNAP23 increased as its concentration in the assay was raised from 0.025 (lane 2) to 0.05 μM (lane 3). No significant increase in Syn4 binding was detected at concentrations from 0.05 to 0.25 μM (lanes 3–6), indicating that saturation had been reached. The binding observed was specific since when GST–SNAP23 was substituted by GST the recovery of Syn4 on the beads was negligible (Figure 7, lane 1). The levels of Syn4 associated with Snapin–SNAP23 complexes were similar to those observed for isolated SNAP23 at all concentrations of Syn4 tested. These results indicate that binding of Syn4 and Snapin to SNAP23 is not competitive, and that Snapin binding does not alter the affinity of Syn4 for SNAP23.

DISCUSSION

In this study we demonstrate that Snapin is a ubiquitously expressed soluble factor that associates with SNAP23 in non-neuronal cells. This data contrasts with the recent report that described Snapin as a brain-specific protein that interacts with SNAP25, but not its broadly expressed homologue [10]. We are unable to account for these disparate findings. In the previous study the tissue distribution of Snapin was investigated by immunoblotting only [10]. Our immunoblotting data showing expression of Snapin outside the brain was obtained using an affinity-purified Snapin antibody, and is supported by Northern blotting experiments as well as the fact that we isolated Snapin from a non-neuronal cell line. Species differences do not account for the disparate findings since in both studies the tissue distribution of Snapin at the protein level was investigated in rat. In the previous report [10], the conclusion that Snapin does not interact with SNAP23 was based on an *in vitro* binding assay where Snapin was shown to associate with SNAP25, but not SNAP23. In contrast, we detected an interaction between Snapin and SNAP23 using the yeast two-hybrid system, as well as *in vitro* and *in vivo* binding assays. Therefore, differences between the experimental conditions or activity of the fusion proteins used in the *in vitro* binding assays are likely to account for the disparate conclusions drawn in these studies. The previous study also reported that Snapin is an integral membrane

protein. In contrast, we obtained multiple lines of evidence from subcellular fractionation, as well as sequence analysis and fluorescence microscopy, indicating that Snapin does not possess a transmembrane domain.

We report that in addition to an N-terminal hydrophobic region (amino acids 1–20) Snapin contains two predicted helical domains, H1 (amino acids 37–65) and H2 (amino acids 81–136). Based on this domain map we investigated the SNAP23-binding site on Snapin by deletion mutagenesis. A mutant [Snapin(36–136)] consisting of both H1 and H2 exhibited SNAP23-binding properties that were similar to the wild-type protein, providing strong evidence that the predicted helical domains are sufficient for interaction with SNAP23. Further analysis of Snapin mutants containing either H1 [Snapin(1–79)] or H2 [Snapin(79–136)] suggested that the C-terminal helical domain constitutes the primary SNAP23-binding site. Although these results should be viewed with caution given the possible compromised structural integrity of the Snapin deletion mutants, our findings are consistent with previous data on Snapin–SNAP25 interaction [10]. The importance of the C-terminus of Snapin in binding to SNAP25 was demonstrated using *in vitro* binding assays where the Snapin(79–136) mutant or a peptide comprising the C-terminal 20 amino acids of Snapin was shown to competitively inhibit the interaction. Furthermore, the Snapin(79–136) mutant was shown to inhibit the association of synaptotagmin with the synaptic SNARE complex, and to block synaptic transmission [10]. These observations underscore the importance of the C-terminal predicted helical domain of Snapin in the regulation of SNARE-mediated vesicle fusion.

We have compared the ability of Syn4 to bind to isolated SNAP23 and pre-formed Snapin–SNAP23 complexes. Syn4 belongs to a subgroup of the Syn family (Syns 1–4) whose members are closely related based on sequence homologies between their SNARE motifs [18], and are involved in vesicle fusion with the plasma membrane. While Syn1 associates with SNAP25 and is required for neurosecretion, Syns 2–4 interact with SNAP23 and are involved in distinct exocytic pathways in non-neuronal cells [2,11]. Our data indicate that Snapin does not affect the ability of Syn4 to bind to SNAP23, implying that the binding sites for Syn4 and Snapin on SNAP23 are distinct. It remains to be determined whether Snapin exerts modulatory effects on VAMP binding to SNAP23–Syntaxin complexes. Nevertheless, the demonstration that Snapin and Syn4 are able to co-associate with SNAP23 is consistent with the previous observation that Snapin is a component of synaptic SNARE complexes containing SNAP25, Syn1 and VAMP2. Like SNAP25, SNAP23 is predicted to comprise two helical domains [2]. In the case of SNAP25 both helices are involved in binding cognate SNAREs [4], whereas the N-terminal helix additionally binds to molecules such as α-SNAP [19], Hrs-2 [20] and SNIP (SNAP25-interacting protein) [21]. It will be interesting to determine whether these molecules compete with Snapin for SNAP23/SNAP25 binding.

SNAP23 has been shown to be involved in membrane-fusion events occurring in multiple trafficking pathways to the plasma membrane, including: transferrin recycling in Madin–Darby canine kidney cells [22]; insulin-stimulated translocation of the Glut4 glucose transporter [23]; compound exocytosis in mast cells [24]; neutrophil exocytosis [25], and lysosome release [26] and dense-core granule secretion [27] from platelets. It is tempting to speculate that Snapin may also function in some or all of these pathways. A role for Snapin in synaptic vesicle exocytosis was established following introduction of a C-terminal peptide or a bacterially expressed truncated Snapin mutant (residues 79–136) into cultured neurons [10]. Since we have shown that the predicted C-terminal helical domain of Snapin contains the major binding site for SNAP23 the Snapin(79–136) mutant may also be a useful reagent for assessing the role of the Snapin–SNAP23 interaction in trafficking. In neurons Snapin has been shown to be a target for direct phosphorylation by cAMP-dependent protein kinase A, suggesting that it may function to modulate synaptic transmission through second-messenger signalling pathways [9]. Phosphorylation of Snapin was demonstrated to promote association of synaptotagmin with the synaptic SNARE complex, and to stimulate the magnitude of both the rapid burst and sustained phases of exocytosis from chromaffin cells. It will be interesting to determine whether Snapin is similarly regulated by phosphorylation in non-neuronal cells.

The observation that Snapin is a broadly expressed protein that associates with SNAP23 has important implications for our understanding of its role in SNARE-mediated vesicle fusion. The previous data suggesting that Snapin is a brain-specific protein that regulates the interaction between the synaptic SNARE complex and the putative Ca^{2+} sensor, synaptotagmin I [9,10], led to speculation that it is involved in Ca^{2+} regulation of synaptic vesicle exocytosis [12]. Our data is not inconsistent with this possibility, although it does suggest that Snapin is unlikely to function exclusively in Ca^{2+}-regulated membrane-fusion events. In this regard it is interesting to note that the synaptotagmin family contains members that are both brain-specific and bind Ca^{2+} with high affinity and those that are expressed ubiquitously and have a low affinity for the divalent cation, suggesting that synaptotagmins perform both Ca^{2+}-dependent and Ca^{2+}-independent functions [8]. Therefore, in non-neuronal cells where exocytosis is not Ca^{2+} regulated, Snapin may be a general component of the SNARE machinery that functions to modulate the Ca^{2+}-independent activities of synaptotagmin(s). The ability to test this hypothesis awaits a better understanding of the functions of the ubiquitously expressed synaptotagmins, and the identification of putative family members that associate with SNARE complexes containing SNAP23. Addressing these issues will provide critical new insights into the mechanisms of SNARE-mediated membrane fusion in non-neuronal cells.

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