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SNAP-25 is present in a SNARE complex in adrenal chromaffin cells

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Abstract SNAP-25 (synaptosomal-associated protein 25 kDa) is a target for botulinum neurotoxins A and E, which both inhibit neurotransmitter release, and was recently identified together with syntaxin and synaptobrevin as receptors for NSF and α-SNAP. We show that SNAP-25 was enriched in the microsomal fraction from adrenal medulla, although the level of SNAP-25 in adrenal medullary microsomes was about 20-fold less than in brain microsomes. Immunocytochemistry confirmed the presence of SNAP-25 in cultured chromaffin cells and showed plasma membrane staining. Using immunoprecipitation, we found that SNAP-25 was present in a complex with syntaxin, synaptobrevin, synaptotagmin, NSF, α-SNAP and other unidentified polypeptides. These data indicate that SNAP-25 in chromaffin cells is present in a complex similar to that identified in brain.

Key words: Exocytosis; Syntaxin; *N*-Ethylmaleimide-sensitive fusion protein (NSF); SNAP; Synaptotagmin; Secretion

1. Introduction

SNAP-25 was originally identified as a neuron-specific protein [1] that is associated with the plasma membrane of the presynaptic nerve terminal [2]. The expression of SNAP-25 correlates with the onset of synaptogenesis [3]. SNAP-25 expression is highly conserved with a high degree of homology between different species [3–5]. Depletion of SNAP-25 expression in cultured rat cortical neurons with antisense oligonucleotides resulted in the prevention of neurite elongation and a role for SNAP-25 in neuronal plasticity has hence been proposed [6].

Although SNAP-25 is tightly associated with the plasma membrane, its amino acid sequence does not predict the existence of a transmembrane domain [1]. Binding to membranes is believed to be via palmitoylation of four clustered cysteine residues [1]. Recently, the chicken gene encoding SNAP-25 was cloned and two isoforms, SNAP-25 a and b, found that are generated by alternative splicing [5]. These isoforms differ in the sequence of the cysteine-rich domain. It is not known whether this affects the efficiency of acylation and hence membrane binding [5,7].

Functional evidence for a role of SNAP-25 in neurotransmitter release has recently emerged from work on clostridial neurotoxins. Botulinum neurotoxins (BoNT) A and E specifically cleave recombinant SNAP-25 and SNAP-25 present in synaptosomes [8–11]. Neurotransmitter release is markedly impaired after intoxification of synapses with BoNT/A or BoNT/E [8–11]. The role of SNAP-25 in neurotransmitter release was further underscored when SNAP-25 was found to be part of the putative docking complex that is implicated in membrane fusion [12,13]. Transport of vesicles through the early secretory pathway requires the presence of NSF (NEM-sensitive fusion protein) and SNAPs)soluble NSF attachment proteins) [14–16]. In an attempt to affinity purify membrane receptors for NSF/SNAPs, Söllner et al. retrieved synaptobrevin, syntaxin and SNAP-25 as SNAREs (SNAP-receptors) from solubilized brain membranes [15]. Synaptobrevin, present on the vesicular membrane [17,18], was termed a v-SNARE (vesicle SNARE) and the plasma membrane proteins syntaxin [19] and SNAP-25 [1] were termed t-SNAREs (target SNAREs). These proteins were found to form a complex and it was proposed that ATP hydrolysis by NSF was a driving force to disintegrate the docking complex and to allow membrane fusion to proceed [13]. The correct binding of v-SNAREs to their appropriate t-SNAREs and the resulting specific targetting of donor and acceptor membranes is known as the SNARE-hypothesis [13]. All the SNAREs found in brain are specific targets for the various clostridial toxins, which inhibit neurotransmitter release [20].

As the SNARE hypothesis as mentioned above puts forward a universal model for the docking of vesicles to their target membranes, it is important to test this model for non-neuronal cell types. Chromaffin cells undergo Ca^{2+}-triggered exocytosis to release catecholamines [21,22]. As neuroendocrine cells they share common features with neuronal cells and two proteins of the SNARE complex, synaptobrevin and syntaxin, have been shown to be present in chromaffin cells [23]. Exocytosis from these cells is sensitive to the clostridial toxins [24–26]. We have examined the localization of SNAP-25 in chromaffin cells using specific antisera. We demonstrate that SNAP-25 is part of a precipitatable complex similar to the SNARE complex from brain.

2. Materials and methods

2.1. Reagents

A first batch of rabbit SNAP-25 polyclonal antiserum [1] used in Fig. 1 was a gift from Michael C. Wilson (The Scripps Research Institute, La Jolla, USA). A second batch of SNAP-25 polyclonal antiserum was generated in rabbits using a similar synthetic peptide (RIDEANQRATKMLGSG) with a C-terminal cysteine added to allow coupling to maleimide-activated keyhole limpet hemocyanin (Pierce). Synaptobrevin antiserum raised in guinea pigs against a synthetic peptide comprising amino acids 33–94 from the cytoplasmic domain of human synaptobrevin was generously provided by Clifford Shone (PHLS, Salisbury, UK). Antiserum directed against the cytoplasmic domain of bovine synaptotagmin was a gift from David Apps (Edinburgh, UK). NSF antibody was prepared using recombinant His_6-NSF and affinity purified from rabbit antiserum on His_6-NSF bound to NTA-agarose. Recombinant His_6-NSF and His_6-α-SNAP were

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Abbreviations: BoNT, botulinum neurotoxin; NSF, *N*-ethylmaleimide-sensitive fusion protein; PNP, post-nuclear pellet; PNS, post-nuclear supernatant; SNAP-25, synaptosomal associated protein 25 kDa; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor.

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expressed from plasmids [27] kindly provided by J.E. Rothman (Sloan Kettering Institute, New York, USA).

2.2. Subcellular fractionation of adrenal medullae

Bovine adrenal medullae were homogenized in 0.3 M sucrose, 1 mM EGTA, 5 mM HEPES, pH 7.3 and centrifuged at 800 × *g* for 15 min. The supernatant was centrifuged at 17,000 × *g* for 20 min at 4°C. The supernatant of this step (PNS) was further centrifuged at 100,000 × *g* for 60 min at 4°C to provide cytosol (supernatant) and microsomal (pellet) fractions. The pellet (PNP) from the 17,000 × *g* centrifugation contained mitochondria as well as crude chromaffin granules. The mitochondria were washed off the top of the pellet. The crude chromaffin granule pellet was resuspended in homogenization buffer, overlayed on a sucrose cushion (1.7 M sucrose, 1 mM EGTA, 1 mM $MgSO_4$, 5 mM HEPES, pH 7.3) and centrifuged at 100,000 × *g* for 60 min at 4°C. The granule pellet was lysed after resuspending in 1 mM $MgSO_4$, 20 mM HEPES, pH 7.3 by freeze-thawing. The granule membranes were obtained by centrifugation at 100,000 × *g* for 60 min at 4°C.

2.3. Immunocytochemistry

Isolation of chromaffin cells was as previously described [28] with modifications [29]. Chromaffin cells were grown for 2 days on glass cover slips in 24-well trays at 10^6 cells per well. Cells were washed in PBS and fixed in 4% formaldehyde in PBS for at least 2 h. After washing twice in PBS, cells were permeabilized in PBT (0.1% Triton X-100, 0.3% BSA in PBS) for 30 min. Cells were then incubated with anti-SNAP-25 antibody (1/100 in PBT) for 1 h at room temperature. Following incubation with biotinylated anti-rabbit IgG antibody (1/100; Amersham) for 1 h, cells were further incubated with streptavidin–Texas red (1/50 in PBT) for 30 min in the dark. Then, cells were washed 3 times in PBT and mounted in antifade glycerol (glycerol/PBS (9/1) containing 0.25% DABCO (1,4-diazabicyclo-[2.2.2]octane) and 0.002% *p*-phenyldiamine).

2.4. Immunoprecipitation

Adrenal medullae were dissected from bovine adrenal glands and homogenized in a Dounce homogenizer in ice-cold buffer A (20 mM Tris-HCl, pH 8.0, 1 M KCl, 250 mM sucrose, 2 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF). The homogenate was filtered through a double layer of muslin and centrifuged at 100,000 × *g* for 1 h at 4°C. The pellet was resuspended in buffer A and the membranes collected by centrifugation at 100,000 × *g* for 20 min at 4°C. The resulting pellet was resuspended in buffer B (10 mM HEPES pH 7.8, 100 mM KCl, 2 mM $MgCl_2$, 1 mM DTT) and centrifuged as above. The membranes were resuspended in buffer B and Triton X-100 was added slowly on ice with stirring to give a final concentration of 4%. Solubilization was allowed to go on for a further 45 min on ice before centrifugation at 100,000 × *g* for 1 h at 4°C. The solubilized proteins were dialyzed against buffer C (20 mM HEPES pH 7.0, 100 mM KCl, 1% PEG (3350) (w/v), 1% glycerol (v/v), 0.9% Triton X-100 (v/v), 1 mM DTT, 0.5 mM ATP and 2 mM EDTA) and re-centrifuged as above after dialysis. Monoclonal anti-syntaxin antibody (HPC-1 clone; Sigma) was covalently bound to protein G-Sepharose fast flow beads (Pharmacia) at 2 mg mouse ascites for 1 ml beads as described [30]. Solubilized membrane proteins from adrenal medulla in buffer C (20 mg) were preincubated on ice for 30 min in the absence or presence of 20 μg His_6-NSF and 15 μg His_6-α-SNAP and precipitated by incubation for a further 2 h with 500 μl anti-syntaxin beads as described [13]. Eluted proteins were precipitated at −20°C with methanol and separated by SDS-polyacrylamide gel electrophoresis for silver-staining and Western blotting.

2.5. SDS-PAGE and Western blotting

Protein samples were separated on 10% SDS-PAGE and either silver-stained or transferred to nitrocellulose and analyzed by Western blotting using antisera at the following concentrations: anti-SNAP-25, 1/1000; anti-synaptotagmin, 1/500; anti-syntaxin, 1/500; anti-synaptobrevin, 1/1000; anti-NSF, 1/200. Proteins were visualized by the ECL system (Amersham).

3. Results and discussion

In order to directly compare the relative abundance of SNAP-25 in adrenal medulla and brain, we compared SNAP-25 immunoreactivity from adrenal medullary microsomes with rat brain microsomes over a range of protein concentrations using an antiserum raised against a synthetic peptide from the C-terminus of SNAP-25 [1] (Fig. 1A). Quantification of the bands showed an approximately 20-fold lower level of SNAP-25 in adrenal medullary microsomes. The specificity of SNAP-25 immunoreactivity was confirmed by preincubating the antiserum with the peptide against which it was raised [1]. Binding of the antiserum to proteins on the nitrocellulose was almost completely abolished when the blocked antiserum was used (Fig. 1B).

To determine the subcellular distribution of SNAP-25, we probed subcellular fractions from bovine adrenal medullae (Fig. 2). A single band was visualized that was most pronounced in the microsomal fraction and to a lesser extent in the homogenate, postnuclear pellet and granule fraction. No SNAP-25 was detected in the cytosol fraction. Immunodecoration with a plasma membrane marker protein for adrenal chromaffin cells, N-CAM [31], gave a similar distribution throughout the fractions (data not shown). The microsomal fraction thus contained the highest amount of plasma membrane compared to the other fractions. Some SNAP-25 was found in the postnuclear pellet and the granule fraction. This, however, is most likely due to some contamination of the granule fraction with plasma membranes as the plasma membrane marker N-CAM was also found to a lesser extent in these fractions.

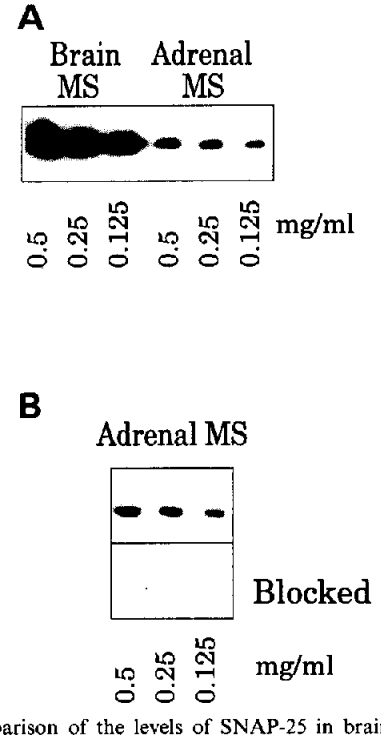

Fig. 1. Comparison of the levels of SNAP-25 in brain and adrenal microsomes. (A) Brain microsomes (MS) from rat forebrain and adrenal medullary microsomes at the indicated protein concentrations were run on a 10% SDS gel (20 μl/lane), transferred to nitrocellulose and SNAP-25 detected with antiserum used at 1/1000. (B) Specificity of the SNAP-25 antiserum. Adrenal microsomes (MS) were separated on SDS-PAGE and transferred for blotting as described above. To block the SNAP-25 antibody, the antiserum was first preincubated for 1 h at 4°C with the SNAP-25 peptide (1 mg/ml) against which the antiserum was raised. Antiserum and peptide were then diluted into PBS and used at 1/1000 for immunoblotting.

Immunoblotting for SNAP-25 across fractions from a subcellular fractionation of adrenal medulla suggested SNAP-25 to be present on the plasma membrane. SNAP-25 is more abundant in brain and neurons, and as interneurons are present in adrenal medulla, it was necessary to confirm that SNAP-25 can be found in chromaffin cells. This was done using immunocytochemistry on cultured chromaffin cells. Essentially all chromaffin cells in culture were stained with anti-SNAP-25 antiserum and a ring of immunofluorescence was visible around the cells, a pattern typical of plasma membrane protein staining (Fig. 3). Thus, the localization of SNAP-25 in adrenal chromaffin cells resembles that of a plasma membrane protein. This localization is analogous to its localization in the presynaptic plasma membrane [1]. It also demonstrates that the SNAP-25a isoform [5] which is the only form apparently present in adrenal medulla [7] and which has a different sequence in the cysteine-rich region, is still able to associate with membranes.

SNAP-25 was recently implicated as a component of the exocytotic machinery forming part of the putative docking complex [12,13], based on an in vitro system using detergent-solubilized brain membrane [12,13]. This complex could be immunoprecipitated, based on an in vitro system using detergent-solubilized brain membranes [12,13], with either syntaxin or SNAP-25 antibodies coupled to beads [13]. If SNAP-25 had a similar function in chromaffin cells to that in neurons, then it should be present in a similar complex. Using solubilized adrenal medullary membranes, we investigated the formation of the SNARE complex in adrenal medulla using immobilized anti-syntaxin antibodies with conditions based on those described by Söllner et al. for brain [13]. A number of polypeptides co-precipitated with syntaxin as revealed on the silver-stained gel (Fig. 4B,C). There was little non-specific binding to protein G-sepharose alone (Fig. 4A). Immunoblotting demonstrated that proteins that are part of the SNARE complex found in brain [12,13] could also be detected in the immunoprecipitate from adrenal medulla (Fig. 4D). Those proteins were namely synaptobrevin, SNAP-25, syntaxin and synaptotagmin.

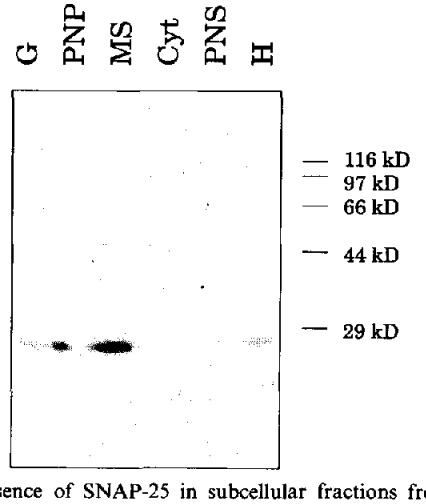

Fig. 2. Presence of SNAP-25 in subcellular fractions from adrenal medulla. Fractions from adrenal medulla were prepared as described in section 2. An equal volume (20 μl/lane) of homogenate (H), postnuclear supernatant (PNS), cytosol (Cyt), microsomes (MS), postnuclear pellet (PNP) and granules (G) was separated on a 10% SDS gel, transferred to nitrocellulose and probed with anti-SNAP-25 antiserum (1/1000).

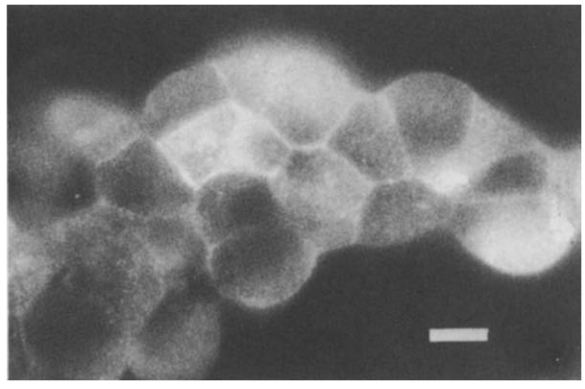

Fig. 3. Immunofluorescence localization of SNAP-25 in cultured adrenal chromaffin cells. Immunocytochemistry was done as described in section 2 with anti-SNAP-25 at 1/100. The scale bar represents 10 μm.

When solubilized adrenal medullary membranes were incubated with exogenous NSF and α-SNAP (Fig. 4C), NSF could also be detected in the immunoprecipitate. Complex formation also takes place in the absence of exogenous NSF and α-SNAP (Fig. 4B). The presence of α-SNAP was not clear from the silverstained gel since α-SNAP comigrates with syntaxin A in our system. In brain, two isoforms of syntaxin, A and B [19], have been identified so far and both are found in the SNARE complex [13]. The monoclonal syntaxin antibody used here recognizes both isoforms [13]. Yet only one band was detected in the immunoprecipitate corresponding to the smaller of the syntaxin isoforms, syntaxin A. However, both syntaxins A and B have been detected in adrenal medulla with antibodies against His_6-syntaxin [23].

The relative amount of NSF was lower than that seen in the complex from brain membranes [13]. At the same time synaptotagmin was also detected in the immunoprecipitate. According to the model proposed by Söllner et al. [13], synaptotagmin should be displaced from the complex when NSF and α-SNAP are present. The presence of synaptotagmin may explain inefficient binding of α-SNAP and NSF and thus account for the low levels of NSF present. In comparison to brain, the overall efficiency of the immunoprecipitation was very low and a several-times higher amount of membrane protein had to be used. Immunoprecipitation with solubilized chromaffin cells from culture revealed a similar pattern on the silver-stained gel to that found from adrenal medullary membranes (data not shown). However, the efficiency was too low to allow detection of proteins by immunoblotting. Again, this might be due to the presence of low levels of the proteins of the putative docking complex in chromaffin cells. This is consistent with the low levels of SNAP-25 found in adrenal medullary microsomes compared to brain microsomes.

Silver-staining of the precipitate from adrenal medullary membranes showed several additional polypeptides including a major 45 kDa component that could not be identified by immunoblotting and which had not been described as part of the immunoprecipitated complex from brain [12,13]. At present we cannot exclude that these polypeptides simply bind non-specifically to the syntaxin antibodies or to the SNARE complex. However, other proteins associating with syntaxin including neurexins [32], N-type Ca^{2+} channels [33] and Munc 18 [23,34,35] have been described.

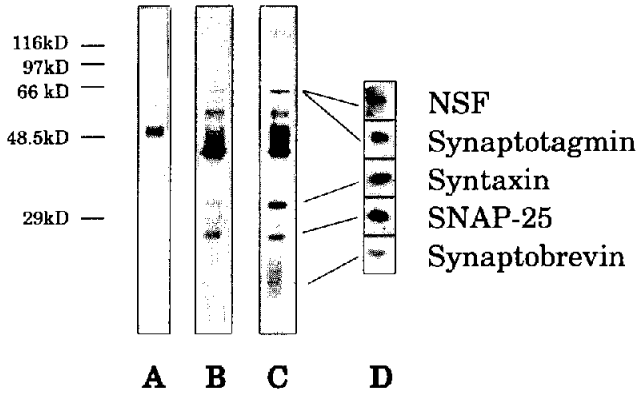

Fig. 4. Immunoprecipitation of the SNARE-complex from adrenal medulla. Solubilized adrenal medullary membranes were immunoprecipitated with protein G-Sepharose alone (A) or with anti-syntaxin antibody coupled to protein G-Sepharose beads (B,C) and the eluate solubilized in 100 μl SDS dissociation buffer. 10 μl of the eluate were run on a 10% SDS gel and silverstained. The membranes had been preincubated without (B) or with (C) the addition of 20 μg NSF and 15 μg α-SNAP. In (D), 20 μl of the eluate from the precipitation with anti-syntaxin antibody was separated on SDS-PAGE and transferred to nitrocellulose for immunoblotting with antisera against the indicated proteins.

We have demonstrated the presence of SNAP-25 in adrenal chromaffin cells and other proteins such as synaptotagmin, synaptobrevin, syntaxin and Munc 18 implicated in the fusion machinery have recently been shown to be present in chromaffin cells [23]. The importance of SNAP-25 in chromaffin cells is shown by the finding that BoNT/A inhibits exocytosis in these cells [24–26]. This toxin gives only a partial inhibition of secretion, but this may be due to it resulting in only partial cleavage of SNAP-25 (Roth et al., unpublished observations). We have extended these observations by showing that proteins found to be part of the docking complex in brain also co-precipitate with syntaxin in chromaffin cells. We have shown that a SNARE complex can be isolated from chromaffin cells and is therefore likely to play a functional role in exocytosis.

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