SNAP-25 is present on chromaffin granules and acts as a SNAP receptor

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Abstract SNAP-25 is located on the plasma membrane and essential for exocytosis of neurotransmitters. It was suggested that SNAP-25 and syntaxin 1 via the interaction with VAMP-2 located on synaptic vesicles mediate the docking of the vesicles with the plasma membrane. In the present study, by means of biochemical and morphological analyses, we showed that SNAP-25 is present on chromaffin granules as well as on the plasma membrane. Reconstitution and immunoprecipitation analyses revealed that SNAP-25 on chromaffin granules has essentially the same properties as does SNAP-25 on the plasma membrane.

Key words: Chromaffin granule; *N*-Ethylmaleimide-sensitive factor; Soluble NSF attachment protein

1. Introduction

Recent progress has revealed that a common principle works for the membrane fusion events in neurotransmitter release and intracellular vesicle-mediated protein transport. Rothman and colleagues identified NSF and SNAPs as factors that mediate intra-Golgi protein transport (for reviews, see Refs. [1,2]). NSF is an ATPase and contains two homologous nucleotide-binding regions [3,4], and SNAPs mediate the association of NSF with membranes [5,6]. Söllner et al. [7] identified VAMP-2 [8,9], syntaxin 1 [10–12], and SNAP-25 [13] as SNAP receptors (SNAREs). NSF, SNAPs and SNAREs are associated to form a 20S complex in membranes, and this complex is disassembled by Mg^{2+}-ATP, whereby NSF is released from the membranes [7,14,15].

Based on the localization of SNAREs [8–10,13,16], VAMP-2 was classified as a v-SNARE, and syntaxin 1 and SNAP-25 as t-SNAREs [7]. SNARE hypothesis predicts that the docking of vesicles with target membranes is mediated by the specific interaction between t- and v-SNAREs [7]. Although the involvement of NSF-SNAPs-SNAREs in various fusion events including exocytosis in chromaffin cells [17–20], recent studies revealed that t-SNAREs are localized not only on the plasma membrane but also on synaptic vesicles [21–23]. These results raised the question of whether or not syntaxin 1 and SNAP-25 act as t-SNAREs. However, syntaxin 1 and SNAP-25 may be incorporated into the vesicles due to incomplete v-SNARE-t-SNARE-dissociation after fusion [22], because recycling of synaptic vesicle proteins from the plasma membrane occurs rapidly and this event is important for the biogenesis of synaptic vesicles [24–26].

Chromaffin granules are organelles involved in the exocytosis of catecholamines, but not in the recycling process. We recently showed that syntaxin 1 exists on chromaffin granules as well as on the plasma membrane [27]. In the present study, we showed that a significant amount of SNAP-25 is present on chromaffin granules as well as on the plasma membrane.

2. Materials and methods

2.1. Materials

Monoclonal antibodies against the following proteins were described previously: brain syntaxin 1B (MAb 6H1) [11], SNAP-25 (MAb BR05) [28,29], and NSF (MAb 2C8) [4]. Antibodies against VAMP-2 (residues 2-19) and SNAP-25 (residues 195-206) were kindly donated by Dr. M. Takahashi (Mitsubishi Kasei Institute of Life Sciences). Polyclonal antibodies against the whole SNAP-25 protein and SNAP were kindly donated by Dr. R. Jahn (Yale University) and Dr. J.E. Rothman (Memorial Sloan-Kettering Cancer Center), respectively. Unless otherwise stated, MAb 6H1, MAb BR05 and anti-VAMP-2 peptide antibody were used for immunoblotting. *Escherichia coli*-expressed NSF and α-SNAP were purified as described by Tagaya et al. [4] and Whiteheart et al. [30], respectively.

2.2. Subcellular fractionation and velocity sedimentation

Chromaffin granules from bovine adrenal medullae were isolated by centrifugation on step gradients of sucrose or Percoll as described previously [27]. The membranes were prepared by centrifugation at $200\,000 \times g_{av}$ for 40 min after hypo-osmotic treatment. Sucrose equilibrium density gradient centrifugation was conducted as described previously [27].

2.3. Immunoelectron microscopy

Purified chromaffin granules were attached to collodion-carbon-coated nickel grids (150 mesh), incubated with buffer A (PBS containing 0.25 M sucrose and 0.5% BSA) for 10 min, and then treated with the first antibodies (10 μg/ml of IgG) in buffer A for 2 h. After washing, the granules were incubated with gold particles (10 nm) conjugated to goat IgG against mouse or rabbit IgG (British BioCell International, Cardiff, UK) in buffer A for 30 min, and then washed several times with 0.1 M cacodylate buffer (pH 7.4) containing 0.25 M sucrose. The granules were then fixed in 2% glutaraldehyde in the same buffer, and washed with distilled water. Absorption staining of the chromaffin granules on the grids was carried out with 0.3% uranyl acetate and 3% polyvinyl alcohol as described previously [31].

2.4. Immunoprecipitation

Chromaffin granule membranes were solubilized with 1% Triton X-100 in EDTA-ATP buffer (25 mM HEPES (pH 7.2), 4 mM EDTA, 0.5 mM ATP, 0.1 M KCl, 0.5 mM dithiothreitol). After centrifugation at $200\,000 \times g_{av}$ for 30 min, the supernatant was incubated with appropriate antibodies at 0°C for 1 h. To this solution was added 30 μl of protein A/G plus-agarose (Santa Cruz Biotechnology). After gentle shaking overnight, the resin was washed, and the bound proteins were eluted with SDS sample buffer with boiling. After SDS-PAGE [32], the proteins were detected by immunoblotting with appropriate antibodies.

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Abbreviations: NSF, *N*-ethylmaleimide-sensitive factor; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor; MAb, monoclonal antibody; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis

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3. Results

3.1. Evidence for the presence of SNAP-25 on chromaffin granules

SNAP-25 is expressed in chromaffin cells, although the expression level is about 20-fold lower than that in brain [33]. When highly purified chromaffin granules were analyzed by immunoblotting, immunoreactivity for SNAP-25 as well as VAMP-2 and syntaxin 1 was detected (Fig. 1). No significant contamination by Na^+,K^+-ATPase, a marker protein of the plasma membrane, was detected on isolated chromaffin granules (Fig. 1), excluding the possibility that the SNAP-25 detected on the granule membranes was derived from contaminating plasma membranes. As shown in Fig. 2, sucrose equilibrium density centrifugation revealed that the amount of SNAP-25 in the precipitate, on which chromaffin granules are located, was comparable to those for fractions 4 and 5, which contained the plasma membrane and microvesicles, when detected with MAb BR05. This suggests that granule-associated SNAP-25 represents a significant portion of the total SNAP-25 pool in chromaffin cells.

We previously reported that SNAP-25 was not detected on chromaffin granules when analyzed with an antibody against the carboxyl-terminal residues (195-206) of brain SNAP-25 [27]. This was probably due to the inefficient immunoblotting. When samples contain large amounts of membranes, the electrophoresis pattern is disturbed and, in addition, the efficiency of immunostaining is considerably lower than that for samples without membranes. In Triton X-100 extracts of chromaffin granules, immunoreactivity was detected with an anti-SNAP-25 peptide antibody, MAb BR05, and an antibody against the whole SNAP-25 protein (Fig. 2B). On the other hand, as observed previously [27], no immunoreactivity was detected with the anti-SNAP-25 peptide antibody when the chromaffin granule fraction was subjected to electrophoresis without Triton X-100 treatment (Fig. 2A).

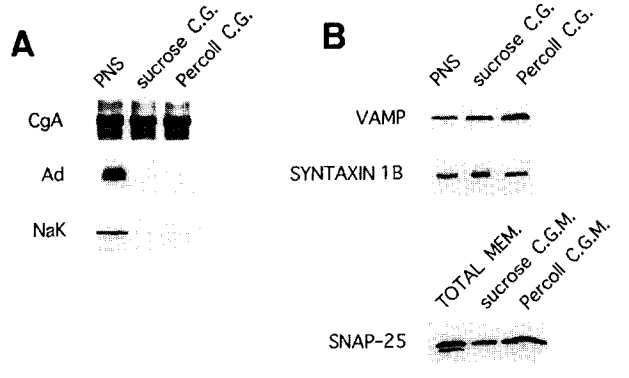

Fig. 1. SNAP-25 is co-purified with chromaffin granules. A postnuclear supernatant (PNS), chromaffin granules isolated by sucrose gradient centrifugation (sucrose C.G.) or Percoll gradient centrifugation (Percoll C.G.), total membranes (TOTAL MEM.), and chromaffin granule membranes (C.G.M.) were subjected to SDS-PAGE, and then immunoblotted. The same amount of proteins was analyzed in each series of SDS-PAGE. (A) Purity of chromaffin granules. Immunoblotting was carried out with antibodies against a chromaffin granule marker protein, chromogranin A (CgA), a mitochondrial marker protein, adrenodoxin (Ad), and a plasma membrane marker enzyme, Na^+, K^+-ATPase (NaK). (B) Presence of SNAP-25 on chromaffin granules. Immunoblotting was carried out with antibodies against SNAREs. MAb BR05 was used for immunostaining for SNAP-25. An immunoreactive band corresponding to a molecular weight lower than that of SNAP-25 was occasionally observed. This band may represent a proteolytic fragment of SNAP-25.

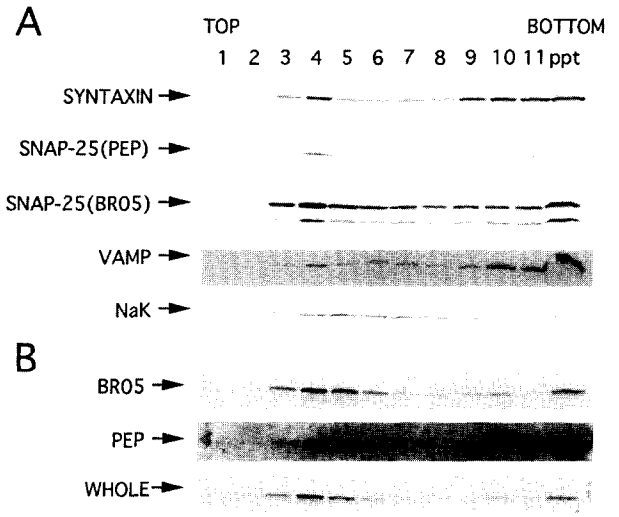

Fig. 2. Subcellular fractionation of adrenal medulla. (A) A postnuclear supernatant was subjected to sucrose equilibrium centrifugation, and then membranes were recovered by centrifugation after hypoosmotic treatment. After SDS-PAGE, proteins were immunoblotted with the following antibodies: MAb (6H1 (SYNTAXIN 1B), anti-SNAP-25 peptide antibody (SNAP-25 (PEP)), MAb BR05 (SNAP-25 (BR05)), anti-VAMP-2 antibody (VAMP), and anti-Na^+,K^+-ATPase antibody (NaK). (B) Membranes were solubilized with Triton X-100, and then the solubilized proteins were subjected to SDS-PAGE, and immunoblotted with MAb BR05 (BR05), anti-SNAP-25 peptide (PEP), and anti-SNAP-25 protein (WHOLE).

To confirm the association of SNAP-25 with chromaffin granules, we carried out immunogold labeling of isolated chromaffin granules. In chromaffin cells, there are two types of granules, one comprising less dense and the other dense-core vesicles. It is known that the former and latter contain adrenalin and nonadrenalin, respectively [34]. Electron microscopy demonstrated that immunoreactivities for VAMP-2 (Fig. 3A) and SNAP-25 (Fig. 3B and C) were observed on both types of granules, although the reactivity for SNAP-25 was less intense. No significant labeling was observed on either granule when a control antibody was used (Fig. 3D). As judged from the density of the organelles, it is obvious that they are not endsomes.

3.2. SNAP-25 on chromaffin granules acts as a SNAP receptor

Syntaxin 1 and SNAP-25 on the plasma membrane [15,35,36] and syntaxin 1 on chromaffin granules [27] can form a complex with VAMP-2. We examined whether or not SNAP-25 on chromaffin granules also forms a complex with VAMP-2 and syntaxin 1. As shown in Fig. 4A, SNAP-25 and syntaxin 1 were co-immunoprecipitated with anti-VAMP-2, whereas none of the three proteins were precipitated when a control antibody was used. This suggests that SNAP-25 on the granules can form a complex with syntaxin 1 and VAMP-2. Consistent with this idea, both SNAP-25 and syntaxin 1 were co-immunoprecipitated with anti-syntaxin 1 or anti-SNAP-25 (Fig. 4B). However, only a small amount of VAMP-2 was co-immunoprecipitated with both antibodies. This contradiction can be reconciled by the idea that the amount of VAMP-2 in chromaffin granules is much higher

than those of syntaxin 1 and SNAP-25, and major portions of syntaxin 1 and SNAP-25, and a minor portion of VAMP-2 form a complex. Perhaps, the efficiency of immunostaining with anti-VAMP-2 was much lower than those with anti-SNAP-25 and anti-syntaxin.

α-SNAP binds to plasma membrane-associated SNAP-25, although it more tightly binds to the complex of syntaxin, SNAP-25 and VAMP-2 [37,38]. To determine whether or not α-SNAP binds to granule-associated SNAP-25, a Triton X-100 extract of chromaffin granule membranes was incubated with *E. coli*-expressed α-SNAP, and then immunoprecipitated with an antibody against SNAP-25 or SNAP. As shown in Fig. 5A and B, α-SNAP was co-immunoprecipitated with the anti-SNAP-25, and conversely SNAP-25 was co-immunoprecipitated with the anti-SNAP, indicating that SNAP-25 can act as a SNAP receptor. Since α-SNAP does not directly bind to VAMP-2 [37,38], co-immunoprecipitation of SNAP with anti-VAMP-2 (Fig. 5A) is consistent with the idea that VAMP-2 can form a complex with syntaxin 1 and SNAP-25 on chromaffin granules. The low efficiency of co-immunoprecipitation of VAMP-2 with anti-SNAP (Fig. 5B) may reflect the fact that the SNAP-25 is a limiting component for the formation of the SNARE complex, as described above.

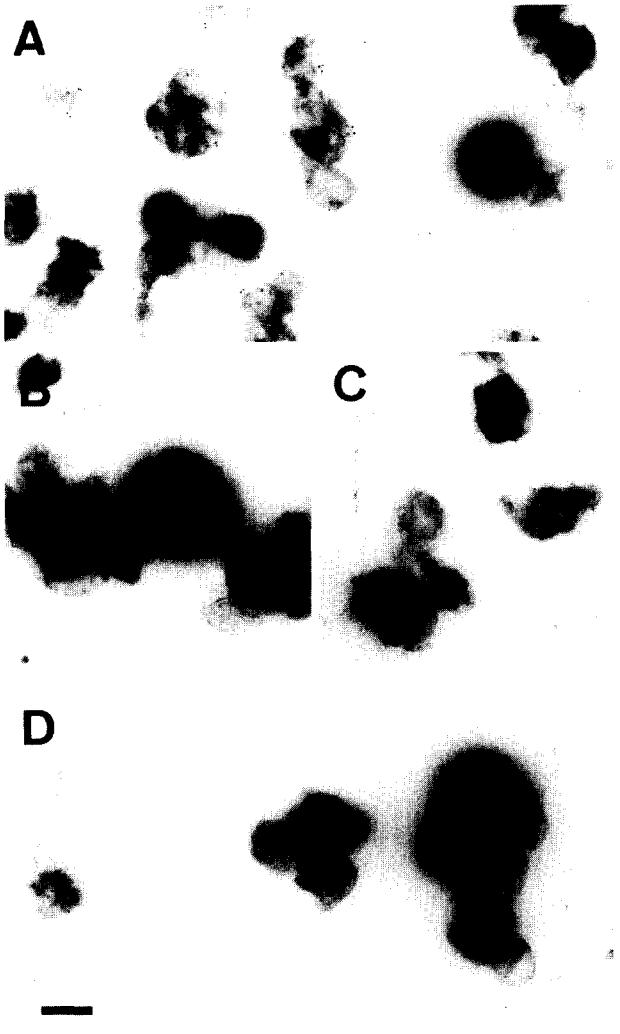

Fig. 3. Immunogold labeling for SNAP-25 and VAMP-2. Immunogold labeling was carried out with anti-VAMP-2 (A), anti-SNAP-25 (MAb BR05) (B and C), and a control mouse IgG (D). Bar, 300 nm.

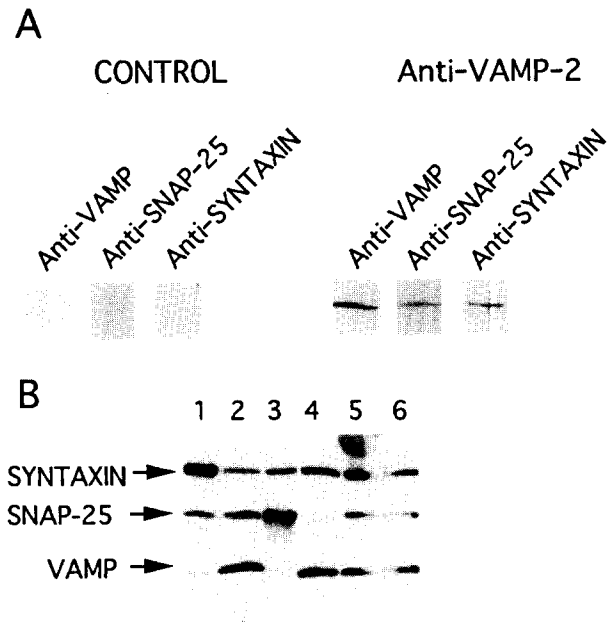

Fig. 4. Co-immunoprecipitation of SNAREs. (A) A Triton X-100 extract of chromaffin granule membranes was immunoprecipitated with a control antibody (CONTROL) and anti-VAMP-2 (Anti-VAMP-2). The precipitates were subjected to SDS-PAGE and immunoblotted with antibodies against SNAREs. (B) Immunoprecipitation was performed with anti-syntaxin 1B (MAb 6H1) (lanes 1 and 2), anti-SNAP-25 (MAb BR05) (lanes 3 and 4), and anti-VAMP-2 (lanes 5 and 6). The precipitates (lanes 1, 3 and 5) and supernatants (lanes 2, 4 and 6) were subjected to SDS-PAGE, and then immunoblotted with a mixture of antibodies against SNAREs.

We next examined whether or not SNAP-25 is a component of the 20S fusion complex. For this purpose, a Triton X-100 extract of chromaffin granules was incubated with *E. coli*-expressed NSF and α-SNAP, and then the complex was immunoprecipitated with anti-NSF (MAb 2C8). As shown in Fig. 5C, SNAP-25 was co-immunoprecipitated with MAb 2C8, but not with a control antibody.

4. Discussion

In a previous study, we showed that syntaxin 1 is present on chromaffin granules as well as on the plasma membrane [27]. In the present study, we have demonstrated that SNAP-25 is also present on chromaffin granules. The amounts of syntaxin 1 and SNAP-25 on chromaffin granules are not trivial portions of the total pool of the two proteins in chromaffin cells. Since chromaffin granules are organelles that exclusively participate in exocytosis, the presence of syntaxin 1 and SNAP-25 on the granules unequivocally excludes the possibility that these proteins are accidentally incorporated into transport vesicles. The present and other results [22,27] raise a question regarding the docking model of vesicles with target membranes, which is based on the different localization of t- and v-SNAREs. It was recently suggested that VAMP-2 and syntaxin play roles after the docking of synaptic vesicles at the active zone of the plasma membrane [39].

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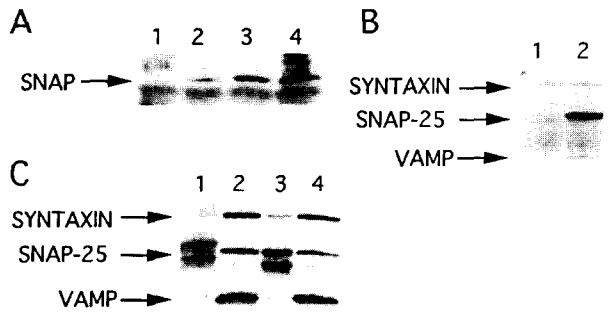

Fig. 5. (A) Co-immunoprecipitation of α-SNAP with SNAREs. A Triton X-100 extract of chromaffin granule membranes was incubated with *E. coli*-expressed α-SNAP, and then immunoprecipitated with a control antibody (lane 1), anti-syntaxin 1 (MAb 6H1) (lane 2), anti-SNAP-25 (MAb BR05) (lane 3), and anti-VAMP-2 (lane 4). The immunoprecipitated proteins were subjected to SDS-PAGE, and then immunoblotted with anti-SNAP. (B) Co-immunoprecipitation of SNAP-25 with α-SNAP. A Triton X-100 extract of chromaffin granule membranes was incubated without (lane 1) or with (lane 2) *E. coli*-expressed α-SNAP. The proteins co-immunoprecipitated with anti-SNAP were immunodetected. (C) Co-immunoprecipitation of SNAP-25 with NSF. A Triton X-100 extract was incubated with NSF and α-SNAP, and then immunoprecipitated with a control antibody (lanes 1 and 2) and MAb 2C8 (lanes 3 and 4). The precipitates (lane 1 and 3) and supernatants (lane 2 and 4) were immunodetected.

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