# Characterization of Vesicular Membrane-Bound α-SNAP and NSF in Adrenal Chromaffin Cells†

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ABSTRACT: α-SNAP and NSF are thought to act as soluble factors, which transiently bind to a complex formed between syntaxin and SNAP-25 located at the plasma membrane and synaptobrevin at the secretory vesicle membrane, at the moment of exocytosis. Here we present data which permit the novel conclusion that α-SNAP and NSF are not soluble in adrenal chromaffin cells but are rather membrane-bound in particular to undocked chromaffin vesicles. Evidence for this new paradigm is derived from several experimental approaches. First, α-SNAP and NSF were found predominantly at cellular membranes and not in the cytosol of cracked chromaffin cells. Second, α-SNAP and NSF were not released from membranes by $Mg^{2+}$ATP, which causes priming of vesicles. Third, immune electron microscopy and immunoblotting of chromaffin vesicles purified by immunoisolation or density gradient centrifugation revealed the presence of α-SNAP and NSF together with typical vesicular proteins such as synaptobrevin and synaptotagmin. In the sucrose gradient 30% α-SNAP and 27% NSF were recovered with chromaffin vesicles. Bound α-SNAP was quantified (14 molecules/vesicle), and binding was characterized with recombinant $his_6$-tagged α-SNAP. Overlay blots revealed that α-SNAP is bound to vesicular SNAP-25 and endogenous NSF. Our data show that mature chromaffin vesicles already contain specifically bound α-SNAP and NSF before docking at the plasmalemma.

NSF[1] is a *N*-ethylmaleimide-sensitive ATPase participating in vesicular transport within the Golgi apparatus. The soluble protein NSF which, in the absence of $Mg^{2+}$ATP, can be bound to Golgi membranes via soluble NSF attachment proteins (SNAPs) is released in the presence of ATP (*1*). Using detergent extracts of a total brain membrane fraction as a source, a complex of synaptobrevin, syntaxin, and SNAP-25 has been identified as a SNAP receptor (SNARE) (*2*). Since these proteins are all targets of clostridial neurotoxins which inhibit neurotransmitter release (cf. ref 3 and 4), an involvement of soluble SNAPs and NSF in exocytosis, in addition to their function in vesicular transport in the Golgi apparatus, appeared to be conceivable.

Synaptobrevin is a membrane protein of synaptic vesicles and thus termed a v-SNARE, while SNAP-25 and syntaxin are found at the plasmalemmal target of synaptic vesicles serving as t-SNAREs during exocytosis. In detergent extracts synaptobrevin forms a heterotrimer with the t-SNAREs syntaxin and SNAP-25, the 7S complex. Upon binding of the soluble proteins α-SNAP and NSF the larger 20S complex is formed which dissociates in the presence of $Mg^{2+}$ATP, a process believed to be essential for exocytotic membrane fusion (*5*).

Studies with clostridial neurotoxins have shown that synaptobrevin, syntaxin and SNAP-25 not only participate in neurotransmitter release but also are essential for hormone secretion (*6*−*12*). Thus similar events may occur during exocytotic fusion of synaptic vesicles and secretory vesicles with the target membrane.

In the present work we analyzed the association of the soluble proteins α-SNAP and NSF with intracellular membranes of adrenal chromaffin cells. We found that the soluble proteins exist predominantly in a membrane-bound form in adrenal chromaffin cells irrespective of the presence of $Mg^{2+}$-ATP. In particular endogenous α-SNAP and NSF were found at the membrane of chromaffin secretory vesicles. Studies with recombinant α-SNAP revealed high affinity. Half-maximal binding was reached with 0.15 μM α-SNAP. Binding sites for α-SNAP were provided by vesicular NSF and SNAP-25. Our data suggest that SNAP-25 serves as a major vesicular SNARE in adrenal chromaffin cells.

## EXPERIMENTAL PROCEDURES

*Analysis of Adrenal Chromaffin Cells.* Bovine adrenal chromaffin cells were prepared as described (*13*). The isolated cells were washed in PBS, an intracellular medium (containing in mM: 150 potassium glutamate, 0.5 EGTA, 5 EDTA, and 10 PIPES, pH 7.2) with or without $Ca^{2+}$ (30 μM free), 2 mM ATP, and $Mg^{2+}$ (1000 μM free), in an extracellular medium (containing in mM: 140 NaCl, 4.7 KCl, 1.2 $KH_2PO_4$, 1.2 $MgSO_4$, 0.5 ascorbic acid, 11 glucose, and 15 PIPES, pH 7.2). Then the cells were broken up by

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[1] Abbreviations: AA, amino acid; BSA, bovine serum albumin; LDH, lactate dehydrogenase; NSF, *N*-ethylmaleimide-sensitive fusion protein; SNAP, soluble NSF attachment protein; SNAP-25, synaptosome-associated protein of 25 kDa.

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freezing and thawing (5×) in liquid nitrogen or cracked mechanically at room temperature by repeated passage (50× during five minutes) through a ball-bearing device (European Molecular Biology Labs, Heidelberg, Germany) at a clearance of 10 $\mu$m (*14*). Following centrifugation at 100,000*g* for 1 h at 4 °C, supernatants (cytosol) were concentrated 4-fold to detect even traces of proteins. Protein (15 $\mu$g) of pellets and supernatants were analyzed by SDS−PAGE and immunoblotting as described below.

*Immunoisolation of Chromaffin Vesicle Membranes.* Dynabeads M-280 (Dynal, Hamburg, Germany) coated with anti-mouse IgG were washed for 15 min at room temperature in PBS containing 2 mM EDTA and 5% (w/v) BSA and blocked by the addition of 40 mg/mL L-α-phoshatidylcholine (Sigma, Munich, Germany) for 1 h at room temperature. The beads, washed twice in PBS containing 2 mM EDTA and 5% (w/v) BSA, were incubated with anti-SNAP-25 antibodies (Sternberger Monoclonals Inc., Baltimore, MD, 3 $\mu$g/$10^7$ Dynabeads) overnight at 4 °C. Bovine adrenal chromaffin cells prepared and cracked mechanically as described above were centrifuged at 2000*g* for 10 min at room temperature to remove the broken cells including their plasma membrane. Only the supernatant containing the intracellular organelles was incubated with the beads first for 30 min at room temperature and then for 1 h at 4 °C. The beads were re-collected by a magnetic particle collector, washed several times, and analyzed by SDS−PAGE and immunoblotting or electronmicroscopy. Dynabeads without anti-SNAP-25 antibody were used as a control.

*Purification of Chromaffin Vesicle Membranes.* Chromaffin vesicles were isolated from bovine adrenal glands as described previously (*15*). Briefly, medullae were homogenized in 20 mM MOPS, pH 7.0, 5 mM EDTA, and 340 mM sucrose in a Teflon-to-glass homogenizer. The homogenate was filtered through four layers of absorbent gauze and centrifuged at 2200*g* for 10 min at 4 °C to remove incompletely homogenized tissue, unbroken cells, and nuclei. The supernatant (S1) was centrifuged at 15000*g* for 20 min at 4 °C to yield a pellet of chromaffin vesicles (P2). The supernatant (S2) was subjected to centrifugation at 100000*g* for 1 h at 4 °C to obtain microsomes as a pellet (P3) and the cytosol (S3). Chromaffin vesicles (P2) were further purified on a discontinuous sucrose gradient each consisting of 0.34 mL of 2.9, 2.6, 2.35, 2.2, and 1.8 M sucrose in 20 mM MOPS, pH 7.0, and 5 mM EDTA. Centrifugation was carried out at 100000*g* for 1 h at 4 °C in a TLS 55 swing-out rotor (Beckman Instruments GmbH, Munich, Germany). Fractions of 4 drops each were collected from the bottom of the gradient by means of a peristaltic pump. Aliquots of pellets, supernatants, and density gradient fractions were analyzed for the presence of the lysosomal enzyme arylsulfatase and lactate dehydrogenase (*16*). α-SNAP and NSF, synaptobrevin, synaptotagmin, syntaxin, SNAP-25 and kinectin, a marker protein for the endoplasmic reticulum (*17*), and connexin-43, a marker for the plasma membrane (*18*), were determined by immunoblotting. The fractions of the density gradient enriched in chromaffin vesicles (fractions 1−9) were pooled and diluted in a 10-fold excess of 20 mM MOPS, pH 7.0, and 5 mM EDTA. Vesicle membranes were harvested by centrifugation at 100000*g* for 1 h at 4 °C and resuspended in 20 mM MOPS, pH 7.0, and 5 mM EDTA.

*Plasmid Construction and Purification of Recombinant Fusion Proteins.* An N-terminal truncated α-SNAP coding sequence was amplified by PCR from the plasmid pQE9α-SNAP bearing the full-length α-SNAP (*19*). The primers used for amplification of α-SNAP (114−295 AA) were as follows: sense, *Bam*HI 5′-ATCAGGATCCCGAGCTCATGGGCCGCT TCACCATCGC-3′; and antisense, *Eco*RI 5′-AATTGAATTCAAGATCCGCGCAGGTCTTCCTCG-3′. The PCR product was cloned into pBluescript SK (Stratagene, Heidelberg, Germany). After digestion with *Bam*HI and *Hin*dIII the insert was subcloned into the pQE-30 vector (Qiagen, Hilden, Germany). pQE9α-SNAP and pQE30α-SNAP (114−295 AA) were transformed in *Escherichia coli* XL1-Blue (Stratagene, Heidelberg, Germany). The fusion proteins were expressed for 4 h after addition of 1 mM isopropyl-thio-$\beta$-D-galactoside. Bacteria were harvested and resuspended in 50 mM sodium phosphate, pH 8.0, and 300 mM NaCl. The cells were disrupted by sonication, and the cell debris was removed by centrifugation at 15000*g* for 20 min at 4 °C. The expressed fusion proteins were purified on a Ni-NTA column (QIAGEN, Hilden, Germany) using a discontinuous gradient of imidazole (80, 160, and 400 mM) according to the instructions of the manufacturer. The eluates were analyzed for the presence of $his_6$α-SNAP or $his_6$α-SNAP (114−295 AA) by SDS−PAGE (12.5%) followed by Coomassie-Blue R staining or immunoblotting. For control experiments the 26 kDa fusion protein $his_6$-dihydrofolate reductase (QIAGEN, Hilden, Germany) was purified under the same conditions as described above.

*SDS−PAGE, Immunoblotting, and Quantification.* The samples to be analyzed were subjected to SDS−PAGE (12.5%) and blotted onto nitrocellulose according to established methods. Binding of monoclonal anti-synaptobrevin antibody (Cl.69.1) diluted to 1:5000 (*20*), anti-synaptotagmin I antibody (Cl.41.1) diluted to 1:2000 (*21*), anti-SNAP-25 antibody (SMI81) diluted to 1:2000 (Sternberger Monoclonals Inc., Baltimore, MD), anti-syntaxin antibody (Cl.HPC-1) diluted to 1:2000 (Sigma, Munich, Germany), anti-α-SNAP antibody (Cl.77.1) diluted to 1:5000 (*22*), anti-NSF antibody (Cl.6E6) diluted to 1:1000 (*23*), anti-$his_6$ antibody diluted to 1:2000 (QIAGEN, Hilden, Germany), anti-connexin-43 antibody diluted to 1:500 (*18*), and anti-kinectin antibody (Cl.CT1) diluted to 1:100 (*24*) was detected by peroxidase-labeled anti-mouse IgG or anti-rabbit IgG antibodies diluted to 1:3000 (Dianova, Hamburg, Germany) and the enhanced chemiluminescense method (Amersham Buchler, Braunschweig, Germany). The films were digitized with a GelPrint2000i system (MWG-BIOTECH, Ebersberg, Germany), and quantitative densitometry was performed using ONE-Dscan software (MWG-BIOTECH, Ebersberg, Germany).

*$His_6$α-SNAP Binding by Chromaffin Vesicle Membranes.* Chromaffin vesicle membranes (100 $\mu$g) were supplemented with different concentrations of recombinant $his_6$α-SNAP (see above) in 20 mM MOPS, pH 7.0, containing 5 mM EDTA. The samples (50 $\mu$L) were incubated for 30 min on ice followed by centrifugation at 100000*g* for 60 min at 4 °C. The supernatants and the pellets, which were washed once in PBS, pH 7.2, were analyzed by Western blotting for the presence of $his_6$α-SNAP with an anti-$his_6$ antibody and for endogenous plus his-α-SNAP with anti-α-SNAP antibody. The immunoreactive bands were quantified den-

sitometrically (see below). Recombinant $his_6$α-SNAP served as a standard. As a control 100 μg of chromaffin vesicle membranes and 10 μg of $his_6$-dehydrofolate reductase were incubated as described above.

*Overlay Assay.* Chromaffin vesicles membranes (25 μg/lane) were blotted onto nitrocellulose membranes and blocked with 5% (w/v) BSA and 5% (w/v) low fat milk powder in TBS-Tween 20 (0.05% (v/v)) overnight. The blots were incubated with recombinant $his_6$α-SNAP (0.1−1 μg in 3 mL TBS-Tween 20) or 1 μg of $his_6$α-SNAP (114−295 AA) in 3 mL of TBS-Tween 20 for 2 h at room temperature followed by extensive washing with TBS-Tween 20. Bound $his_6$α-SNAP fusion proteins were detected using anti-$his_6$ antibody and enhanced chemiluminescence. As a control blots were incubated with 1 μg of the recombinant $his_6$-dehydrofolate reductase in 3 mL of TBS-Tween 20 as described above. NSF and SNAP-25 were identified in the same blots as described above.

*Immunolocalization at the Ultrastructural Level.* Chromaffin vesicle membranes were fixed for 20 min with 3% (w/v) paraformaldehyde and 1.5% (v/v) glutaraldehyde in PBS and recovered by centrifugation. Pellets were incubated for 15 min in 1% (w/v) glycin in PBS, washed in PBS, and resuspended in warmed 1.5% (w/v) standard agarose (Biozym, Oldendorf, Germany) in PBS. Agarose films were produced between microscopic slides by cooling at 4 °C. Then the films were blocked with 5% (w/v) BSA in PBS for 30 min followed by incubation with monoclonal antibodies directed against synaptobrevin, α-SNAP, and NSF (dilutions: 1:200 in 1% (w/v) BSA, 0.01% (w/v) sodium azide in PBS) overnight at room temperature. Controls consisted of incubations of the films without the specific antibody or with polyclonal anti-connexin-43 antibodies (dilutions: 1:200 in 1% (w/v) BSA, 0.01% (w/v) sodium azide in PBS). After several washes in PBS the ABC peroxidase method was applied (*25*). Specimens were postfixed with 1% (w/v) $OsO_4$ in 0.1 M phosphate buffer, pH 7.2, and embedded in Epon 812 (Merck, Darmstadt, Germany). Ultrathin sections were counterstained with 2% uranyl acetate and lead citrate and examined in a Zeiss EM 10 electron microscope.

## RESULTS

Previous analyses of soluble and membrane-bound pools of α-SNAP and NSF in endocrine cells provided inconsistent results. In a pancreatic B-cell line the proteins were mainly detected in the cytosol and the association of the proteins with the cellular membranes was not influenced by ATP (*26*). In adrenal chromaffin cells permeabilized with digitonin NSF and α-SNAP were found to be predominantly membrane-bound but slowly released in part by ATP (*27*). In the absence of detergents and ATP the amounts of α-SNAP in the membrane fraction of adrenal tissue were much larger than in the cytosol (*28*). In pheochromocytoma cells (PC12) binding of NSF to the Golgi apparatus but not to other cellular membranes was affected by ATP (*29*).

For clarification we first reinvestigated the membrane association of α-SNAP and NSF in adrenal chromaffin cells broken up with different techniques and a variety of media in order to define more exactly membrane and soluble pools of these proteins. For comparison we also determined the distribution of the membrane proteins SNAP-25 and syntaxin,

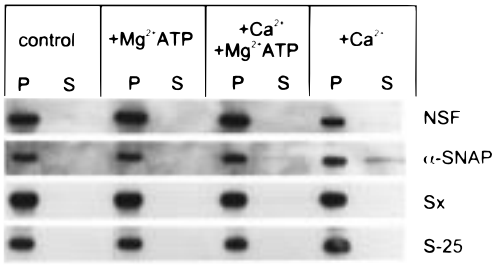


FIGURE 1: α-SNAP and NSF are not soluble in adrenal chromaffin cells. Chromaffin cells were broken up by cracking in an intracellular medium supplemented with the indicated compounds followed by ultracentrifugation (see Experimental Procedures). Independent of the presence or absence of $Ca^{2+}$, $Mg^{2+}$, and ATP, the soluble proteins α-SNAP and NSF, similar to the membrane proteins syntaxin (Sx) and SNAP-25 (S-25), remained in the pellets (P). In the supernatants (S), only traces of these proteins were detected. Densitometric analysis of each pellet in comparison to the control pellet revealed 94%−99% identity of the signal.

which bind α-SNAP, the attachment protein of NSF (*22*, *30*). When bovine adrenal chromaffin cells were broken up by repeated freezing and thawing in PBS or an intracellular medium (see Experimental Procedures) followed by ultracentrifugation (100000*g*, 1 h, 4 °C), the membrane-bound proteins SNAP-25 and syntaxin were recovered within the pellet as expected. Surprisingly the soluble proteins α-SNAP and NSF were also found in the pellet and not in the supernatant (data not shown). Also, if intracellular organelles and the cytosol were released from chromaffin cells by a cell cracker in an extracellular (not shown) or in an intracellular medium followed by ultracentrifugation, α-SNAP and NSF, like SNAP-25 and syntaxin, were identified in the pellet (Figure 1).

Two sequential steps can be distinguished during exocytosis of catecholamines. Priming, caused by $Mg^{2+}$ATP, is followed by $Ca^{2+}$-triggered exocytosis (*31*). Since $Mg^{2+}$-ATP releases α-SNAP and NSF from Golgi and possibly other intracellular membranes, we investigated whether $Mg^{2+}$ATP and/or $Ca^{2+}$ modifies the distribution of α-SNAP and NSF between cellular membranes and the cytosol of adrenal chromaffin cells. However, α-SNAP and NSF as well as the membrane-bound proteins syntaxin and SNAP-25 were detected in the pellet (P) irrespective of the addition of $Ca^{2+}$ (30 μM free), 2 mM ATP and $Mg^2$ (1000 μM free), or $Mg^2$ATP plus $Ca^{2+}$ to the intracellular medium (Figure 1).

Since we noticed α-SNAP and NSF by immunogold labeling at the membranes of undocked chromaffin vesicles (not shown) we carried out immunoisolation experiments. For this purpose we used antibodies directed against SNAP-25 and syntaxin. These proteins have previously been shown to be present not only at the plasmalemma but also at adrenal chromaffin vesicle membranes (*7*, *32*, *33*). Moreover SNAP-25 and syntaxin are known to specifically bind α-SNAP in vitro (*22*, *30*). First chromaffin cells were broken up with a cell cracker in order to release intracellular organelles (see Experimental Procedures). Then the broken cells including their plasma membrane (as observed at light microscopical and electron microscopical levels) were removed by low speed centrifugation. Anti-SNAP-25 or anti-syntaxin coated Dynabeads allowed to immunoisolate from the supernatant intracellular organelles which, according to their size (data not shown) and the presence of synaptotagmin (Figure 2),

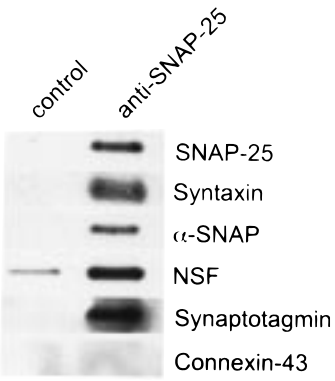


FIGURE 2: Immunoisolation of undocked chromaffin vesicle membranes. Dynabeads coated with anti-SNAP-25 IgG were used to immunoisolate undocked adrenal chromaffin vesicles from mechanically cracked chromaffin cells after low speed centrifugation to remove the cracked plasma membrane. The re-collected magnetobeads were analyzed by Western blotting showing the presence of SNAP-25, syntaxin, α-SNAP, and NSF in the isolated adrenal chromaffin vesicles characterized by the presence of synaptotagmin and the absence of connexin-43, a marker for plasma membrane. Dynabeads without anti-SNAP-25 antibody were used as a control.

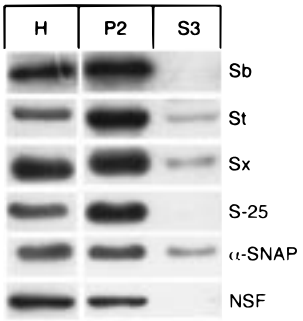


FIGURE 3: Distribution of α-SNAP, NSF, and membrane proteins in fractions obtained by differential centrifugation. The membrane proteins synaptobrevin (Sb), synaptotagmin (St), syntaxin (Sx), SNAP-25 (S-25), α-SNAP, and NSF were analyzed in the adrenal medullary homogenate (H). All of these proteins were found to be enriched in the chromaffin vesicles fraction (P2). The cytosol (S3) obtained by centrifugation at 100000*g* for 60 min contained only small amounts of α-SNAP and NSF as well as the other membrane proteins analyzed. The relative specific activity of the proteins (ratio of the percent of antigen to the percent of protein in the fractions) was determined. In the chromaffin vesicles (P2) we observed an enrichment of the vesicle membrane proteins synaptobrevin and synaptotagmin (relative specific activity of 1.3) as well as of the membrane-bound proteins SNAP-25 and syntaxin (relative specific activities of 1.5 and 1.2 respectively) and, surprisingly, also of the soluble proteins α-SNAP and NSF (relative specific activity of 1.3).

were chromaffin vesicles. No plasma membrane fragments could be detected with an antibody directed against connexin-43. As would be expected the isolated vesicles contained besides SNAP-25 and syntaxin also α-SNAP and NSF (Figure 2).

Further evidence for the membrane-bound nature of α-SNAP and NSF in adrenal chromaffin cells was obtained during subfractionation studies (Figure 3). Chromaffin vesicles (P2) were obtained by differential centrifugation of homogenized bovine adrenal medullae (H). When equal amounts of proteins were separated by SDS−PAGE followed by immunoblotting, the membrane proteins synaptobrevin, synaptotagmin, syntaxin, and SNAP-25 but also the soluble α-SNAP and NSF were found to be enriched in the fraction

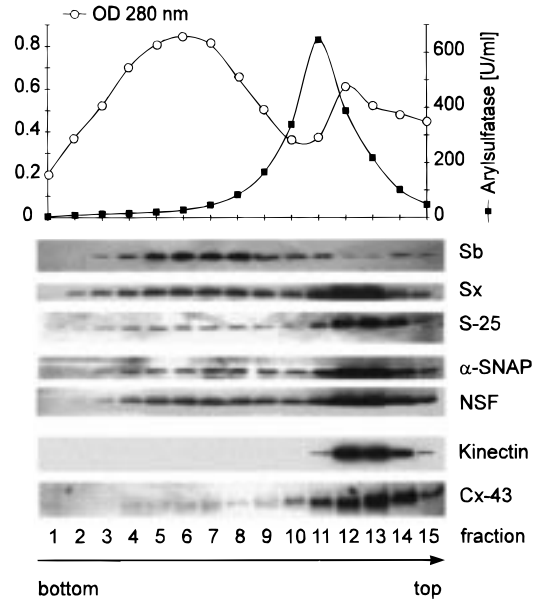


FIGURE 4: Purification of chromaffin vesicles on a sucrose gradient. Synaptobrevin and absorption at 280 nm in the TCA supernatant identified chromaffin vesicles in the lower part of the gradient (fractions 1−9). Significant amounts of α-SNAP, NSF, syntaxin (Sx), and SNAP-25 (S-25) were also detected in vesicle fractions. The distribution of lysosomes was evaluated by the determination of arylsulfatase activity in the fractions. Kinectin served as a marker for endoplasmic reticulum derived membranes and connexin-43 (Cx-43) for the plasma membrane.

P2 (Figure 3). Only small amounts of the proteins analyzed were detected in the high speed supernatant (S3) (Figure 3). Quantitatively 0.4%−5% of the membrane proteins, 7% of α-SNAP, and 6% NSF were found in the cytosol (S3). These values are to be compared with the percentage of lactate dehydrogenase, an established marker for the cytosol which amounted to 89% ± 3%. Together, these observations as well as the data on cracked cells shown above indicate that the putative soluble proteins α-SNAP and NSF are predominantly membrane-bound in adrenal chromaffin cells.

Chromaffin vesicles, due to their high density, can be successfully purified from other organelles and subcellular membranes by sucrose density gradient centrifugation. As shown in Figure 4 markers for the plasmalemma, microsomes, and lysosomes are found at the top of the gradient whereas chromaffin vesicles, characterized by high concentrations of material absorbing light at 280 nm in the TCA supernatant (indicating vesicular catecholamines and ATP) and enriched in synaptobrevin, are found as a broad band at higher density. The distribution of the other proteins examined in the gradient is clearly bimodal. The presence of significant amounts of syntaxin and SNAP-25 in chromaffin vesicles is in accordance with previous ultrastructural examinations and subfractionation studies (*7*, *32*, *33*). The large amounts of syntaxin and SNAP-25 in the microsomal fractions are in agreement with their presence in the plasmalemma (*34*, *35*). The distribution of α-SNAP and NSF, when compared to that of syntaxin and SNAP-25, is very similar (Figure 4).

The amounts of α-SNAP and NSF in the collected fractions (1−9) containing the chromaffin vesicles were determined by densitometric evaluations. In the gradient

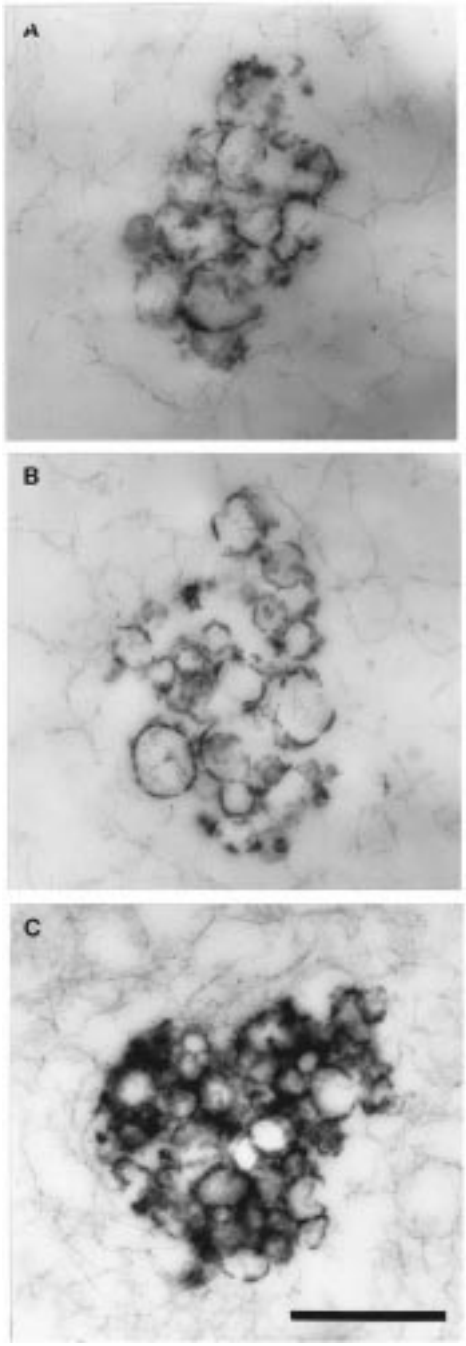


FIGURE 5: Immunolocalization of α-SNAP, NSF, and synaptobrevin in isolated chromaffin vesicle membranes. Chromaffin vesicle membranes prepared by hypotonic lysis of chromaffin vesicles recovered from the density gradient (see Experimental Procedures) were embedded in agarose and subjected to immunolabeling using the pre-embedding technique. Chromaffin vesicle membranes were immunostained with antibodies directed against α-SNAP, NSF, and synaptobrevin: bar 0.5 $\mu$m.

30% of α-SNAP and 27% of NSF, respectively, were found in the vesicle fractions. Since α-SNAP binds both NSF and the SNAREs syntaxin and SNAP-25 in vitro (*22*, *30*), we conclude that these proteins may associate also at chromaffin vesicle membranes.

Membranes were prepared by hypotonic lysis of chromaffin vesicles recovered from sucrose gradients (see Experimental Procedures). Then we investigated the presence of α-SNAP and NSF by immunocytochemistry at the ultrastructural level. We found strong labeling of the chromaffin

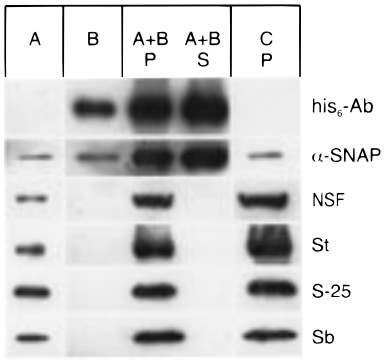


FIGURE 6: Binding of his$_6$α-SNAP by chromaffin vesicle membranes. Chromaffin vesicle membranes (10 $\mu$g/lane) contain α-SNAP, NSF, synaptotagmin, SNAP-25, and synaptobrevin (A). Recombinant his$_6$α-SNAP (1 $\mu$g/lane) is recognized by anti-his$_6$- and anti-α-SNAP antibodies (B), while anti-his$_6$ antibody does not detect endogenous α-SNAP (A). Bound his$_6$α-SNAP in the pellet (P) was determined after incubation of 100 $\mu$g of vesicle membranes with 10 $\mu$g of his$_6$α-SNAP for 30 min on ice followed by centrifugation at 100000*g* for 60 min and washing A + B (P). Free his$_6$α-SNAP remains in the supernatant (10 $\mu$L/lane) A + B (S). The pellet, after incubation with his$_6$-dehydrofolate reductase, shows no unspecific binding of the control protein C (P).

vesicle membranes, using an antibody against synaptobrevin, an established membrane protein of chromaffin vesicles (Figure 5). In support of the data described above, chromaffin vesicle membranes were also immunostained for α-SNAP and NSF (Figure 5). In controls without the specific antibody or with an antibody directed against connexin-43, a plasma membrane marker, no labeling was observed (data not shown). These observations and the analysis of the same antigens in fractions recovered from the sucrose gradient (see above) clearly show that α-SNAP and NSF, as syntaxin and SNAP-25 (*7*, *32*, *33*), are associated with chromaffin vesicle membranes.

The interaction of α-SNAP with chromaffin vesicle membranes was further investigated with the recombinant fusion protein his$_6$α-SNAP. In the experiment shown in Figure 6 we incubated vesicle membranes with his$_6$α-SNAP and harvested them by centrifugation followed by washing (see Experimental Procedures). We found his$_6$α-SNAP and endogenous α-SNAP in the vesicle membrane pellet along with synaptobrevin, synaptotagmin, and SNAP-25. Unbound his$_6$α-SNAP but no vesicular membrane proteins remained in the supernatant. If his$_6$α-SNAP was replaced by the recombinant fusion protein his$_6$dihydrofolate reductase as a control, no immunoreactivity could be detected with the anti-his$_6$ antibody in the pellet (Figure 6). These experiments indicate that α-SNAP is specifically bound by chromaffin vesicle membranes; moreover they document that isolated chromaffin vesicles possess additional unoccupied binding sites.

The binding of recombinant his$_6$α-SNAP by chromaffin vesicle membranes was also analyzed as a function of his$_6$α-SNAP concentration (Figure 7). Bound his$_6$α-SNAP was determined by immunoblots with anti his$_6$ antibody followed by densitometry. The evaluations showed that binding of his$_6$α-SNAP was saturated at concentrations higher than 0.3 $\mu$M. Half-maximal binding was observed with about 0.15 $\mu$M his$_6$α-SNAP. To quantify the endogenous amount of α-SNAP and the binding capacity, we incubated chromaffin vesicle membranes with the saturating concentrations of

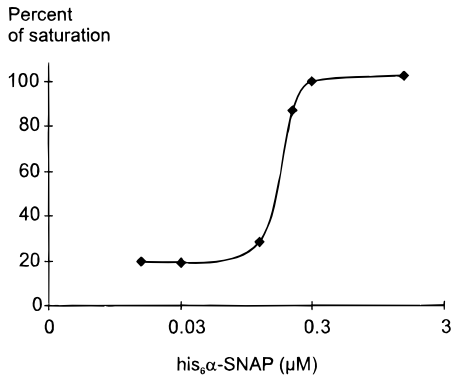


FIGURE 7: Concentration dependence of $his_6$α-SNAP binding by chromaffin vesicle membranes. Chromaffin vesicle membranes (100 $\mu$g) were incubated with 0.02, 0.03, 0.1, 0.2, 0.3, and 1.7 $\mu$M $his_6$α-SNAP in 50 $\mu$L of PBS for 30 min on ice and centrifuged at 100000*g* as described above. The pellets were subjected to SDS−PAGE and blotted onto nitrocellulose. Bound $his_6$α-SNAP was determined using an anti-$his_6$ antibody and quantified by densitometry. Saturation of binding occurred with concentrations higher than 0.3 $\mu$M $his_6$α-SNAP, half-maximal binding around 0.15 $\mu$M.

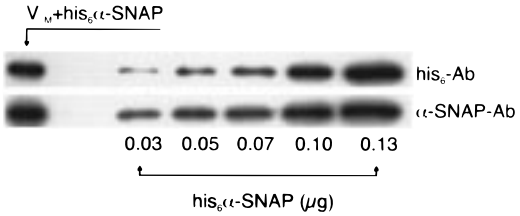


FIGURE 8: Quantification of $his_6$α-SNAP binding by chromaffin vesicle membranes. Chromaffin vesicle membranes ($V_M$, 5 $\mu$g/lane), incubated with $his_6$α-SNAP (see Experimental Procedures), were centrifuged and washed. The amounts of total α-SNAP and $his_6$α-SNAP were determined in immunoblots with an anti-$his_6$ antibody and an anti-α-SNAP antibody, respectively. Intensities of immunoreactive bands were quantified and compared with the signal obtained with different concentrations of recombinant $his_6$α-SNAP (see table below).

$his_6$α-SNAP. We calibrated the immunoblots with known concentrations of recombinant α-SNAP. Bound $his_6$α-SNAP was determined with the antibody interacting with the $his_6$ tag but not with α-SNAP. Endogenous plus $his_6$α-SNAP was measured with anti-α-SNAP antibody (Figure 8). Quantification of the immunoblots by densitometry revealed that the binding capacity for α-SNAP on chromaffin vesicle membranes was about 10 times higher than the endogenous amount. We detected 2.2 ± 0.4 $\mu$g of endogenous α-SNAP per 1 mg of membrane protein equivalent to 14.0 ± 2.4 molecules per vesicle and found that 21.4 ± 2.9 $\mu$g of $his_6$α-SNAP can be bound per 1 mg of vesicle membrane. The total amount of α-SNAP present in the pellet after incubation with $his_6$α-SNAP was 23.4 ± 3.7 $\mu$g/mg of vesicle membrane (Table 1).

We also investigated whether the association of recombinant or endogenous α-SNAP is influenced by ATP (as binding to Golgi membranes) or by $Ca^{2+}$, the second messenger triggering exocytosis. However, as shown in Figure 9, binding of neither exogenous nor or endogenous α-SNAP was influenced by these treatments.

To reveal the nature of the binding sites of α-SNAP in chromaffin vesicle membranes, we investigated binding of recombinant $his_6$α-SNAP to blotted vesicle membranes by an overlay assay. Two prominent $his_6$α-SNAP binding bands at 76 and 25 kDa were detected which were further identified

Table 1: Amounts of Endogenous α-SNAP and Binding of $His_6$α-SNAP by Chromaffin Vesicle Membranes[a]

| preparation | addition of $his_6$α-SNAP [$\mu$g/$V_M$ mg] | detected by α-SNAP-Ab [$\mu$g/$V_M$ mg] | detected by His-Ab [$\mu$g/$V_M$ mg] |
|---|---|---|---|
| 1 | | 2.91 ± 0.32 | |
| | 100 | 22.52 ± 4.05 | 20.02 ± 1.45 |
| 2 | | 1.63 ± 0.59 | |
| | 100 | 22.10 ± 4.34 | 23.94 ± 3.21 |
| 3 | | 2.02 ± 0.20 | |
| | 100 | 25.63 ± 2.75 | 23.20 ± 4.11 |

[a] Anti-α-SNAP antibody was used to determine both endogenous α-SNAP and $his_6$α-SNAP. After addition of $his_6$α-SNAP the bound protein was investigated with an anti-$his_6$ antibody. Three different preparations of vesicle membranes ($V_M$) were analyzed, and 6 affinity assays were carried out. Values are mean ± SD. Since 1 mg of total vesicle protein is equivalent to $5.8 \times 10^{11}$ chromaffin vesicles (*56*), it can be calculated that 14 ± 2.4 molecules of α-SNAP is bound per chromaffin vesicle. Under saturating conditions 140.8 ± 23.4 additional molecules of α-SNAP can be bound per vesicle.

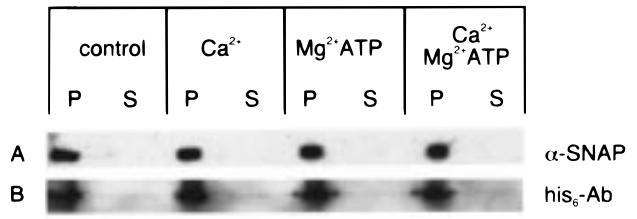


FIGURE 9: Effect of $Ca^{2+}$, $Mg^{2+}$, and ATP on the association of endogenous and recombinant α-SNAP with chromaffin vesicle membranes. (A) Chromaffin vesicle membranes isolated in intracellular media containing 5 mM EDTA were incubated for 15 min with $Ca^{2+}$ (30 $\mu$M free), 2 mM ATP, and $Mg^{2+}$ (1000 $\mu$M free), or $Mg^2$ATP plus $Ca^{2+}$ at room temperature. Following ultracentrifugation α-SNAP was found in the pellets (P) and not in the supernatant (S) irrespective of the composition of the medium used. (B) Binding of recombinant $his_6$α-SNAP to chromaffin vesicle membranes determined after 30 min of incubation on ice was not influenced by addition of $Ca^{2+}$ (30 $\mu$M free), 2 mM ATP and $Mg^2$ (1000 $\mu$M free), or $Mg^2$ATP plus $Ca^{2+}$.

as NSF and SNAP-25 (Figure 10 A). No binding of $his_6$-dihydrofolate reductase, used as a control, could be detected. To confirm the specificity of $his_6$α-SNAP binding to vesicular SNAP-25 in the overlay assay, we incubated the blotted vesicle membranes with an amino-terminal truncated $his_6$α-SNAP (114−295 AA) shown in Figure 10 B. As expected, binding to NSF (*36*), but not to SNAP-25 (*37*), was observed. These data demonstrate the presence of two specific binding proteins for α-SNAP at chromaffin vesicle membranes. Binding to NSF would be expected from the presence of NSF in chromaffin vesicles purified by density gradient centrifugation or immunoisolation (see above). Binding of α-SNAP to SNAP-25 but not to syntaxin or other membrane proteins of chromaffin vesicles indicates that SNAP-25 serves as the major v-SNARE on the vesicle membrane.

## DISCUSSION

The SNARE hypothesis (*38*) of vesicular transport describing the sequence of events during exocytosis is based on the identification of a complex of synaptobrevin, SNAP-25, and syntaxin as SNAP receptors (SNAREs) in brain membranes (*2*), and is supported by the observations that the same proteins are substrates of inhibitory clostridial neurotoxins (see refs *3*, *4*). In brain detergent extracts

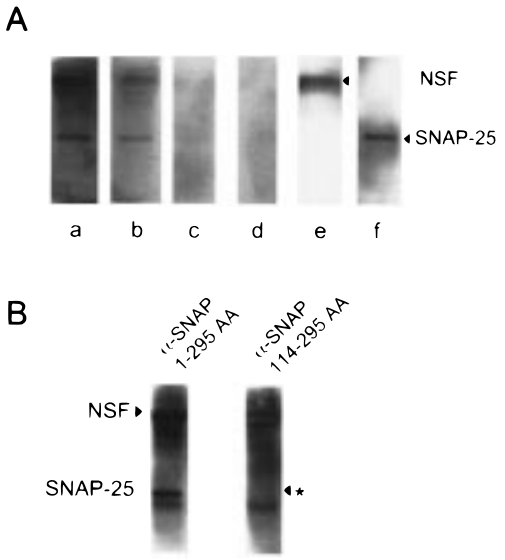


FIGURE 10: Identification of SNAP receptor proteins in chromaffin vesicle membranes using an overlay assay. (A) Chromaffin vesicle membranes (25 $\mu$g/lane) subjected to SDS−PAGE and blotted onto nitrocellulose were incubated with (a) 1 $\mu$g, (b) 0.5 $\mu$g, and (c) 0.1 $\mu$g of his$_6$α-SNAP in 3 mL of TBS-Tween. Bound his$_6$α-SNAP was detected using anti-his$_6$ antibody. The two prominent bands were identified as NSF (e) and SNAP-25 (f) using monoclonal antibodies against these proteins. As a control 1 $\mu$g of his$_6$-dehydrofolate reductase was used instead of his$_6$α-SNAP (d). (B) An amino-terminal trucated his$_6$α-SNAP (114−295 AA) protein did not bind to vesicular SNAP-25 (marked by the asterisk) in contrast to the full-length his$_6$α-SNAP showing the specific binding of his$_6$α-SNAP by SNAP-25 of chromaffin vesicle membranes.

SNAREs form complexes which recruit α-SNAP and NSF and dissociate upon the addition of $Mg^{2+}$ATP (*5*), processes suggested to occur during the docking of vesicles at their target membrane at the moment of exocytosis.

α-SNAP has recently been shown to enhance exocytosis of synaptic vesicles in the squid giant synapse (*39*). Investigation of the temperature-dependent paralysis of comatose mutants of Drosophila revealed a mutation in the NSF gene and provided first functional evidence for a possible role of NSF in neurotransmission (*40*). Furthermore peptides derived from NSF modulate the synaptic function of the giant synapse of the squid (*41*). Thus soluble α-SNAP and NSF may act together in regulated exocytosis of neurotransmitters in a way similar to the way that they act in constitutive membrane fusion events within the Golgi apparatus and between intracellular organelles (*1*).

The functional domains involved in the interaction of soluble and membrane-bound proteins during assembly of the SNARE complexes have been well-established in studies with recombinant proteins in vitro, but little is known about the properties in biological membranes. More recent findings regarding the location of SNAREs, the formation and the disassembly of complexes in intact cells add important information on the arrangement of the cognate proteins and the order of events predicted by the original SNARE hypothesis (*38*).

Syntaxin and SNAP-25, which bind α-SNAP in vitro (*22*, *30*) and which were previously proposed to be exclusively present at the plasmalemma where they act as target-SNAREs, have also been found in addition at the membrane of synaptic and adrenal chromaffin vesicles (*7*, *32*, *33*, *42*, *43*). Thus syntaxin and SNAP-25 at both the vesicular membrane and the plasmalemma may attach NSF via α-SNAP. Importantly SNARE complexes thought to be formed only during interactions of the secretory organelles and the plasmalemma have recently been detected to pre-exist in synaptic and chromaffin vesicles (*7*, *44*). Presumably SNARE complexes are already formed soon after their biosynthesis because they have been found not only in mature undocked secretory vesicles and in the growth cone (*45*) but also in the axon (*46*) where vesicles formed in the Golgi apparatus of the perikaryon are transported to the periphery.

One likely function of vesicular syntaxin and SNAP-25 is to bind α-SNAP which in turn associates NSF. The first evidence for such a role was provided by the observation that SNARE complexes formed in the detergent extract of chromaffin vesicle membranes bind exogenous α-SNAP (*33*). Although the presence of endogenous α-SNAP and NSF has been reported in synaptic and chromaffin vesicles (*47*, *48*), no data regarding the amounts, the affinity, or the receptors were available. In this contribution we show for the first time that secretory vesicles have significant amounts of endogenous α-SNAP. α-SNAP (2 $\mu$g) was found to be bound by 1 mg of purified chromaffin vesicle membranes. From this value it can be calculated that $14.0 \pm 2.4$ molecules of α-SNAP are bound per undocked chromaffin vesicle. We found that 10 times more α-SNAP is specifically bound in vitro by chromaffin vesicle membranes under saturating conditions. Addition of α-SNAP to digitonin-permeabilized adrenal chromaffin cells enhances (by a factor of 1.5) calcium-induced catecholamine release in the presence of ATP (*27*, *49*). Binding of α-SNAP to unoccupied SNAREs may have contributed to this effect. The overlay assays presented in this study revealed that α-SNAP predominantly binds to vesicular SNAP-25 and not to syntaxin. This indicates that SNAP-25 probably serves as a relevant v-SNARE of undocked adrenal chromaffin vesicles.

Secretion can be divided into two sequential steps, that is, $Mg^{2+}$ATP-dependent priming which is followed by $Ca^{2+}$-triggered exocytosis (*31*). It is likely that ATP cleavage by NSF plays an important role in priming (*27*, *50*, *51*). We found NSF to exist predominantly in a membrane-bound form in adrenal chromaffin cells, and no release occurred in the presence of $Mg^{2+}$ATP (see above). NSF is also associated with chromaffin and synaptic vesicles (*47*, *48*). However, in contrast to Golgi membranes, NSF bound to synaptic vesicles or chromaffin vesicles remains attached to the membranes in the presence of $Mg^{2+}$ATP, suggesting a different mode of binding (*29*, *47*, *48*). While there is agreement that $Mg^{2+}$ATP does not influence binding of NSF to chromaffin or synaptic vesicles, the effect of $Mg^{2+}$ATP on the membrane association of α-SNAP is a matter of debate. A complete removal of endogenous α-SNAP with $Mg^{2+}$ATP was reported (*47*). We did not find an effect of $Mg^{2+}$ATP on the membrane association of endogenous α-SNAP in chromaffin cells, on endogenous α-SNAP at chromaffin vesicle membranes, or on the binding of recombinant binding α-SNAP to chromaffin vesicle membranes. Thus it is possible that α-SNAP like NSF remains bound at the membrane of chromaffin vesicles in the presence of $Mg^{2+}$ATP in concentrations found in the cytosol. Interestingly, during docking of vesicles to the plasmalemma, α-SNAP and NSF are released by $Mg^{2+}$ATP (*50*), that is, prior to calcium-induced vesicle fusion and catecholamine

release, which is independent of α-SNAP and NSF (*27*, *49*). Thus it appears that interaction of chromaffin vesicles with the plasmalemma is essential for the release of α-SNAP.

The presence of a full set of SNARE proteins (*7*, *32*, *33*, *42*, *43*), SNARE heteromers (*7*, *44*), and membrane-bound NSF and α-SNAP in undocked chromaffin and synaptic vesicles suggests that the vesicles carry NSF and α-SNAP to the site of exocytosis. Although an effect of α-SNAP and NSF in undocked vesicles has not been described, the proteins may function prior to docking as observed during NSF driven events of yeast vacuoles, clathrin-coated vesicles, and phagosomes (*51*−*54*). The secretory organelles, for example, may acquire competence for docking to the target membrane or be primed for subsequent steps involved in rapid exocytosis. After $Mg^{2+}$ATP-driven release of α-SNAP and NSF from their receptors during interaction of the secretory vesicles with the plasmalemma (*50*), complexes between vesicular and plasmalemmal proteins may be formed which again can interact with α-SNAP and NSF (*1*, *2*) or they may proceed to pore formation which is independent of α-SNAP and NSF (*55*).

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