Characterization of α-Soluble N-Ethylmaleimide–Sensitive Fusion Attachment Protein in Alveolar Type II Cells

Implications in Lung Surfactant Secretion

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N-ethylmaleimide–sensitive fusion protein (NSF) and soluble NSF attachment protein (α-SNAP) are thought to be soluble factors that transiently bind and disassemble SNAP receptor complex during exocytosis in neuronal and endocrine cells. Lung surfactant is secreted via exocytosis of lamellar bodies from alveolar epithelial type II cells. However, the secretion of lung surfactant is a relatively slow process, and involvement of SNAP receptor and its cofactors (NSF and α-SNAP) in this process has not been demonstrated. In this study, we investigated a possible role of α-SNAP in surfactant secretion. α-SNAP was predominantly associated with the membranes in alveolar type II cells as determined by Western blot and immunocytochemical analysis using confocal microscope. Membrane-associated α-SNAP was not released from the membrane fraction when the cells were lyzed in the presence of Ca^{2+} or Mg^{2+}ATP. The alkaline condition (0.1 M Na_2CO_3, pH 12), known to extract peripheral membrane proteins also failed to release it from the membrane. Phase separation using Triton X-114 showed that α-SNAP partitioned into both aqueous and detergent phases. NSF had membrane-bound characteristics similar to α-SNAP in type II cells. Permeabilization of type II cells with β-escin resulted in a partial loss of α-SNAP from the cells, but cellular NSF was relatively unchanged. Addition of exogenous α-SNAP to the permeabilized cells increased surfactant secretion in a dose-dependent manner, whereas exogenous NSF has much less effects. An α-SNAP antisense oligonucleotide decreased its protein level and inhibited surfactant secretion. Our results suggest a role of α-SNAP in lung surfactant secretion.

Lung surfactant is secreted through exocytosis that involves the fusion of lamellar bodies with the plasma membrane (1). Previous studies have shown that several second messengers such as Ca^{2+}, cyclic AMP, and several protein kinases such as protein kinase A and C, and Ca^{2+}- and calmodulin-dependent protein kinase, are involved in the stimulation of surfactant secretion (2, 3). Substrates for these protein kinases in alveolar type II cells are unclear, but could be specific targeting, docking, and/or fusion proteins including annexin II and soluble N-ethylmalimide–sensitive fusion (NSF) attachment protein (SNAP) receptors (SNAREs). Our previous studies have shown that annexin II promotes membrane fusion of lamellar bodies of type II cells (4–6). We have also identified several other proteins that participate in various stages of exocytosis in type II cells, including caplain (7, 8), phospholipidase A_2 (9), protein kinase A (10), and Ca^{2+}- and calmodulin-dependent protein kinase (11).

The SNARE hypothesis was proposed by Rothman and coworkers to explain vesicular targeting, docking, and fusion with the target membrane (12). The hypothesis postulates that vesicles are specifically targeted and docked onto the plasma membrane via the interaction between vesicle SNAREs (v-SNAREs) and target SNAREs (t-SNAREs). An example of v-SNARE is VAMP (vesicle-associated membrane protein). t-SNAREs include syntaxin and SNAP-25/23. The interaction of v- and t-SNAREs results in the formation of 7S complex via a coil–coiled structure (13). This complex then provides binding sites for soluble cofactors such as α-SNAP and NSF to form a complex now referred to as 20S complex (14). NSF is an ATPase capable of binding and hydrolyzing ATP (15). The hydrolysis of ATP provides energy to disassemble the complex (16, 17). This is accompanied by a transient alteration of conformation of syntaxin such that it no longer binds to VAMP (18, 19). It is also required for recycling of the complex components to different membranes (20) as well as release of NSF and α-SNAP into the cytosol. From the point of view of this hypothesis, NSF and α-SNAP are cytosolic proteins that can only associate with the membrane through the docking complex.

NSF and α-SNAP have widespread involvement in vesicular transport pathways leading to exocytosis. In mammalian cells, NSF is thought to function in the fusion of essentially all types of vesicles, including endoplasmic reticulum, Golgi, and endocytic vesicles, as well as secretory vesicles undergoing regulated exocytosis (for review *see* Ref. 12). NSF is also required for the transport of vesicles from the endoplasmic reticulum to Golgi complex (21). Its role in exocytosis was explicitly confirmed by the fact that mutant NSF (Drosophila homolog, dNSF-1) conferred an apparent failure of synaptic transmission at an elevated temperature (22). The role of α-SNAP in regulated exocytosis was supported by the observation that its injection into the squid giant synapse (23) or its addition to digitonin-permeabilized

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Abbreviations: fetal bovine serum, FBS; fluorescein isothiocyanate, FITC; minimum essential medium, MEM; N-ethylmaleimide–sensitive fusion protein, NSF; phosphate-buffered saline, PBS; soluble NSF attachment protein, SNAP; SNAP receptor, SNARE; Tris-buffered saline, TBS.

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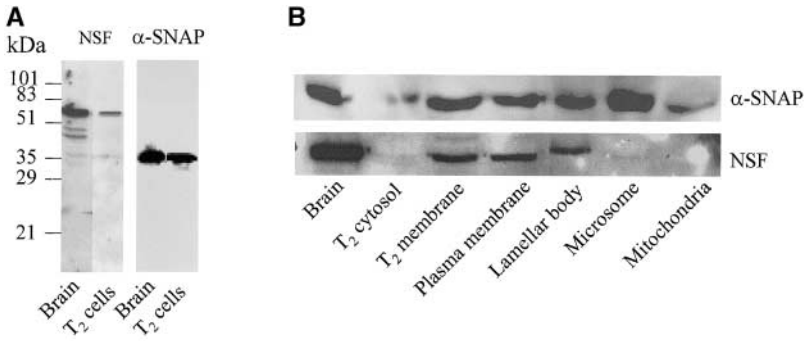

Figure 1. α-SNAP and NSF are primarily associated with the membrane fraction of type II cells. (*A*) Alveolar type II cells isolated from rat lung were lyzed in hypotonic lysis buffer containing 5 mM EGTA, 5 mM EDTA, 2 mM DTT, 2 μg/ml aprotonin, 2 μg /ml leupeptin, and 20 mM Tris-HCl, pH 7.4. Total cell lysate (70 μg) was analyzed by Western blot using anti-NSF or anti–α-SNAP antibodies. (*B*) Type II cell lysate was centrifuged at 100,000 × *g* for 1 h to separate cytosolic from membrane fraction. The cytosolic and the membrane fractions as well as plasma membrane, lamallar body, microsome, and mitochondria (50 μg protein each) were analyzed by SDS-PAGE and Western blot using specific antibodies against NSF and α -SNAP. Rat brain homogenate (50 μg) was used as positive control.

chromaffin cells (24) stimulated Ca^{2+}-dependent exocytosis. Moreover, Nagamatsu and colleagues demonstrated that treatment of MIN6 cells with an antisense phosphorothioate oligonucleotide complementary to α-SNAP mRNA suppressed the translation of this protein and subsequently inhibited insulin secretion (25).

Although the role of NSF and α-SNAP in membrane fusion and exocytosis appears to be conserved in a variety of cell systems, their role in surfactant secretion, a relatively slow process of exocytosis, has not been demonstrated. In this study, we investigated the conditions under which α-SNAP and NSF associate with lung type II cell membranes. Furthermore, we attempted to establish a functional role of α-SNAP by reconstituting surfactant secretion from permeabilized type II cells with recombinant α-SNAP and by decreasing protein level in intact type II cells using α-SNAP antisense oligonucleotide. To our knowledge, this is the first study to investigate the role of SNAREs in surfactant secretion.

Materials and Methods

Materials

Leupeptin, aprotinin, and ATP were obtained from Sigma (St. Louis, MO). Horseradish peroxidase–conjugated goat anti-mouse and anti-rabbit IgG, gelatin, prestained molecular mass standard were obtained from BioRad (Melville, NY). Nitrocellulose membranes were from Scheilcher and Schuell (Keene, NH). Eagle's minimal essential medium (MEM) was from ICN (Costa Mesta, CA). Fetal bovine serum (FBS) was from GIBCO (Grand Island, NY). Elastase was from Worthington Biochemical Co. (Freehold, NJ). Enhanced chemiluminescence was from Amersham Pharmacia Biotech (Arlington Height, IL). Monoclonal antibodies for NSF and α-SNAP were obtained from StressGen (Victoria, BC, Canada). Monclonal antibodies against α-SNAP used for one of Western Blots (Figure 1A) and immunocytochemistry/histochemistry (Figures 2 and 3) were from BD Biosciences (San Diego, CA). Anti–SP-C goat polyclonal antibodies were from Santa Cruz (Santa Cruz, CA). CY3-conjugated anti-mouse IgG and fluorescein isothiocyanate (FITC)-conjugated anti-goat IgG were from Jackson Immunoresearch Laboratories (West Grove, PA). FITC-conjugated MPA (*Maclura pomifera*) was from EY laboratories (San

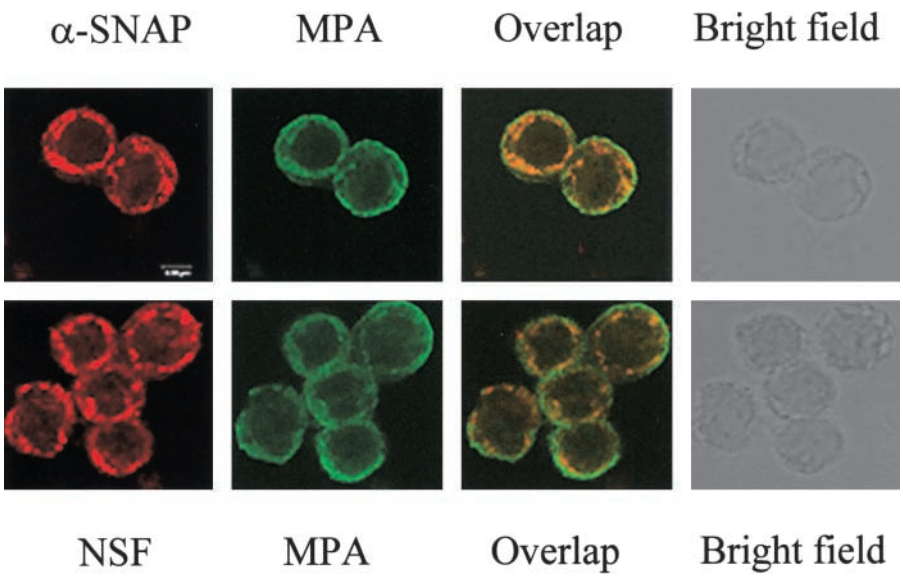

Figure 2. Immunocytochemical localization of α-SNAP and NSF. Paraformaldehyde-fixed alveolar type II cells were permeabilized with 0.5% Triton X-100 for 20 min and incubated with or without anti-NSF (3:100) or anti–α-SNAP (1:50) antibodies and CY3-conjugated goat anti-mouse IgG (1:200), followed by incubation with FITC-MPA (0.1 μg/ml). Cells were examined with a confocal laser scanning fluorescent microscope. *Scale bar:* 4 μm.

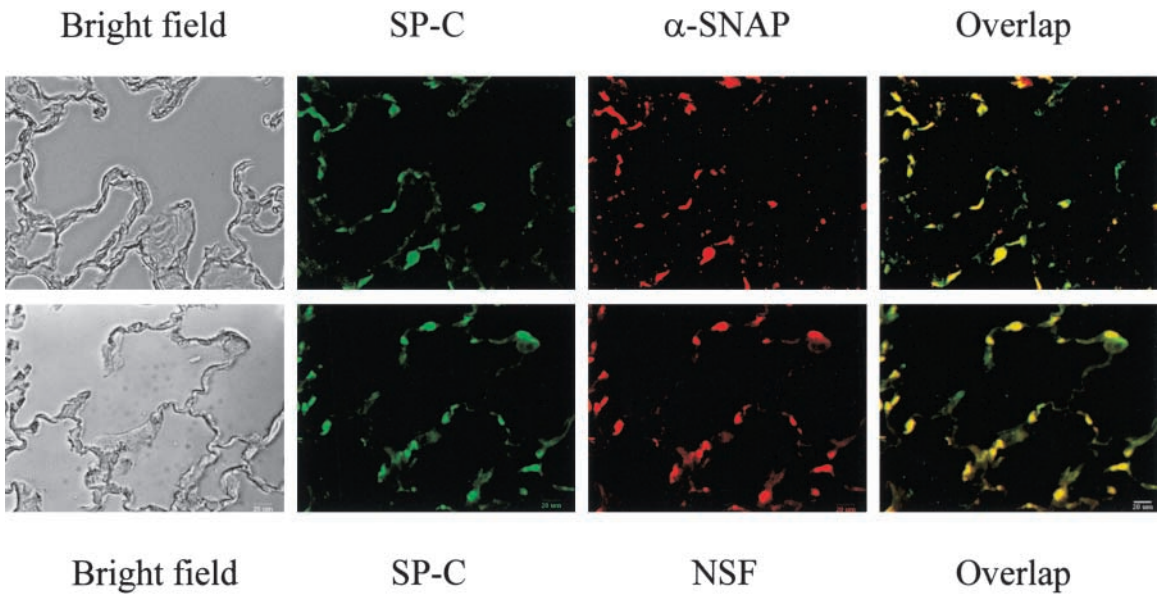

Figure 3. Immunohistochemical localization of α-SNAP and NSF. Paraffin-embedded rat lung tissues were dually labeled with anti–α-SNAP or NSF antibodies and anti–SP-C antibodies, followed by CY3-conjugated anti-mouse and FITC-conjugated anti-goat antibodies. The results show the colocalization of α-SNAP or NSF with SP-C, a marker of alveolar type II cells. *Scale bar:* 20 μm.

Mateo, CA). FuGene 6 was from Roche Molecular Biochemicals (Indianapolis, IN). Phosphorothioate oligonucleotides for α-SNAP were synthesized by MWG-Biotech (High Point, NC). cDNA clones encoding His_6-tagged NSF and α-SNAP were kindly provided by Dr. J. E. Rothman (Memorial Sloan-Kettering Cancer Center, NY).

Isolation and Culture of Alveolar Type II Cells

Type II cells were isolated from the lungs of adult male Sprague-Dawley rats (180–200 g) according to the method of Dobbs and coworkers (26) as previously described by Liu and colleagues (5). Isolated type II cells were either directly lyzed for the α-SNAP and NSF identification or plated on 35- or 100-mm plastic tissue culture dishes and cultured overnight in 1.5 ml or 10 ml MEM supplemented with 10% FBS for other analyses. The purity of overnight cultured type II cells were greater than 95% as evaluated by modified Papanicolaou staining.

Preparation of Membrane and Cytosolic Fractions of Type II Cells

Freshly isolated type II cells were lyzed by sonication in 100 μl lysis buffer (5 mM EGTA, 5 mM EDTA, 2 mM DTT, 2 μg/ml aprotonin, 2 μg/ml leupeptin, and 20 mM Tris-HCl, pH 7.4) and centrifuged at 100,000 × *g* for 1 h. The supernatant was then separated from the pellet and designated as cytosolic and membrane fractions, respectively.

Lamellar Body Purification

Lamellar bodies were isolated from rat lung by the upward floatation method according to Chander and coworkers (27). Rat lungs were cleared of blood by perfusion and homogenized in 10 ml of 1 M sucrose. The homogenate was filtered through four layers of gauze cloth, placed under a discontinuous sucrose gradient (0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2 M), and centrifuged at 80,000 × *g* for 3 h. The lamellar body fraction recovered at the 0.4 and 0.5 M interface was diluted to 0.24 M sucrose and centrifuged at 20,000 × *g* for 30 min. The pellet containing lamellar bodies was re-suspended in 10 mM Hepes-Tris buffer (pH 7.4) containing 0.24 M sucrose for further analysis.

Plasma Membrane Preparation

Plasma membrane was isolated according to Maeda and coworkers (28). Rat lung was homogenized in 10 ml of buffer B (10 mM Na-Pi, pH 7.4, 30 mM NaCl, 1 mM $MgCl_2$, 5 μM PMSF, 0.02% NaN_3, 10 μg/ml DNase, and 0.32 M sucrose). The homogenate was filtered through four layers of gauze cloth and loaded on a sucrose gradient (0.5, 0.7, 0.9, 1.2 M). The gradient was centrifuged at 95,000 × *g* for 60 min, and plasma membrane collected from 0.9 and 1.2 M interface. This fraction was diluted to 0.32 M sucrose with cold buffer A (buffer B minus 0.32 M sucrose) and centrifuged at 95,000 × *g* for 30 min. The pellet containing plasma membrane was re-suspended in buffer B for further analyses.

Western Blot Analysis

Protein samples were resolved on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) under reducing conditions and electrophorically transferred to a nitrocellulose membrane. The blot was transiently stained with Ponceau S to monitor transfer efficiency of proteins and blocked with Tris-buffered saline (TBS) containing 2% gelatin for 1 h. The blot was then incubated with the appropriate primary antibodies (anti-NSF, 1:1,000 dilution; and anti–α-SNAP, 1:500 dilution) in TTBS (TBS plus 0.05% Tween 20) containing 1% gelatin for 3–12 h and then incubated with secondary antibodies (horseradish peroxidase–conjugated IgG, 1:5,000 dilution) for 1 h. Finally, the blot was vis-

ualized by incubating it in enhanced chemiluminescence Western blot detection reagent for 1 min and exposed to Kodak Biomax light film from Eastman Kodak company (Rochester, NY) for 10–30 s.

Immunocytochemistry

Alveolar type II cells were cytospined to a glass slide and fixed in 4% paraformaldehyde, and then permeabilized with 0.5% Triton X-100 for 20 min. Fixed cells were incubated for 30 min with 0.5% bovine serum albumin plus 2% goat serum in phosphate-buffered saline (PBS) to block nonspecific binding and incubated with or without anti-NSF (3:100) or anti–α-SNAP (1:50) antibodies in PBS at 4°C for 1 h. The cells were then washed three times with PBS and incubated with CY3-conjugated goat anti-mouse IgG (1:200) for 1 h. After washing twice, the slides were incubated with FITC-labeled MPA (0.1 μg/ml) for 2 min, washed once, and then mounted in 0.1 M n-propyl gallate to prevent photobleaching. Cells were examined with a Leica confocal laser scanning fluorescent microscope equipped with an argon-krypton laser. Images of the cells were created with standard objectives and photomultiplier tubes dedicated to fluorescent excitation and emission spectra for CY3 and FITC.

Immunohistochemistry

Rat lungs were infused with 4% formaldehyde in PBS at 20 cm gauge pressure and embedded in paraffin. The sections were dewaxed with xylene and rehydrated. The slides were treated with 0.05% Triton X-100 in PBS for 20 min and blocked with 10% FBS for 1 h. The sections were double-labeled with anti-NSF (1:1,000 dilution) or anti–α-SNAP monoclonal antibodies (1:50 dilution) and anti–SP-C goat polyclonal antibodies (1:1,000 dilution) overnight at 4°C in a humid chamber, followed by the incubation with Cy3-conjugated anti-mouse and FITC-conjugated anti-goat antibodies (1:2,500 dilution) for 1 h. The sections were added antifade mounting medium (1.5% n-propylgalate and 60% glycerol in PBS) and examined using Nikon Eclipase E-600 fluorescent microscope.

Detergent Extraction of NSF and α-SNAP

Type II cell or brain cell lysate (5 mg/ml) in homogenization buffer (2 mM EGTA, 1 mM PMSF, 150 mM NaCl, 1 mM benzamidine, 1 μg/ml aprotonin, 10 μg/ml leupeptin, and 20 mM Tris-HCl, pH 7.4) were incubated with varying concentrations of SDS (0%, 0.01%, 0.1% and 1%) or Triton X-100 (0%, 0.5%, 1%, and 2%) for 30 min on ice. The samples were centrifuged at 100,000 × *g* for 1 h to separate cytosolic plus extracted proteins (supernatant) from membrane or detergent resistant proteins (pellet). Alternatively, cell lysates were first centrifuged at 100,000 × *g* for 1 h to separate the cytosol from the membrane fraction. The pellet (membrane fraction) was re-suspended and treated with varying concentrations of Triton X-100 or SDS for 30 min on ice. The samples were then centrifuged at 100,000 × *g* for 1 h to separate the extracted (supernatant) from the detergent resistant (pellet) proteins. Both supernatant and pellet were analyzed for the presence of NSF and α-SNAP using SDS-PAGE and Western blot.

Phase Partitioning

Alveolar type II cells were lyzed by sonication in hypotonic lysis buffer containing 2.5% Triton X-114 and centrifuged at 2,000 rpm for 10 min to remove the cell debris. The supernatant was then warmed to 30°C to induce phase separation. Proteins in the upper aqueous phase (hydrophilic proteins) and lower detergent phase (hydrophobic proteins) were precipitated with 1:1 acetone plus ethanol. Precipitates were analyzed by SDS-PAGE and Western blot using antibodies against NSF and α-SNAP.

Alkaline Extraction

Alveolar type II cells were lyzed by sonication in hypotonic lysis buffer containing 0.1 M Na_2CO_3, pH 12 for 1 h at 4°C and then centrifuged at 100,000 × *g* for 1 h. Seventy micrograms of protein in the supernatant (cytosolic and peripheral membrane proteins) and pellet (integral membrane proteins) were analyzed by SDS-PAGE and Western blot as described above.

Mg^{2+}ATP Priming

Freshly isolated alveolar type II cells were lyzed for 30 min by sonication in hypotonic lysis buffer containing 1 mM Ca^{2+}, 2 mM Mg^{2+}ATP, 2 mM Mg^{2+}ATPγS, 1 mM Ca^{2+} plus 2 mM Mg^{2+}ATP or 2 mM Mg^{2+}GTPγS for 30 min. The samples were then centrifuged at 100,000 × *g* for 1 h to separate protein in the supernatant (cytosol plus released proteins) from pellet (membrane attached). Seventy micrograms of proteins in the supernatant and pellet were analyzed for the presence of NSF and α-SNAP by SDS-PAGE and Western blot.

Purification of Recombinant His_6-NSF and His_6–α-SNAP

Recombinant His_6-NSF and His_6–α-SNAP were expressed in *Escherichia coli* XLI blue and purified using Ni^{2+}-NTA resin as described (29, 30).

Permeabilization of Alveolar Type II Cells

Isolated type II cells (10×10^6/dish) were cultured overnight on 100-mm cell culture dishes. The dishes were washed three times with 10 ml MEM and two times with 10 ml permeabilization buffer (118 mM NaCl, 5 mM KCl, 25 mM $NaHCO_3$, 1 mM KH_2PO_4, 2 mM $MgCl_2$, 1 mM EGTA, 10 mM glucose, and 30 mM HEPES, pH 7.4). The cells were equilibrated for 30 min in permeabilization buffer. At the end of incubation, the medium was removed and cells incubated for 10 min in fresh permeabilization buffer (5 ml) containing 40 μM β-escin to permeabilize the cells followed by further incubation in permeabalization buffer without β-escin for 0, 20, or 50 min. The cells were lyzed by sonication in lysis buffer and the amount of NSF and α-SNAP remaining in the cell after leakage analyzed by SDS-PAGE and Western blot.

Reconstitution of Surfactant Secretion by Purified α-SNAP

Introduction of exogenous proteins into permeabilized type II cells were essentially done according to our previously published method (5). Type II cells (1×10^6) were pre-labeled overnight with MEM containing 10% FBS, 1 μCi [^{3}H]choline, washed, and permeabilized 10 min with permeabilization buffer containing 40 μM β-escin. Fresh permeabilization buffer with or without NSF or α-SNAP (1–40 μg/ml) was added. One set of dishes were removed to establish *time zero* value, and the remaining dishes incubated for 20 min. A quantity of 0.5 μM Ca^{2+} was added to stimulate the cells to secrete for 30 min. Lipids in the medium and cells were extracted and analyzed for radioactivity. Secretion was expressed as dpm in the medium/dpm in medium + dpm in cells × 100% and corrected by subtracting the *time zero* value.

Antisense Oligonucleotide and Surfactant Secretion

The antisense phosphorothioate oligonucleotide complementary to α-SNAP (5′-TCC ATG ACG TCG CGC AGC TGC-3′) surrounding the initiation codon and corresponding sense oligonucleotide were synthesized based on rat α-SNAP cDNA sequence. The transfection agent, FuGene 6, was used to facilitate the efficient transfer of oligonucleotide into cells. One hundred microliters of MEM was incubated with 6 μl of FuGene 6 for 5 min at room temperature. Antisense or sense oligonucleotide was added to the mixture and further incubated for 15 min. The mixture was added dropwise to type II cells (1×10^6) at the time of plating. After overnight culture, cells were washed six times with MEM and stimulated with 1 μM PMA for 2 h to enhance surfactant secretion. At the end of incubation, surfactant secretion was measured as described above. Similar experiments were performed to determine α-SNAP protein levels in the antisense or sense oligonucleotide-treated cells except that 100 mm dishes were used for this purpose. α-SNAP protein levels were quantitated by Western blot, followed by densitometry.

Statistics

All secretion studies were performed in duplicate and the individual results were averaged. Statistical significance was evaluated by the Student's *t* test. The level of significance was taken as $P < 0.05$.

Results

Identification of α-SNAP and NSF in Alveolar Type II Cells

As a first step to establishing the roles of α-SNAP and NSF in lung surfactant secretion, we investigated their presence in alveolar type II cells by Western blot. As shown in Figure 1A, α-SNAP and NSF are detected in type II cell lysate.

Subcellular Localization of α-SNAP and NSF in Type II Cells

The subcellular localization of α-SNAP and NSF is important because it provides information regarding the arrangement of cognate proteins and the order of events predicted by the original SNARE hypothesis. Figure 1B shows the distribution of α-SNAP and NSF in different subcellular membrane fractions. According to the original SNARE hypothesis, α-SNAP and NSF are cytosolic proteins. However, we observed that α-SNAP and NSF are primarily located on the membrane fraction of type II cells. Only trace amounts of α-SNAP and NSF were detectable in the cytosolic fraction. When subcellular membrane fractions were examined, α-SNAP and NSF were present in both plasma membrane and lamellar bodies. α-SNAP was also detected in microsome and mitochondria, where no NSF was seen.

Immunocytochemistry coupled with confocal fluorescent microscopy is a powerful tool for establishing the localization of proteins in intact cells because it eliminates the possible artifacts and contamination during subcellular fractionation. We double-labeled type II cells with antibodies against α-SNAP or NSF with the type II cell marker, MPA. As shown in Figure 2, α-SNAP and NSF showed strong immunofluoresecnce signals on the plasma membrane of type II cells. The control cells in which primary antibody was omitted did not show any fluorescence (data not shown). As another control, primary antibodies were pre-incubated with purified recombinant proteins before being added to the cells. Once again, this did not generate fluorescent signals (data not shown). These results indicate that the fluorescence observed was not due to nonspecific binding of secondary antibodies. Stimulation of cells with secretagogues (1 μM PMA or 1 mM ATP) for 2 h did not cause change in α-SNAP and NSF distribution (data not shown).

To further examine cellular locations of NSF and α-SNAP in lung tissue, we performed immunohistochemical analysis. The rat lung tissue sections were double-labeled with anti-NSF or anti–α-SNAP monoclonal antibodies and anti–SP-C goat polyclonal antibodies. SP-C is a specific marker of alveolar type II cells. As shown in Figure 3, α-SNAP or NSF is colocalized with SP-C, suggesting its location in alveolar type II cells. The controls that primary antibodies were omitted or pre-blocked with purified recombinant proteins did not generate fluorescent signals (data not shown).

ATP Priming Failed to Release α-SNAP and NSF from the Membrane Fractions

Addition of ATP and Ca^{2+} is known to cause priming and triggering of fusion that disrupt SNARE complex, thereby releasing its soluble components (α-SNAP and NSF) and recycling SNAREs to their specific subcellular membranes. To examine whether these conditions change the distribution of α-SNAP and NSF, we lyzed cells in the presence of ATP plus or minus Ca^{2+}, or ATPγS to mimic priming and fusion conditions. None of these conditions caused a significant release of α-SNAP and NSF from the membrane (Figure 4). Because Rab proteins have been shown to interact with SNAREs (31), we also included GTPγS in our lysis buffer to observe its effect on α-SNAP and NSF localization. As shown in Figure 4, we did not detect any changes of the distribution of these proteins.

Detergent Extraction of α-SNAP and NSF from Type II Cell Membranes

Most amphiles in biological membranes, including phospholipids, stereods, and membrane proteins, are insoluble and form liquid crystals or insoluble precipitates in aqueous media (32). Detergents are soluble amphiphiles that form micelles of various sizes and shapes at specific temperatures and certain critical concentrations. At such conditions, they solubilize proteins, thereby releasing them from the membrane. We used this knowledge to characterize the association of α-SNAP and NSF with the membranes of type II cells compared with brain cells. We treated type II cells and brain lysate with SDS as well as Triton X-100 to observe whether there is a differential extraction of α-SNAP and NSF in type II cells as compared with those in the brain. As shown in Figure 5, 0.01% SDS failed to extract α-SNAP in both brain and type II cells. However, 0.1–1% SDS extracted all α-SNAP in both cases. A difference between type II cell and brain α-SNAP was observed during Triton X-100 treatments. Triton X-100 (0.5–2%) completely extracted brain α-SNAP, whereas only part of α-SNAP was extracted in type II cells under the same conditions. These results suggest that α-SNAP in type II cells is more tightly bound to the membrane than that in brain. Similar results were observed with NSF, except that NSF was more resis-

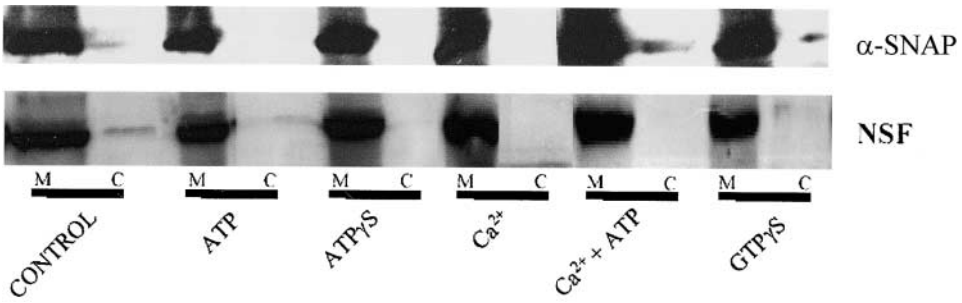

Figure 4. ATP, ATPγS, GTPγS, or Ca^{2+} did not change the distribution of α-SNAP and NSF. Freshly isolated alveolar type II cells were lyzed for 30 min in hypotonic lysis buffer containing 1 mM Ca^{2+}, 2 mM ATPγS, 1 mM Ca^{2+} plus 2 mM ATP or 2 mM GTPγS. Lysates were centrifuged at 100,000 × *g* for 1 h. The supernatant (cytosolic fraction, C) was separated from the pellet (membrane fraction, M). Both fractions were analyzed by SDS-PAGE and Western blot using antibodies against α-SNAP and NSF. The results shown were representative of two independent experiments.

tant to Triton X-100 extraction in both type II cells and brain (data not shown).

We also investigated the effect of Triton X-100 and SDS on α-SNAP and NSF associated with type II cell and brain membranes that have been isolated from the cytosolic fraction. This was intended to investigate the possibility that some cytosolic factors may re-enforce the association of these proteins with the membrane, thereby causing them to re-associate with the membrane even after extraction by detergents. It was surprising to observe that α-SNAP in the isolated membranes became more resistant to both SDS and Triton X-100 extraction in both brain and type II cells (Figure 6). The same was observed in NSF extraction (data not shown).

Triton X-114 Phase Separation

To determine whether α-SNAP and NSF have the characteristics of integral or peripheral membrane proteins, we treated type II cell membrane fraction with 0.1 M Na_2CO_3, pH 12, known to extract peripheral but not integral, membrane proteins. As shown in Figure 7A, neither α-SNAP nor NSF were extracted from the membrane by high pH treatment, indicating that they are not peripherally attached to the membrane. We also subjected type II cell lysate to temperature-induced phase separation in Triton X-114 known to partition proteins into hydrophobic and hydrophilic components. This treatment revealed that α-SNAP and NSF partitioned into both aqueous and detergent phases, implying that they exist in both hydrophobic and hydrophilic pools in type II cells. More NSF was detected in the hydrophobic pool. However, α-SNAP was equally distributed between the two phases (Figure 7B).

Addition of Exogenous α-SNAP into Permeabilized Type II Cells Increases Surfactant Secretion

To examine the effect of exogenous α-SNAP and NSF on surfactant secretion, alveolar type II cells were permeabilized using β-escin to enable the leakage of cytosolic pro-

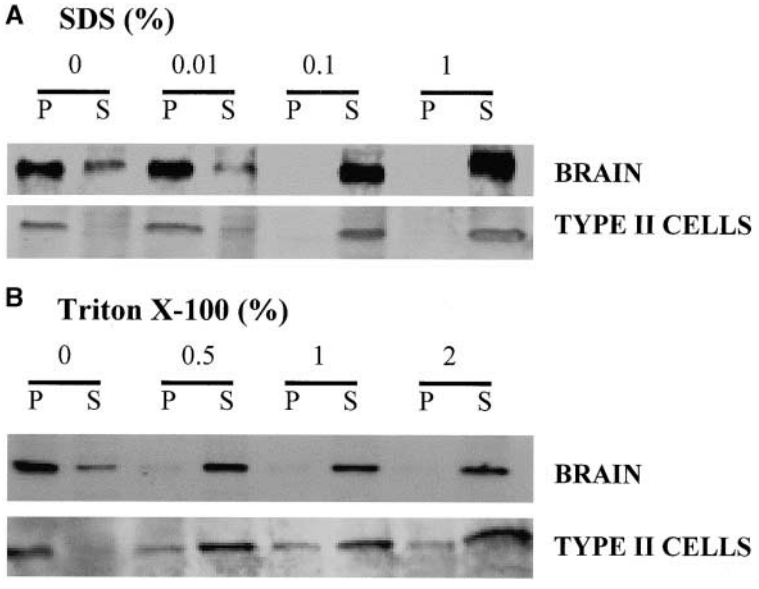

Figure 5. Type II cell α-SNAP is more resistant to Triton X-100 than brain's. Quantities of 5 mg/ml brain or type II cell lysate in homogenization buffer were treated with varying concentrations of SDS (0%, 0.01%, 0.1%, and 1%) or Triton X-100 (0%, 0.5%, 1%, and 2%) and incubated for 30 min on ice. The lysates were centrifuged at 100,000 × *g* for 1 h to separate supernatant (S) (cytosolic plus extracted proteins) from pellet (P) (membrane or detergent resistant proteins). Both supernatant and pellet were analyzed for the presence of NSF and α-SNAP using SDS-PAGE and Western blot. The results shown were representative of two (brain) or three (type II cells) independent experiments.

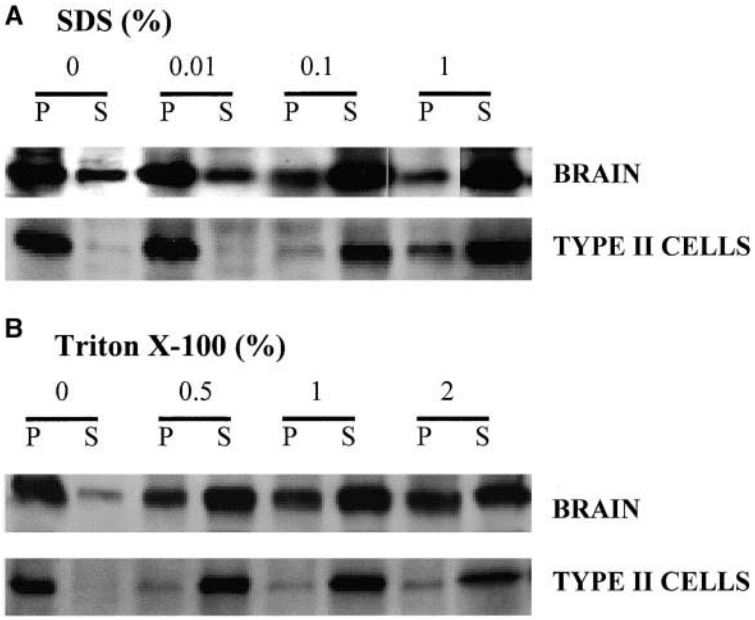

Figure 6. α-SNAP in isolated cell membranes is more resistant to Triton X-100 and SDS extraction than those in cell lysates. Brain or type II cell lysates were centrifuged at 100,000 × *g* for 1 h to separate the cytosolic from the membrane fraction. The pellet (membrane fraction) was treated with varying concentrations of Triton X-100 (0%, 0.5%, 1%, and 2%) or SDS (0%, 0.01%, 0.1%, 1%) for 30 min on ice. The samples were then centrifuged at 100,000 × *g* for 1 h to separate the extracted (supernatant [S]) from the detergent-resistant (pellet [P]) proteins. Both supernatant and pellet were analyzed for the presence of α-SNAP using SDS-PAGE and Western blot. The results shown were representative of two (brain) or three (type II cells) independent experiments.

teins. The leakage-insensitive proteins were detected by Western blot. As shown in Figure 8A, no apparent leakage of α-SNAP was observed after a 10-min permeabilization. However further 30- or 60-min incubation in the absence of β-escin resulted in 39 ± 11 and 47 ± 12% of α-SNAP leakage ($n = 3$, $P < 0.05$). On the other hand, the quantity of NSF did not significantly decrease under these conditions.

Such β-escin–permeabilized type II cells were reconstituted with purified recombinant NSF and α-SNAP and assayed for Ca^{2+}-stimulated surfactant secretion. The purity of the recombinant NSF and α-SNAP used in this study is shown in Figure 7B. The addition of exogenous NSF into the permeabilized cells slightly affected surfactant secretion, but this effect was not statistically significant. (Figure 8C). The addition of α-SNAP caused a significant increase in surfactant secretion in a dose-dependent manner.

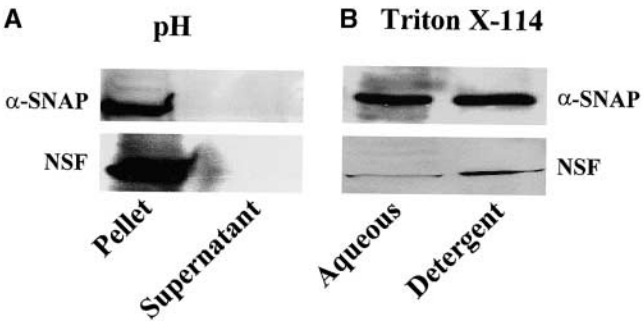

Figure 7. Alkaline condition does not affect the distribution of α-SNAP and NSF, whereas Triton X-114 partitioned them into hydrophobic and hydrophilic components. (*A*) Type II cells were treated with 0.1 M Na_2CO_3, pH 12 for 1 h at 4°C and then centrifuged at 100,000 × *g* for 1 h. The pellet (P) and supernatant (S) fraction were analyzed by SDS-PAGE and Western blot. (*B*) Type II cell lysate was partitioned using Triton X-114. Proteins in the upper aqueous phase (hydrophilic proteins) and lower detergent phase (hydrophobic proteins) were evaluated for the presence of NSF and α-SNAP by SDS-PAGE and Western blot. The results shown were representative of two independent experiments.

α-SNAP Antisense Oligonucleotide Inhibits Surfactant Secretion

Antisense oligonucleotides can interfere with both transcription and translation of target mRNA, and thus decrease the level of proteins expression (33). To further examine the role of α-SNAP in surfactant secretion, we suppressed the expression of endogenous α-SNAP in type II cells by incubating the cells with α-SNAP antisense oligonucleotide in the presence of the transfection reagent, FuGene 6 overnight. The corresponding sense oligonucleotide was used in parallel as control. Surfactant secretion and α-SNAP protein level was determined. As shown in Figure 9, α-SNAP antisense oligonucleotide treatment decreased surfactant secretion to 68 ± 5.4% of the untreated cells ($n = 4$, $P < 0.05$). The sense oligonucleotide had no significant effects. Western blot analysis showed a decrease of α-SNAP protein level in the antisense oligonucleotide–treated cells compared with the untreated cells. Quantitation of the bands by densitometry revealed that the α-SNAP protein decreased to 62 ± 17% of the untreated cells ($n = 3$, $P < 0.05$). The slightly higher level of α-SNAP proteins in the sense oligonuleotide–treated cells than in the untreated cells may be due to the variations of protein determination and protein loading. To ensure that the oligonucleotide effect observed was not due to FuGene 6 toxicity, we treated cells with FuGene 6 and tested for cell viability using trypan blue dye exclusion method. The result indicated that the viability of the treated and untreated cells was the same (93–95%). Neither the cell morphology nor the PC secretion was affected during FuGene 6 treatment (data not shown).

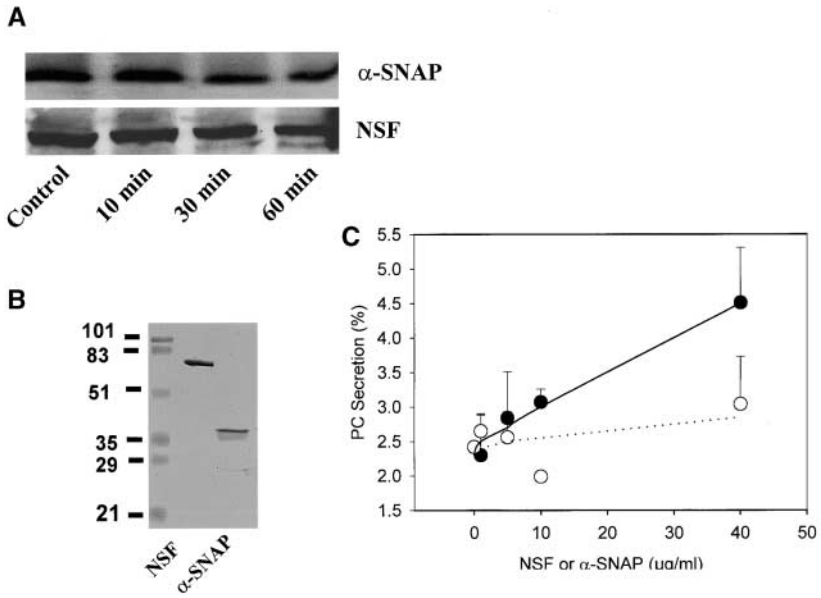

Figure 8. Reconstitution of surfactant secretion with exogenous α-SNAP. (*A*) α-SNAP partially leaks out from the β-escin–permeabilized type II cells: Alveolar type II cells were permeabilized for 10 min in permebilization buffer containing 40 μM β-escin. Cells were washed and further incubated in permeabilization buffer for 0, 20, and 50 min. Cells were then lyzed in hypotonic lysis buffer and analyzed by SDS-PAGE and Western blot using antibodies against α-SNAP and NSF. The results shown are representative of three independent experiments. (*B*) Purity of recombinant NSF and α-SNAP as analyzed by SDS-PAGE and stained with commassie blue. (*C*) α-SNAP increases lung surfactant secretion from permeabilized type II cells: Type II cells were permeabilized with 40 μM β-escin for 10 min. Recombinant NSF (1–40 μg/ml) or α-SNAP (1–40 μg/ml) was added and incubated for 20 min. Subsequently, 0.5 μM Ca^{2+} was added to stimulate surfactant secretion for 30 min. Lung surfactant secretion was expressed as a percentage of radioactivity in the medium divided by that in the medium and the cell. Data shown are mean ± SE ($n = 3$). *Filled circles*, α-SNAP; *open circles*, NSF.

Discussion

Regulation of exocytosis in alveolar type II cells differs in some aspects from exocytosis in synapse or endocrine cells, because lung surfactant secretion is a relatively slow process compared with neurotransmitter or hormone secretion. It is therefore not obvious that the same mechanism of exocytosis is used in both cases. Even though the late steps of exocytotic pathway in a variety of secretory cells appear to involve the common proteins, some differences have been noted with respect to early stages of the process, especially targeting, docking, and priming. It was therefore important to investigate whether SNARE model is an appropriate mechanism for lamellar body exocytosis. In the present study, we examined the distributions and possible roles of α-SNAP and NSF in alveolar type II cells. We demonstrated for the first time that α-SNAP and NSF are present in type II cells and that they are primarily associated with membrane using Western blot and immunocytochemistry. We also provided strong evidence that α-SNAP participates in exocytosis in type II cells using reconstitution and antisense oligonucleotide approaches.

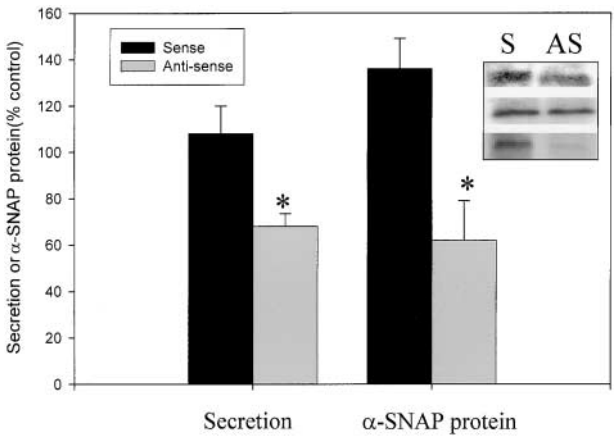

Figure 9. Inhibition of surfactant secretion by an antisense oligonucleotide complementary to α-SNAP in type II cells. Type II cells (1×10^6) were treated with α-SNAP antisense (*grey bars*) or sense (*black bars*) oligonucleotide (1 μM) in the presence of the transfection reagent, FuGene 6. The cells were washed and stimulated with 1 μM PMA to enhance surfactant secretion. Surfactant secretion was expressed as a percentage of the untreated cells. Data shown are mean ± SE ($n = 4$), $^*P < 0.05$ versus sense-treated or untreated cells. α-SNAP protein levels were determined by Western blot and densitometry. The results were expressed as a percentage of the untreated cells. Data shown are mean ± SE ($n = 3$), $^*P < 0.05$ versus sense-treated or -untreated cells. The insert shows Wesrtern blots. S, sense-treated, AS, antisense-treated.

The SNARE hypothesis model suggests that the formation of NSF/α–SNAP/SNARE complex activates NSF ATPase. This results in the conversion of NSF into an ADP-bound state that is unable to interact with α-SNAP/SNAREs, and thus releases NSF and α-SNAP from the complex (18, 19). ATP hydrolysis by NSF also leads to the disassembly of SNARE complex (16). This model implies that NSF and α-SNAP are cytosolic proteins. In agreement with this hypothesis, Kiraly-Borri and coworkers reported the presence of these proteins in the cytosolic component of brain and pancreatic β-cells (34). However, extremely low levels of NSF and α-SNAP were observed in the cytosol of liver or chromaffin cells (34, 35). Those results depicted that the localizations of these proteins are tissue or cell specific. On the same context, we detected most of NSF and α-SNAP in the membrane of type II cells by immunocytochemistry and by Western blot of subcellular fractions. Treatment of intact type II cells with ATP or PMA to stimulate fusion of lamellar body with plasma membrane or

treatment of membrane fractions with ATP to effect priming yielded no change in their distribution. Banaschewski and colleagues observed that α-SNAP and NSF were not released from the membrane by Mg^{2+}ATP (35). Steel and coworkers also showed that the treatment of clathrin-coated vesicles with Mg^{2+}ATP did not result in the release of NSF (36). The fact that both α-SNAP and NSF are not released from the membrane under priming conditions raises the possibility that these proteins may interact directly with membrane lipids or proteins rather than SNAREs in type II cells. Interaction of α-SNAP and NSF with other proteins such as Rab 6, GluR2, or β-arrestin has been reported (37).

Although the suspicion that NSF and α-SNAP might have a hydrophobic pool capable of traversing the membrane in the absence of SNAREs has been reported (38), the possibility that NSF and α-SNAP might have some hydrophobic aspects has been overlooked. We investigated this possibility by subjecting type II cell membrane to high pH that extract peripheral membrane proteins and Triton X-114 treatment known to separate integral membrane proteins from soluble or peripheral membrane proteins. High pH totally failed to extract α-SNAP and NSF in type II cells. The fact that both proteins were detected in the detergent phase of Triton X-114 indicates that they have a hydrophobic component. Hydrophobic proteins have the capability of traversing cell membranes, hence it is likely that both α-SNAP and NSF have some sort of direct insertion into the membrane of type II cells. The hydrophobic pool of these proteins may therefore not require the assistance of the SNAREs to interact with the membrane. Another possibility is that Triton X-114 or high pH condition does not disrupt the association of α-SNAP and NSF with the SNARE complex, which partition into the detergent phase. In agreement with our data is the observation that a substantial amount of NSF bind to endosomal membrane in the absence of α-SNAP (39). Furthermore, Steel and associates demonstrated that NSF and α-SNAP have the capacity to bind membranes on which SNAREs have been inactivated, and that α-SNAP is capable of binding to liposomes (38). These observations should instigate a search for alternative ways by which these proteins interact with membranes during exocytosis.

Permeabilized cells are known to lose cytosolic components and their secretory response (40). We took advantage of this phenomenon to investigate whether α-SNAP and NSF have a role in surfactant secretion in type II cells. Our results demonstrate that ~ 53% α-SNAP remain in the β-escin–permeabilized type II cells. However, the majority of NSF did not leak out, probably because it forms a hexamer that is too large to pass through the pores created by β-escin. The amount of α-SNAP leaked out of type II cells during permeabilization is not consistent with the quantity we detected in the cytosolic fraction of lyzed cells. It is possible that more α-SNAP disassociates from the membrane as trace amount of cytosolic α-SNAP leak out, thereby shifting the equilibrium from membrane bound form to cytosolic form. Another possibility is that β-escin may extract α-SNAP from type II cell membrane, although we used a low concentration of β-escin in the permeabilization experiment. Addition of exogenous NSF did not cause major change of surfactant secretion, whereas α-SNAP significantly increased Ca^{2+}-dependent surfactant secretion. Such reconstitutive effects of α-SNAP rather than NSF have also been reported for insulin secretion in Streptolysin O–permeabilized HIT-T15 cells (a pancreatic β-cell line) (34) and catecholamine release in digitonin-permeabilized chromaffin cells (24). Inability of NSF to stimulate surfactant secretion from permeabilized type II cells suggests that the cells may already contain adequate amount of membrane-bound NSF, and hence are not capable of using an exogenous source, whereas α-SNAP might function by acting on the leakage-insensitive NSF. Because NSF detected in type II cells was predominantly membrane-associated, it raises the question: why would additional α-SNAP be necessary to cause an increase in surfactant secretion if NSF is already recruited into the 20S complex by endogenous α-SNAP? Does this imply that the membrane bound NSF has an extra binding site for α-SNAP, or is there an alternative mechanism by which these two proteins independently interact with membrane? We favor the latter, because α-SNAP can directly interact with membrane lipid (38).

Synthetic phosphorothioate modified antisense oligonucleotides have proved to be a powerful technique for inhibiting many cell function both *in vivo* and *in vitro* (41, 42). These oligonucleotides are resistant to nuclease degradation (43) and hence are an important tool for inhibition of gene expression and protein expression without enzymatic interferences. The antisense oligonucleotides have been used by several authors to study the exocytotic function of SNAREs (25, 44, 45). We used this technique to further confirm the role of α-SNAP in lung surfactant secretion. Indeed, α-SNAP antisense oligonucleotide–treated type II cells exhibited a significant decrease in protein expression and surfactant secretion. A similar result was observed in insulin secretion by MIN6 cells (25)

In summary, we identified α-SNAP and NSF and established their distributions in type II cells. We also provided evidence to support a functional role of α-SNAP in exocytosis of lung surfactant by alveolar type II cells.

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