# Syntaxin 2 and SNAP-23 Are Required for Regulated Surfactant Secretion†

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ABSTRACT: The secretion of lung surfactant in alveolar type II cells is a complex process involving the fusion of lamellar bodies with the plasma membrane. This process is somewhat different from the exocytosis of hormones and neurotransmitters. For example, it is a relatively slower process, and lamellar bodies are very large vesicles with a diameter of ~1 $\mu$m. SNARE proteins are the conserved molecular machinery of exocytosis in the majority of secretory cells. However, their involvement in surfactant secretion has not been reported. Here, we showed that syntaxin 2 and SNAP-23 are expressed in alveolar type II cells. Both proteins are associated with the plasma membrane, and to some degree with lamellar bodies. An antisense oligonucleotide complementary to syntaxin 2 decreased its mRNA and protein levels. The same oligonucleotide also inhibited surfactant secretion, independent of secretagogues. A peptide derived from the N-terminus of syntaxin 2 or the C-terminus of SNAP-23 significantly inhibited $Ca^{2+}$- and GTP$\gamma$S-stimulated surfactant secretion from permeabilized type II cells in a dose-dependent manner. Furthermore, introduction of anti-syntaxin 2 or anti-SNAP-23 antibodies into permeabilized type II cells also inhibited surfactant release. Our results suggest that syntaxin 2 and SNAP-23 are required for regulated surfactant secretion.

Early autoradiographic studies using labeled precursors of surfactant indicated that pulmonary surfactant components are synthesized in the endoplasmic reticulum of alveolar type II cells (*1*). Newly synthesized surfactant lipids and proteins are transported directly from the trans-Golgi bodies to immature lamellar bodies and multivesicular bodies, respectively. Fusion of these two vesicles results in the formation of composite lamellar bodies, which are eventually converted into mature lamellar bodies. This is a critical step in the biosynthesis and secretion of surfactant as it accomplishes the mixing of surfactant components. Mature lamellar bodies are then targeted and fused with the plasma membrane, and the surfactant content is released into the alveolar air space. After secretion, surfactant forms a monolayer at the air−water interface and thereby reduces surface tension.

Surfactant secretion is regulated by surfactant secretagogues such as $\beta$-adrenergic agonists, purinoceptor agonists, $Ca^{2+}$ ionophore A23187, vasopressin V1, arachidonic acid and its metabolites, histamine, and serum lipoproteins (*2*−*4*). These agonists activate protein kinases A and C and $Ca^{2+}$- and calmodulin-dependent kinase. The downstream effects of these protein kinases may include the phosphorylation of the proteins directly involved in trafficking, targeting, docking, and fusion of lamellar bodies with the plasma membrane. We have previously shown that annexin II participates in the fusion of lamellar bodies with the plasma membrane (*5*−*7*). However, relatively little is known about the molecular mechanisms of this process.

The SNARE[1] hypothesis was proposed by Rothman and colleagues to explain vesicle targeting, docking, and fusion (*8*). The vesicle SNARE (v-SNARE), VAMP, is localized on the secretory vesicles, whereas target SNAREs (t-SNARE), including syntaxin and SNAP-25 or SNAP-23, are localized on the plasma membrane. During regulated secretion, the vesicles are targeted and docked onto the plasma membrane. The interaction of t- and v-SNAREs via a coiled-coil structure leads to the formation of the SNARE core complex and membrane fusion (*9*, *10*). The binding of α-SNAP to the SNARE core complex drives a conformational change that enables NSF to be recruited. NSF is an ATPase capable of binding and hydrolyzing ATP. The hydrolysis of ATP provides energy that disrupts the complex to recycle the SNARE for another round of fusion (*11*).

Syntaxins 1−4 have been detected on the plasma membrane of various cells (*12*). Ashton and Dolly demonstrated that treatment of rat synaptosomes with botulinum toxins removed the majority of intact syntaxin 1A/1B and inhibited $K^+$ depolarization-induced noradrenaline secretion (*13*). Chen et al. (*14*) reported that syntaxins 2 and 4 work in concert with SNAP-23 to stimulate the release of lysosomes from streptolysin O-permeabilized platelets. Syntaxin 4 is essential for translocation of glucose transporter GLUT4 to the plasma membrane of glucose responsive cells (*15*). The homozygotic disruption of the syntaxin 4 gene results in early embryonic

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[1] Abbreviations: NSF, soluble *N*-ethylmaleimide-sensitive fusion protein; SNAP, NSF attachment protein; SNARE, SNAP receptor; v-SNARE, vesicle SNARE; t-SNARE, target SNARE; PMA, phorbol 12-myristate 13-acetate; MEM, Eagle's minimal essential medium; FBS, fetal bovine serum; ECL, enhanced chemiluminescence; TBS, Tris-buffered saline; oligo, oligonucleotide; PBS, phosphate-buffered saline.

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lethality (*16*). However, heterozygous knockout mice, with or without syntaxin 4, had normal viability with no significant impairment in growth, development, or reproduction. These mice manifested impaired glucose tolerance with a 50% reduction in whole-body glucose uptake due to a defect in insulin-stimulated GLUT4 translocation.

SNAP-25 is a neuron-specific protein encoded by a 6.5 kb gene. Alternative splicing leads to the expression of two isoforms, SNAP-25 a and b (*17*), with a difference of nine amino acid residues in the palmitoylation domain. SNAP-23 and syndet are the ubiquitously expressed SNAP-25 isoforms (*18*, *19*). A newly identified family member, SNAP-29, is localized on the intracellular membranes (*20*, *21*). Unlike syntaxin, the SNAP-25 family is associated with the membrane through palmitoylation that occurs in a conserved cysteine rich sequence found in the linker at the center of N- and C-terminal helices (*22*, *23*). The cleavage of SNAP-25 by botulinum toxin A inhibits synaptic vesicle exocytosis (*24*). Botulinum toxin E cleaves SNAP-25 at the Ile181−Gly197 sequence and completely blocks $Ca^{2+}$-dependent exocytosis in ATP-primed PC-12 cells (*25*). SNAP-23 also has been shown to play a role in exocytosis of other cells (*14*, *26*−*32*).

Although SNAREs have been implicated in regulated exocytosis in a variety of cells, including pancreatic $\beta$ cells (*33*), eosinophils (*34*), chromaffin cells (*35*), platelets (*14*, *27*, *36*), etc., their role in surfactant secretion has not been investigated. We hypothesized that SNAREs are part of the molecular machinery required for surfactant secretion. In this paper, we reported the presence of syntaxin 2 and SNAP-23 in alveolar type II cells. We also examined their subcellular localization and established functional roles of syntaxin 2 and SNAP-23 in surfactant secretion using antisense oligonucleotide, peptide inhibition, and antibody neutralization.

## EXPERIMENTAL PROCEDURES

*Materials.* Leupeptin, aprotinin, phorbol 12-myristate 13-acetate (PMA), and ATP were obtained from Sigma (St. Louis, MO). Horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit IgG, gelatin, and prestained molecular mass standards were obtained from Bio-Rad (Melville, NY). Nitrocellulose membranes were from Scheilcher & 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 (ECL) reagents were from Amersham Pharmacia Biotech (Arlington Heights, IL). Anti-syntaxin 2 antibodies were obtained from StressGen (Victoria, BC). Antibodies against SNAP-25, SNAP-23, and syntaxin 1 were obtained from Synaptic Systems GmbH (Göttingen, Germany). Anti-syntaxin 3 antibodies were a generous gift from M. Knepper [National Heart, Lung, and Blood Institute (NHLBI), National Institutes of Health, Bethesda, MD]. Anti-syntaxin 4 antibodies were from Transduction Laboratory (Lexington, KY). FITC-conjugated anti-rabbit secondary antibodies were from Jackson Immunoresearch Laboratories (West Grove, PA). FuGene 6 was from Roche Molecular Biochemicals (Indianapolis, IN). Phosphorothioate oligonucleotides for syntaxin 2 were synthesized by MWG-Biotech (High Point, NC). Syntaxin 2 and SNAP-23 antibodies used for function studies were raised in rabbits using synthetic peptides corresponding to N-terminal amino acids 1−19 of rat syntaxin 2 (MRDRLPDLTACRKS-DDGDN) or C-terminal amino acids 199−210 of rat SNAP-23 (CANTRAKKLIDS) (Genmed Synthesis Inc., South San Francisco, CA). These antibodies were affinity-purified using peptide-conjugated beads. Scrambled peptides [syntaxin 2 (DLPCDGADRSMLRTDNKDR) and SNAP-23 (NIRKSALTKCAD)] were synthesized by Gemed Synthesis Inc. TRI reagents were from MRC (Cincinnati, OH). Taq polymerase, DNase, and the ImProm-II reversed transcription system were from Promega (Madison, WI). The pGEX1$\lambda$ T, pGEX 2, or pGEX-KG expression vectors encoding thrombin-cleavable GST fusion proteins were as follows: cytoplasmic domains of syntaxin 1A (amino acids 1−265), syntaxin 2 (amino acids 1−265), syntaxin 3 (amino acids 1−263), and syntaxin 4 (amino acids 1−272) (*37*) (a kind gift of V. M. Olkkonen, National Public Health Institute, Helsinki, Finland) and full-length SNAP-23 and -25 (38) (a kind gift of A. Klip, The Hospital for Sick Children, Toronto, ON). All recombinant proteins were expressed in *Escherichia coli* BL-21(DE3)pLysS and affinity-purified using glutathione−Sepharose beads.

*Isolation and Culture of Alveolar Type II Cells.* Type II cells were isolated from the lungs of adult male Sprague-Dawely rats (180−200 g) according to the method of Dobbs et al. (*39*) as previously described by Liu et al. (*5*). Lungs were lavaged and digested with elastase at 37 °C. Lung tissue was then chopped three times using a tissue chopper. The cell suspension was filtered through a series of filters (pore sizes of 160, 37, and 15 $\mu$m), and the filtrate was centrifuged at 1100 rpm for 10 min. The cells were resuspended in MEM and panned for 1 h on a rat IgG-coated bacteriological plate to remove alveolar macrophages. The unattached type II cells were centrifuged, resuspended in MEM containing 10% FBS, and counted using a hemocytometer. The viability of isolated cells was >93% as evaluated by trypan blue exclusion. Isolated type II cells were either directly lysed for the SNARE isoform identification or plated on 35 mm plastic tissue culture dishes and cultured overnight for the surfactant secretion assay. The purity of overnight cultured type II cells was greater than 95% as evaluated by the modified Papanicolaou staining.

*Lamellar Body Purification.* Lamellar bodies were isolated from rat lung by the upward flotation method described by Chander et al. (*40*). 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, and 0.2 M), and centrifuged at 80000*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 20000*g* for 30 min. The pellet containing lamellar bodies was resuspended 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 the method of Maeda et al. (*41*). Rat lung was homogenized in 10 mL of buffer B [10 mM $NaP_i$ (pH 7.4), 30 mM NaCl, 1 mM $MgCl_2$, 5 $\mu$M PMSF, 0.02% $NaN_3$, 10 $\mu$g/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, and 1.2 M). The

gradient was centrifuged at 95000*g* for 60 min and plasma membrane collected from the interface between 0.9 and 1.2 M. This fraction was diluted to 0.32 M sucrose with cold buffer A (buffer B without 0.32 M sucrose) and centrifuged at 95000*g* for 30 min. The pellet containing plasma membrane was resuspended in buffer B for further analyses.

*Western Blot Analysis.* Protein samples were resolved via 10% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) under reducing conditions and electrophoretically transferred to a nitrocellulose membrane. The blot was transiently stained with Ponceau S to monitor the 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 in TTBS (TBS with 0.05% Tween 20) containing 1% gelatin for 3−12 h and then incubated with secondary antibodies (horseradish peroxidase-conjugated IgG, 1:5000 dilution) for 1 h. Finally, the blot was developed with ECL reagents.

*Immunocytochemistry.* Alveolar type II cells were seeded on a coverslip at a density of $1.2-3.75 \times 10^5$ and incubated overnight in MEM with 10% FBS in an atmosphere of 5% $CO_2$ at 37 °C. Cells were washed three times with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde, and permeabilized with 0.5% Triton X-100 for 20 min. The fixed cells were incubated for 30 min with 0.5% bovine serum albumin and 2% goat serum in PBS to block nonspecific binding. The cells were incubated with anti-syntaxin 2 (1:50) or anti-SNAP-23 (1:50) antibodies in PBS at 4 °C for 1 h. The coverslips were washed three times with PBS and incubated with FITC-conjugated goat anti-rabbit IgG (1:200) for 1 h. After being washed three times, the coverslips were 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−drypton laser.

*RT-PCR.* Total RNA was extracted using TRI reagents. After being treated with DNase, RNA (2 $\mu$g) was reverse-transcripted into cDNAs using the ImProm-II reverse transcription system. The cDNAs were amplified with Taq DNA polymerase and specific primers. The primer sequences were as follows: syntaxin 1, 5′-GGATCATCATGGACTCCAGC-3′ (forward) and 5′-TATCCAAAGATGCCCCCGATG-3′ (reverse); syntaxin 2, 5′-GTTTGTCGAGACTCAGGGT-GAAAT-3′ (forward) and 5′-TGGCTTTCTTAGTCTCTTC-CTTGG-3′ (reverse); and syntaxin 3, 5′-AGCCATCTTCACT-TCTGGGA-3′ (forward) and 5′-GCCCAACGGACAATCC-AATAATC-3′ (reverse). PCR was performed using the following conditions: 1 cycle of 95 °C for 2 min, 25 cycles of 94 °C for 30 s, 53 °C for 1 min, and 72 °C for 30 s, followed by a final elongation step of 72 °C for 5 min.

*Syntaxin 2 Antisense Oligonucleotide and Surfactant Secretion.* The antisense phosphorothioate oligonucleotides (oligo) complementary to syntaxin 2 (5′-CAG CCG GTC CCG CAT-3′) surrounding the initiation codon and corresponding sense oligo were synthesized on the basis of the rat syntaxin 2 cDNA sequence. The transfection agent, FuGene 6, was used to facilitate the efficient transfer of oligo into cells. MEM (100 $\mu$L) was incubated with 6 $\mu$L of FuGene 6 for 5 min at room temperature. Antisense or sense oligo (0.1−2.5 $\mu$M) was added to the mixture and further incubated for 15 min. The mixture was added dropwise to type II cells ($1 \times 10^6$) at the time of plating. After overnight culture, cells were washed six times with MEM and stimulated with 1 $\mu$M PMA, 10 $\mu$M terbutaline, 0.1 $\mu$M $Ca^{2+}$ ionophore A23187, or 1 mM ATP for 2 h to enhance surfactant secretion. At the end of the incubation, surfactant secretion was assessed as described above. Similar experiments were performed to determine mRNA and protein levels of syntaxin 2 in antisense or sense oligo-treated cells except that 100 mm dishes instead of 35 mm dishes were used for this purpose. The mRNA and protein levels were determined by RT-PCR and Western blotting, respectively.

*Inhibition of Surfactant Secretion from Permeabilized Type II Cells by Syntaxin 2 and SNAP-23 Peptides and Antibodies.* Type II cells ($1-2 \times 10^6$) were prelabeled overnight with MEM containing 10% FBS and 1 $\mu$Ci of [$^3$H]choline. The dishes were washed three times with MEM and two times with 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 the incubation, the medium was removed and cells were incubated for 10 min in fresh permeabilization buffer containing 40 $\mu$M $\beta$-escin to permeabilize the cells as previously described (*5*). Fresh permeabilization buffer with or without peptides (0−120 $\mu$g/mL) or antibodies (10 $\mu$g/mL) against syntaxin 2 or SNAP-23 was added. One set of dishes was moved to establish the time zero value. For the remaining dishes, 0.5 $\mu$M $Ca^{2+}$, 0−120 $\mu$M GTP$\gamma$S, or both were added to stimulate PC secretion for 30 min. Lipids in the medium and cells were extracted and analyzed for radioactivity. Secretion was expressed as [disintegrations per minute in medium/(disintegrations per minute in medium + disintegrations per minute in cells)] × 100% and corrected by subtracting the time zero value.

*Data Analysis.* All secretion studies were carried out in duplicate, and the individual results were averaged. Statistical significance was evaluated by the Student's *t* test. The level of significance was taken to be a $P$ of $<0.05$.

## RESULTS

*Identification and Subcellular Localization of t-SNAREs in Alveolar Type II Cells.* SNAREs are crucial for exocytosis in various cell systems. Their presence in type II cells and their role in surfactant secretion have not been reported. As a first step in establishing the roles of t-SNAREs in lung surfactant secretion, we investigated their presence in alveolar type II cells by Western blotting. As shown in Figure 1A, syntaxins 2 and 3 and SNAP-23 were detected in type II cell lysates. Conversely, syntaxin 1 and SNAP-25 were present in very small quantities, while syntaxin 4 was undetectable.

Using a subcellular fractionation technique followed by Western blotting, we determined whether syntaxin 2 and SNAP-23 are present in lamellar bodies and plasma membrane. As expected, SNAP-23 and syntaxin 2 were present on the plasma membrane isolated from type II cells. However, a large portion of SNAP-23 and a small amount of syntaxin 2 were also detected on the lamellar bodies (Figure 1B). Immunocytochemistry coupled with confocal fluorescent microscopy showed that the staining pattern of syntaxin 2 and SNAP-23 was cortical in type II cells (Figure 2), consistent with their plasma membrane localization as

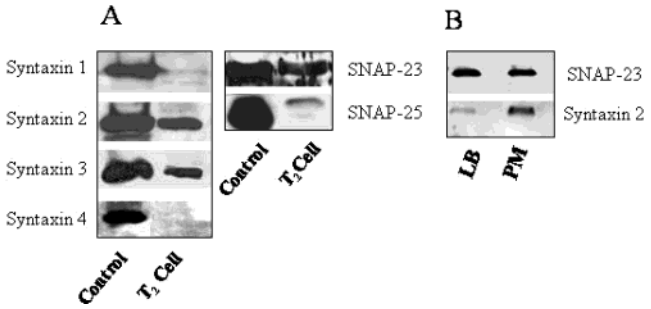


FIGURE 1: Syntaxin 2 and SNAP-23 are present in alveolar type II cells. (A) Type II cells isolated from rat lung were lysed in hypotonic lysis buffer containing protease inhibitors. The cell lysate (35 $\mu$g) was analyzed for the presence of various t-SNARE isoforms using SDS−PAGE and Western blotting. Brain homogenate (35 $\mu$g) was used as a positive control. For syntaxin 4, human endothelial cell lysate (10 $\mu$g) was used as a positive control. (B) Lamellar bodies (LB) were isolated from rat lung homogenate and plasma membrane (PM) from rat type II cells. LB and PM (25 $\mu$g of protein each) were analyzed by Western blotting.

revealed by Western blotting. SNAP-23 staining also showed the appearance of granules, presumably lamellar bodies. The control without primary antibodies did not generate signals.

*Syntaxin 2 Antisense Oligonucleotide Inhibits Stimulated Surfactant Secretion.* An antisense oligonucleotide (oligo) complementary to rat syntaxin 2 was introduced into alveolar type II cells to inhibit the expression of the syntaxin 2 protein. Western blot analysis revealed that the antisense oligo decreased the syntaxin 2 protein level (Figure 3A). RT-PCR indicated a reduction in the syntaxin 2 mRNA level in the antisense oligo-treated cells compared to the sense oligo-treated cells (Figure 3B). Syntaxin 2 antisense oligo did not affect mRNA levels of syntaxins 1 and 3, thereby confirming the specificity of the oligo. Furthermore, syntaxin 2 antisense oligo inhibited PMA-stimulated surfactant in a dose-dependent manner, whereas the sense oligo had no significant effects on the secretion (Figure 4).

Various secretagogues can stimulate surfactant secretion through different signal transduction pathways. To test whether the inhibition of surfactant secretion by syntaxin 2 antisense oligo is pathway-specific, antisense and sense oligo-treated cells were stimulated with ATP, terbutaline, and $Ca^{2+}$ ionophore A23187. As shown in Figure 5, the antisense oligo inhibited all the secretagogue-stimulated secretion, suggesting that syntaxin 2 is required for a common step during regulated surfactant secretion.

*Syntaxin 2 and SNAP-23 Peptides and Antibodies Inhibit Surfactant Secretion from Permeabilized Cells.* We have previously shown that $Ca^{2+}$ modestly stimulaties surfactant secretion from permeabilized type II cells (*5*). To achieve maximal stimulation, we further examined the effect of a combination of $Ca^{2+}$ and GTP$\gamma$S on the permeabilized cells. The level of surfactant secretion increased with increasing concentrations of GTP$\gamma$S and appeared to reach saturation at 20 $\mu$M (data not shown). Inclusion of 0.5 $\mu$M $Ca^{2+}$ further increased the level of GTP$\gamma$S-stimulated surfactant secretion. The level of secretion was increased by 137 ± 15 and 202 ± 27% ($n$ = 3) with 20 $\mu$M GTP$\gamma$S and 0.5 $\mu$M $Ca^{2+}$ with 20 $\mu$M GTP$\gamma$S, respectively.

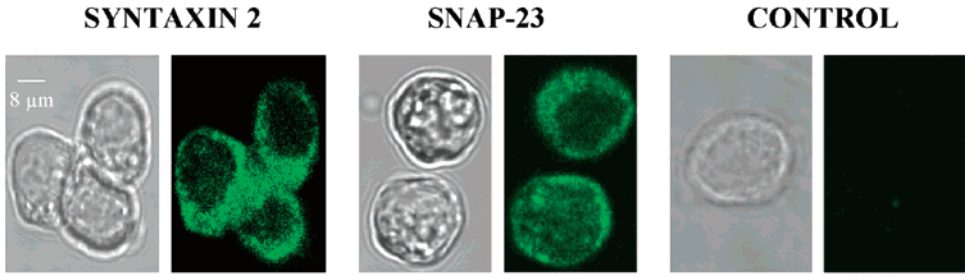


FIGURE 2: Immunocytochemical detection of t-SNAREs in type II cells. Paraformaldehyde-fixed alveolar type II cells were permeabilized with 0.5% Triton X-100 and probed with anti-SNAP-23 or anti-syntaxin 2 antibodies, and FITC-conjugated goat anti-rabbit IgG. The control was treated the same way except that primary antibodies were omitted.

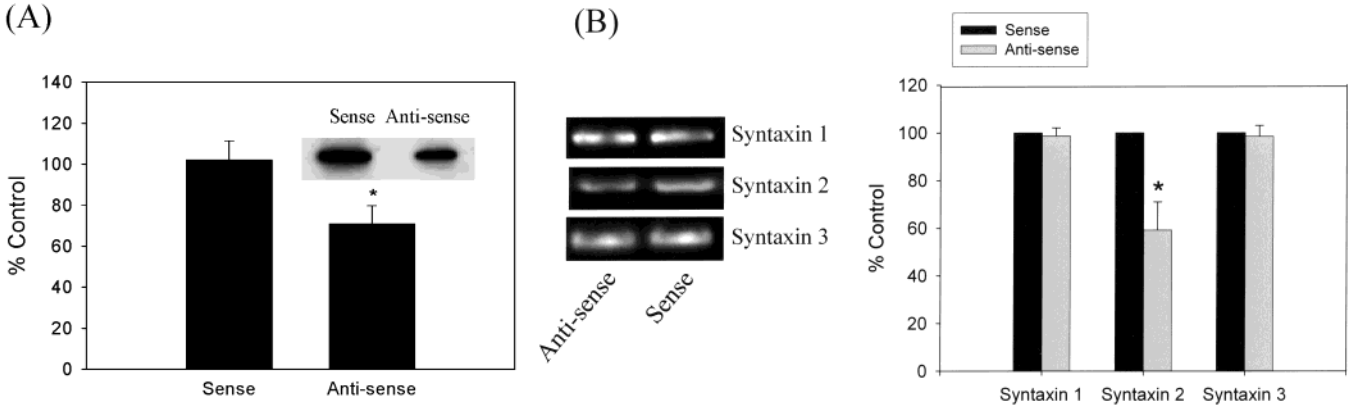


FIGURE 3: Syntaxin 2 protein and mRNA levels in antisense oligo-treated type II cells. Type II cells ($1 \times 10^6$) were treated with syntaxin 2 antisense or sense oligo (0.5 $\mu$M) in the presence of the transfection reagent FuGene 6. The cells were washed and stimulated with 1 $\mu$M PMA. (A) The protein levels of syntaxin 2 were determined by Western blotting followed by densitometry. Data are means ± the standard error ($n$ = 3). The asterisk denotes a $P$ of <0.05 vs sense-treated. The inset is a representative of the Western blots. (B) mRNA levels of syntaxins 1−3 were determined by RT-PCR. Data are means ± the standard error ($n$ = 3). The asterisk denotes a $P$ of <0.05 vs sense-treated. A representative RT-PCR is shown.

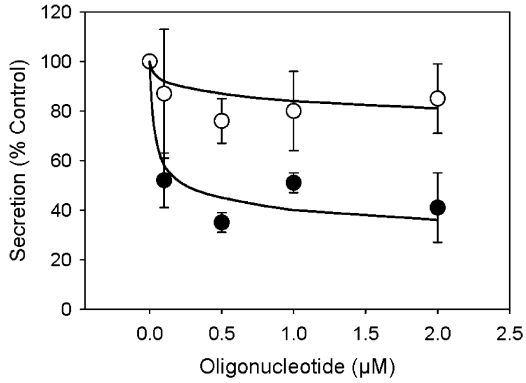


FIGURE 4: Dose-dependent inhibition of PMA-stimulated surfactant secretion by syntaxin 2 antisense oligo. Type II cells ($1 \times 10^6$) were treated with syntaxin 2 antisense or sense oligo (0.1−2 $\mu$M) in the presence of the transfection reagent FuGene 6. The cells were stimulated with 1 $\mu$M PMA for 2 h. Surfactant secretion was expressed as a percentage of the control cells (stimulated with PMA in the absence of oligos). The actual secretion value of the control cells was 4.26 ± 0.35%. Data are means ± the standard error ($n$ = 3): (●) antisense oligo and (○) sense oligo.

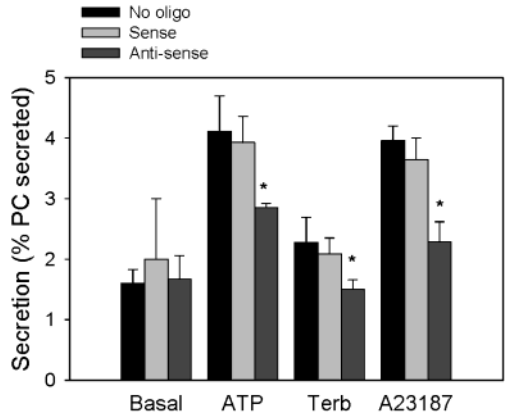


FIGURE 5: Surfactant secretion stimulated by various secretagogues is inhibited by a syntaxin 2 antisense oligo. Type II cells ($1 \times 10^6$) were treated with syntaxin 2 antisense or sense oligo (0.5 $\mu$M) in the presence of the transfection reagent FuGene 6. The cells were stimulated with 1 mM ATP, 10 $\mu$M terbutaline (Terb), or 0.1 $\mu$M $Ca^{2+}$ ionophore A23187 for 2 h. Surfactant secretion was expressed as [disintegrations per minute in medium/(disintegrations per minute in medium + disintegrations per minute in cells)] × 100%. Data are means ± the standard error ($n$ = 3). The asterisk denotes a $P$ of <0.05 vs sense-treated cells: No oligo, basal secretion in the absence of secertagogues; Sense, sense oligo; and Anti-sense, antisense oligo.

Using $Ca^{2+}$ and GTP$\gamma$S as stimuli, we studied whether introduction of peptides or antibodies of t-SNAREs into the permeabilized cells inhibits surfactant secretion. We treated permeabilized type II cells with varying concentrations of peptides containing an amino acid sequence corresponding to N-terminal amino acids 1−19 of syntaxin 2 or C-terminal amino acids 199−210 of SNAP-23. Both peptides caused a significant dose-dependent decrease in the level of surfactant secretion (Figure 6). The inhibition was more sensitive to the SNAP-23 peptide than to the syntaxin 2 peptide. However, the scrambled peptides with identical compositions, but sequences different from those of syntaxin 2 and SNAP-23 peptides, had no effects on the surfactant secretion. The syntaxin 2 and SNAP-23 antibodies generated from these peptides only recognized syntaxin 2 and SNAP-23, respectively, but not other syntaxin isoforms or SNAP-25 (Figure 7A), indicating that these antibodies were isoform-specific. The syntaxin 2 and SNAP-23 antibodies inhibited PC secretion by 84 and 47%, respectively (Figure 7B). Rabbit IgG used as a control did not inhibit surfactant secretion.

## DISCUSSION

Even though the late steps of the exocytotic pathway in a variety of secretory cells appear to involve common proteins, some differences have been noted with respect to early stages of the process, especially targeting, docking, and priming. It is important to investigate whether the SNARE model operates in exocytosis of lamellar bodies, considering the difference between surfactant secretion and exocytosis of hormones and the neurotransmitter. In this study, we for the first time demonstrate that t-SNARE, syntaxin 2, and SNAP-23 are present in type II cells. We also provide strong evidence that syntaxin 2 and SNAP-23 participate in exocytosis in type II cells using peptide inhibition, antibody neutralization, and antisense oligo.

We observed that alveolar type II cells, like neuronal cells, contained at least one isoform of t-SNAREs. SNAP-23 and syntaxin 2 were the major t-SNARE isoforms detected in these cells as opposed to syntaxin 1 and SNAP-25 in brain cells. The subcellular localization of SNARE proteins in type II cells is the key to establishing the patterns of protein flow and sorting of the lamellar bodies. As expected, syntaxin 2 was predominately detected in the type II cell plasma membrane, while a large amount of SNAP-23 was also observed in lamellar bodies in addition to its plasma membrane localization. The presence of t-SNARE in secretory granules has also been observed by others. Tagaya et al. (*42*, *43*) reported the presence of syntaxin 1 and SNAP-25 in adrenal chromaffin granules as well as membranes of synaptic vesicles. Feng et al. (*44*) showed that syntaxin 2 is evenly distributed between the plasma membrane and granules and suggested that this distribution might be necessary for determining whether a vesicle is capable of undergoing homotypic or heterotypic fusion. Hansen et al. (*45*) reported that the zymogen granule membrane contains more syntaxin 3 than the plasma membrane of pancreatic acinar cells. Although syntaxin 3 is also present in type II cells, its function remains to be determined.

SNAREs self-assemble into extremely stable four-helix bundles composed of two cognate parts during the interaction of two membrane bilayers or within the same bilayer. For fusion to occur, all the t-SNARE must reside in one bilayer, and the v-SNARE must reside in the other, a phenomenon termed topological restriction (*46*). v-SNARE on the vesicle links with their cognate matching t-SNAREs on the target membrane to generate the SNARE pins that force the two lipid bilayers into close apposition, resulting in membrane fusion (*9*, *10*). The presence of t-SNAREs on lamellar bodies may indicate that the SNARE core complex pre-exists in the granules in the cis conformation and the cis conformation must convert to the trans conformation before membrane fusion.

The role of syntaxin and the SNAP-25 family of proteins in membrane fusion and exocytosis has been reported in a variety of cell systems (*12*). Antisense oligo has been used to study the role of syntaxin 1 in exocytosis (*47*). The study presented here shows that type II cells treated with antisense

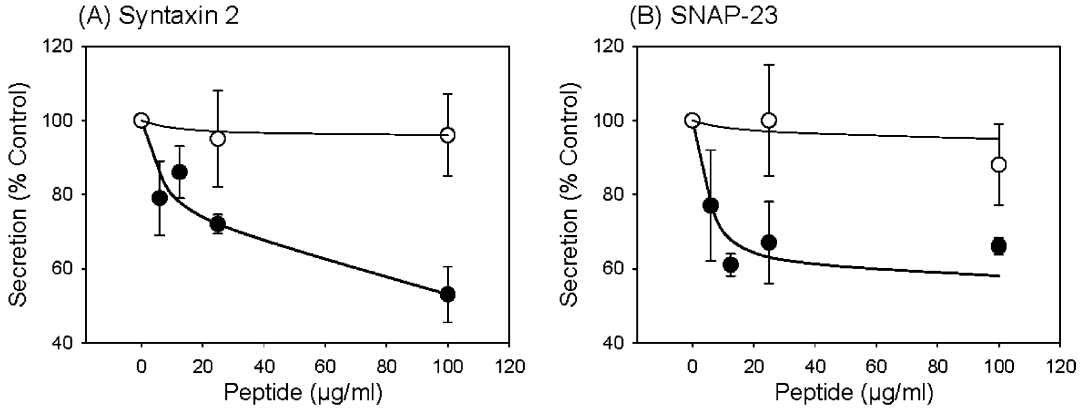


FIGURE 6: Inhibition of GTP$\gamma$S- and $Ca^{2+}$-stimulated surfactant secretion by syntaxin 2 and SNAP-23 peptides. Type II cells ($1 \times 10^6$) were permeabilized with 40 $\mu$M $\beta$-escin and preincubated with the peptide corresponding to N-terminal amino acids 1−19 of syntaxin 2 (A, ●), the scrambled peptide with a composition identical to that of the syntaxin 2 peptide (A, ○), the peptide corresponding to C-terminal amino acids 199−210 of SNAP-23 (B, ●), or the scrambled peptide with a composition identical to that of the SNAP-23 peptide (B, ○). The cells were then stimulated with 20 $\mu$M GTP$\gamma$S and 0.5 $\mu$M $Ca^{2+}$. Surfactant secretion was expressed as a percentage of the control cells (stimulated with GTP$\gamma$S and $Ca^{2+}$ in the absence of the peptides). The actual surfactant secretion value for the control cells was 3.23 ± 0.65%. Data are means ± the standard error ($n = 3$).

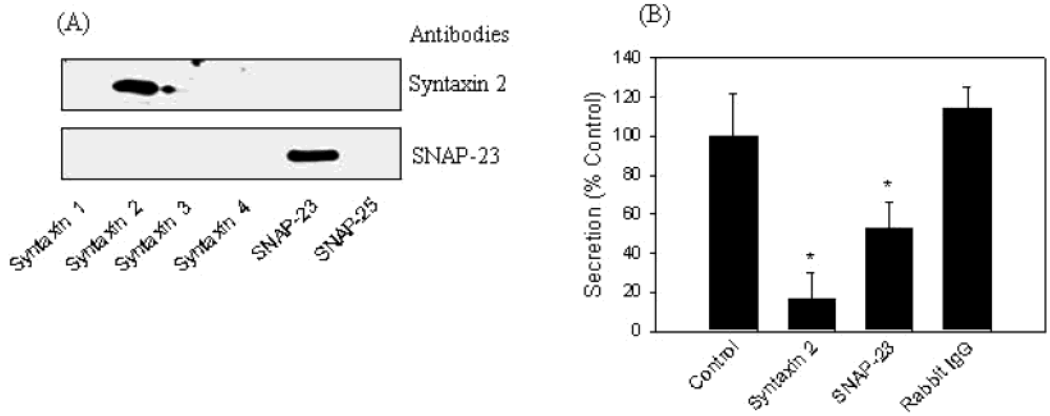


FIGURE 7: Inhibition of surfactant secretion by antibodies against SNAP-23 and syntaxin 2. (A) The anti-syntaxin 2 and anti-SNAP-23 antibodies reacted only with syntaxin 2 and SNAP-23, respectively. Recombinant syntaxins 1−4, SNAP-23, and SNAP-25 (100 ng each) were probed with anti-syntaxin 2 or anti-SNAP-23 antibodies on Western blots. (B) Type II cells ($1 \times 10^6$) were permeabilized with 40 $\mu$M $\beta$-escin and pretreated with 10 $\mu$g/mL SNAP-23 or syntaxin 2 antibodies. The cells were stimulated with 20 $\mu$M GTP$\gamma$S and 0.5 $\mu$M $Ca^{2+}$. Rabbit IgG (10 $\mu$g/mL) was used as a control. Surfactant secretion was expressed as a percentage of the control cells (stimulated with GTP$\gamma$S and $Ca^{2+}$ in the absence of the peptides). The actual surfactant secretion value for the control cells was 3.67 ± 0.82%. Data are means ± the standard error ($n = 3$). The asterisk denotes a $P$ of $<0.05$ vs control cells.

oligo to syntaxin 2 showed a remarkable dose-dependent decrease in the level of PMA-stimulated surfactant secretion. Syntaxin 2 antisense oligo also caused a significant decrease in its mRNA and protein, indicating that the decrease in the level of surfactant secretion was due to a reduction of syntaxin 2 levels in the cells. Syntaxin 2 antisense oligo also inhibited surfactant secretion stimulated by other secretagogues, including ATP, terbutaline, and $Ca^{2+}$ ionophore, implying that syntaxin 2 may act at the later stage of exocytosis, i.e., the fusion of lamellar bodies with the plasma membrane. How syntaxin is regulated is still not clear, but it could be a target protein of protein kinases, including protein kinase C (PKC), protein kinase A (PKA), and $Ca^{2+}$- and calmodulin-dependent protein kinase (CaMK). These protein kinases are activated by secretagogues used in our studies. Ohyama et al. (*48*) demonstrated that microinjection of the CaMKII-binding domain of syntaxin 1A specifically modulated exocytosis in chromaffin cells and in neurons. *In vitro* studies using purified proteins have shown that syntaxins 1A and 4 can be phosphorylated by casein kinase II, and syntaxin 3 and SNAP-25 by CaMKII and PKA, respectively (*49*). Protein phosphorylation changes the interaction of SNARE proteins. Phosphorylated syntaxin 4 is less able to bind SNAP-25. On the other hand, the phosphorylation of syntaxin 1A enhanced its interaction with synaptotagmin I (*49*). However, another report has shown that PKA, but not casein kinase II, phosphorylation of syntaxin 4 disrupts its ability to interact with SNAP-23 (*38*). A novel kinase called SNARE kinase has been identified. The phosphorylated SNAP-23 via this kinase cannot assemble into the t-SNARE complexes (*50*).

We have previously established an $\beta$-escin-permeabilized type II cell model for studying the functions of cytoplasmic proteins. $Ca^{2+}$ stimulates surfactant secretion in the permeable cells, but this stimulation is modest. The studies presented here reveal that GTP$\gamma$S, a nonhydrolyzable GTP analogue, also enhanced the secretion and a combination of $Ca^{2+}$ and GTP$\gamma$S caused a further increase in the level of secretion from the $\beta$-escin-permeabilized cells. Syntaxin exists in a closed conformation, hindering it from interacting with SNAP-25. This closed state is maintained through its interaction with nSec1/munc18. The activation of GTPases

leads to the release of nSec1 from syntaxin and the conversion of a closed conformation to an open conformation. This enables the formation of the SNARE core complex (*51*). The addition of a synthetic peptide derived from the N-terminus of syntaxin 2 and its antibodies to permeabilized type II cells inhibited GTP$\gamma$S- and $Ca^{2+}$-stimulated surfactant secretion, further supporting a role of syntaxin 2 in surfactant secretion. Similarly, a peptide derived from the C-terminus of SNAP-23 as well as its antibodies caused a suppression of the secretion. Our data agree with the reports by Martin-Martin et al. that SNAP-23 and syntaxin 6 antibody significantly inhibited GTP$\gamma$S- and $Ca^{2+}$-dependent exocytosis in permeabilized human neutrophils (*31*) and by Lemons et al. that antibodies against syntaxins 2 and 4 and SNAP-23 inhibited $Ca^{2+}$-stimulated release of platelet factor 4 (PF4) (*52*). In summary, we show that syntaxin 2 and SNAP-23 are expressed in type II cells. We also provide evidence to support functional roles of syntaxin 2 and SNAP-23 in exocytosis of lung surfactant by alveolar type II cells.

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