Physical and Functional Interactions of SNAP-23 with Annexin A2

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Lung surfactant is secreted through the fusion of lamellar bodies with the plasma membrane of alveolar epithelial type II cells. Annexin A2, a Ca^{2+}- and phospholipid-binding protein, promotes the fusion of lamellar bodies with the plasma membrane. Soluble N-ethylmaleimide–sensitive factor attachment protein receptors (SNAREs) are known to have an essential role in surfactant secretion. We hypothesized that annexin A2 acts as a Ca^{2+} sensor and mediates membrane fusion via its interaction with SNAREs. Both purified or endogenous annexin A2 in type II cells specifically bound with SNAP-23 in a Ca^{2+}-dependent manner, as determined by pull-down experiments using recombinant glutathione S-transferase-tagged SNAP-23. A deletion study identified the cysteine-rich region (CRR) of SNAP-23 as the binding site for annexin A2. Mutations of cysteine residues in the CRR dramatically decreased the binding. SNAP-23 also co-immunoprecipitated with annexin A2; however, a SNAP-23 mutant failed to co-immunoprecipitate with annexin A2. Immunofluorescence revealed a co-localization of SNAP-23 and annexin A2 in type II cells. Furthermore, anti–SNAP-23 antibody significantly inhibited annexin A2–mediated fusion between lamellar bodies and the plasma membrane. These data suggest that annexin A2 and SNAP-23 are involved in the same pathway in the regulation of lung surfactant secretion.

Keywords: SNAP-23; annexin A2; lung surfactant; alveolar type II epithelial cells; membrane fusion

Lung surfactant is a surface active material, which forms a monolayer at the air–liquid interface and reduces the surface tension of alveoli, thus preventing alveoli from collapse. Deficiency of surfactant at the alveolar surface is associated with infant and acute respiratory distress syndromes. Lung surfactant is synthesized and secreted by alveolar type II cells. It is composed of phospholipids, mainly dipalmitoylphosphatidylcholine (DPPC), and surfactant proteins A, B, and C. Most components of surfactant are synthesized in the endoplasmic reticulum and stored in the specified organelles, lamellar bodies. Secretion of surfactant involves the translocation, docking, and fusion of lamellar bodies with the apical plasma membrane. This process is very complicated and the underlying mechanism is still poorly understood (1–4).

Soluble N-ethylmaleimide–sensitive fusion protein attachment protein receptors (SNAREs) are a protein family that exist ubiquitously in eukaryotic cells and play crucial roles in membrane targeting, docking, and fusion (5–7). The vesicle SNARE (v-SNARE), VAMP, is located on the membrane of secretory vesicles, while the target SNAREs (t-SNARE), syntaxin and SNAP-25/SNAP-23, are located on the plasma membrane. SNARE proteins contain the characteristic coil-coiled domains, termed as SNARE motifs, which are approximately 60 to 70 residues in length. The interaction of the SNARE motifs from cognate SNARE proteins in two adjacent membranes forms a trans-SNARE complex to pull the membranes into close apposition, and eventually leads to membrane fusion. Reconstitution of SNARE proteins into liposomes simulates the membrane fusion *in vitro*, suggesting that the SNARE proteins may provide the minimal fusion machinery (8, 9). However, the speed is too slow to be physiological. Furthermore, two recent studies have demonstrated that inter-membrane SNARE interactions in the SNARE-reconstituted liposome systems with more native SNARE densities do not drive membrane fusion (10, 11). An elevation of cytosolic Ca^{2+} concentration is required to trigger regulated exocytosis. Synaptotagmin I has been found to increase the fusion of liposomes reconstituted with SNAREs in a Ca^{2+}-dependent manner, demonstrating its role in Ca^{2+}-triggered exocytosis (12). On the other hand, the hippocampal neurons from synaptotagmin I knockout mice can still release neurotransmitter although the kinetics of the release is affected (13). With the studies of the Ca^{2+}-triggered fusion of sea urchin cortical vesicles *in vitro*, it is proposed that SNARE proteins may just play a modulatory role, acting at the upstream targeting and docking stages (14–16). Disruptions of these steps by attacking SNARE proteins with neurotoxins, antibodies, or other chemicals may alter the overall exocytotic process, while some other proteins associated with SNARE proteins actually mediate the final fusion step(s). V-ATPase V0 sector has been reported as a membrane fusogen acting downstream and independent of trans-SNARE complex formation (17). These data suggest the possible existence of additional proteins that may act as Ca^{2+} sensor and/or fusogenic proteins.

Annexin A2 is a Ca^{2+}-dependent phospholipid-binding protein, which plays an important role in various aspects of vesicular trafficking (18). It has been shown to be involved in Ca^{2+}-dependent membrane fusion during exocytosis and endocytosis in a variety of cells, including chromaffin cells and alveolar type II cells (19–23). Annexin A2 exists as a 36-kD monomer (AIIm) or as a heterotetramer (AIIt) in which two monomers bind to a pair of p11, an S100 family protein (24). Annexin A2 binds to negatively charged phospholipids and mediates the aggregation and fusion of liposome in the presence of Ca^{2+} (20, 25). The modifications of cysteine or tyrosine residues of annexin A2 by nitric oxide or peroxynitrite abolishes its capability to mediate liposome aggregation (26, 27). The depletion of annexin A2 from alveolar type II cell cytosol reduces its membrane fusion activity (28). The silencing of the annexin A2 gene by RNAi

CLINICAL RELEVANCE

Our work suggests that SNAP-23 and annexin A2 are involved in the same pathway in the regulation of lung surfactant secretion. These observations add to our knowledge of the mechanism of secretion and disease formation in type II epithelial cells.

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significantly decreases the secretion of surfactant in isolated alveolar type II cells (29).

Based on our previous studies, SNARE proteins and annexin A2 are required in lung surfactant secretion (20, 28, 30–32). We propose that SNARE proteins and annexin A2 function in the same pathway in regulating surfactant secretion, with annexin A2 acting as a Ca^{2+} sensor and mediating the final membrane fusion. In this study, we first determined the physical interactions between SNARE proteins and annexin A2 by using the *in vitro* Glutathione S-Transferase (GST) pull-down assay and co-immunoprecipitation. We also identified the annexin A2 binding site of SNAP-23. We then investigated their functional interactions with the usage of an *in vitro* biological membrane fusion model. Our results demonstrate that SNARE proteins and annexin A2 not only have physical interactions, but they are also functionally linked together.

MATERIALS AND METHODS

Reagents and Chemicals

Octadecyl rhodamine B chloride (R18) was obtained from Molecular Probes (Eugene, OR). Maclura pomifera agglutinin gel was from EY Laboratories (San Mateo, CA). Fetal bovine serum (FBS), trypsin-EDTA, Dulbecco's modified Eagle's medium (DMEM), Opti-MEM, and Lipofactamine 2000 were from Invitrogen Life Technologies (Carlsbad, CA). Enhanced chemilluminescence (ECL) reagent, glutathione sepharose 4B beads were from Amersham Pharmacia Biotech (Arlington Heights, IL). N-Ethylmaleimide (NEM) was obtained from Sigma-Aldrich (St. Louis, MO). S-Nitroso-L-glutathione (GSNO) was from Cayman Chemicals (Ann Arbor, MI). Anti–SNAP-23 antibodies were raised using the synthetic peptide corresponding to C-terminal residues 199–210 (CANTRAKKLIDS) of rat SNAP-23 (Genmed Synthesis Inc., South San Francisco, CA). These antibodies were affinity-purified using peptide-conjugated beads, as previously described (31). Anti–annexin A1, A4, A5, A6 antibodies, and Protein G PLUS-Agarose, were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti–annexin A2 antibodies were from Santa Cruz Biotechnology and Zymed Laboratories Inc. (South San Francisco, CA). Anti–annexin A3 antibody was a kind gift from Dr. J. D. Ernst of the University of California in San Francisco. Anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was from BD Biosciences (Palo Alto, CA). Anti–green fluorescent protein (GFP) antibody was from Abcam Inc. (Cambridge, MA). Anti-FLAG antibody was from Cell Signaling Technology, Inc. (Danvers, MA). Goat anti-rabbit secondary antibody (horseradish peroxidase–conjugated IgG) was from Bio-Rad Laboratories (Hercules, CA). Rat anti-mouse secondary antibody was from Jackson Immunoresearch Laboratories (West Grove, PA). *Escherichia coli* BL21 (DE3) pLysS was from EMD Biosciences, Inc. (Novagen Brand, Madison, WI). 293A HEK and A549 lung epithelial cell line were from ATCC (Manassas, VA). The mammalian two-hybrid assay kit was from Stratagene (La Jolla, CA). Dual-luciferase reporter assay system was from Promega (Madison, WI).

Plasmids

The pGEX expression vectors encoding GST-tagged SNARE proteins were as follows (33, 34): Cytoplasmic domains of syntaxin 1A (residues 1–265), syntaxin 2 (1–265), syntaxin 3 (1–263), and syntaxin 4 (1–272) were provided as a kind gift from Dr. V. M. Olkkonen (National Public Health Institute, Helsinki, Finland); and full-length SNAP-23 and SNAP-25 were kindly provided from Dr. A. Klip (The Hospital for Sick Children, Toronto, ON, Canada). Cytosolic domains of VAMP-2 (1–94), and VAMP-8 (1–75) were from Dr. Richard H. Scheller of Stanford University. To construct SNAP-23 deletion mutants, various fragments of SNAP-23 were amplified from the plasmid containing full-length SNAP-23 and inserted into the same expression vector. The overexpression vector for annexin A2-GFP was constructed as described (29). For overexpression of SNAP-23, full-length SNAP-23 or SNAP-23ΔCRR fragments were amplified with FLAG tag added at C-terminus via the 3′ primer. For the mammalian two-hybrid assay, full-length SNAP-23, p11, or Rab14 was inserted into the bait vector pCMV-BD. For target construct of pE/CMV-AII-NLS-AD, the GFP gene in pE/CMV-AII-GFP was replaced with the fragment amplified from target vector pCMV-AD, containing SV40 nuclear localization signal, NF-κB activation domain and SV40 polyA (nt 660–1783). All the constructs were confirmed by DNA sequencing.

Purification of Bovine Annexins

Annexin A1, A2 monomer and tetramer, A4, A5, and A6 were purified from bovine lung tissue through sequential column chromatography by using DEAE-Sepharose CL6B, Sephacryl S100, and Mono S columns as described previously (32).

Preparation of Alveolar Type II Cell lysate

Alveolar type II cells were isolated from 180- to 200-g Sprague-Dawley rats as described previously (32). Freshly isolated cells were lysed in lysis buffer (40 mM Hepes, pH 7.0, 100 mM KCl, 1 mM EGTA, 2 mM $MgCl_2$, 1% NP40 and a protease inhibitor cocktail including 1 mM PMSF, 10 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml benzamidine, and 10 μM pepstatin), followed by sonication on ice for 15 seconds. The lysate was centrifuged at 100,000 × *g* for 1 hour at 4°C, and the supernatant was collected.

Expression and Purification of GST-Tagged SNARE Proteins

The GST-SNARE cDNA clones were transformed into *E. coli* BL21 (DE3) pLysS and GST recombinant proteins were purified. Briefly, a single colony was inoculated in Luria-Bertani (LB) medium containing 100 μg/ml of ampicillin. When the A_{660} reached 0.6–0.8, 1 mM Isopropyl β-D-thiogalactopyranoside was used to induce the protein expression for 4 to 5 hours at 37°C. The cells were harvested by low-speed centrifugation and washed three times with cold PBS. The cells were then sonicated and centrifuged at 20,000 × *g* for 15 minutes. The cell lysate was incubated with Glutathione Sepharose 4B beads. The recombinant protein then was eluted with 10 mM reduced glutathione and stored at −80°C.

In Vitro GST Pull-Down Experiments

Glutathione Sepharose 4B beads were first incubated with 0.5% BSA to block nonspecific binding for 3 hours. Ten microliters (bed volume) of the beads were then mixed with 0.2 nmole of GST or GST-SNARE protein in 100 μl of reaction volume and incubated for 4 hours at 4°C. The beads were washed with the binding buffer (40 mM Hepes, pH 7.0, 100 mM KCl, 1 mM EGTA, 2 mM $MgCl_2$, 1% NP40, and varying concentrations of Ca^{2+}) and incubated with either purified AIIt (2, 4, 8, 16, 32, or 64 μg), other annexins, or alveolar type II cell lysate (500 μg). After end-to-end rotation at 4°C for 4 hours, beads were washed six times with binding buffer and boiled with SDS sample buffer. The soluble fractions were detected for the presence of annexins by Western blotting. To modify AIIt, 10 μg of AIIt was incubated with 1 mM NEM or 1 mM GSNO in 100 μl of 0.1 M phosphate buffer (pH 7.0) for 15 minutes. Samples were dialyzed against binding buffer to remove NEM or GSNO. The control was treated the same, except that NEM and GSNO were omitted. The treated AIIt then was used for pull-down assays.

Co-Immunoprecipitation of Annexin A2 and SNAP-23

To overexpress annexin A2-GFP and SNAP-23-FLAG, 0.5×10^6 of 293A cells were seeded in 6-well plates in 2 ml of complete DMEM containing 10% FBS and nonessential amino acid. Transfection was performed when the cells reached 80% confluence. A quantity of 500 ng of plasmid pE/CMV-annexin A2-GFP was mixed with 500 ng of either pE/CMV-SNAP23-FLAG or pE/CMV-SNAP23ΔCRR-FLAG in 50 μl of Opti-MEM. The transfection reagent Lipofectamine 2000 was used with a DNA-reagent ratio of 1:3 (mass:volume). Forty-eight hours after transfection, cells were washed with PBS, and lysed in lysis buffer (40 mM Hepes, pH 7.0, 100 mM KCl, 1 mM EGTA, 2 mM $MgCl_2$, 1% NP40, and a protease inhibitor cocktail). A quantity of 1.5 μl of anti-GFP antibody was added into 150 μl of cell lysate, and $CaCl_2$ was supplemented to a final concentration of 1 mM. The mixture was then incubated at 4°C overnight. Fifteen microliters of Protein G PLUS-Agarose was added and incubated for an additional 4 hours at room temperature. The resin was then washed with binding buffer for four

times, and boiled with SDS sample buffer. The soluble fractions were detected by Western blotting.

Luciferase Assay

A549 cells (0.5×10^6) were seeded in 96-well plates in 2 ml of complete DMEM containing 10% FBS and nonessential amino acid. Transfection was performed at 80% cell confluence. A total of 775 ng of total DNA (bait vector pCMV-BD-SNAP-23, p11 or Rab14, target vector pE/CMV-AII-NLS-AD, and report vector pFR-Luc, 250 ng each; second reporter vector pRL-TK, 25 ng) was used. Forty-eight hours after transfection, luciferase activity was measured with a dual-luciferase reporter assay system. In brief, cells were washed with PBS and lysed. Ten out of 50 μl of lysate was mixed with Luciferase Assay Reagent II (LAR II) and firefly (*Photinus pyralis*) luciferase activity was measured. This reaction was stopped and the Renilla (*Renilla reniformis*) luciferase reaction was started simultaneously by adding Stop and Glo Reagent to the same sample. The firefly luciferase activity was normalized against that of Renilla luciferase. The plates were read with the FLUOstar OPTIMA Multifunction Microplate reader (BMG Biotech, Durham, NC).

Western Blotting

Protein samples were fractionated on 10% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane. The blot was visualized with Ponceau S staining and blocked with 5% fat-free milk in TTBS (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween 20). The blot was incubated with appropriate primary antibodies (annexin A1, 1:1,000; annexin A2, Santa Cruz 1:500; annexin A2, Zymed 1:1,000; annexin A3, 1:500; annexin A4, 1:1,000; annexin A5, 1:1,000; annexin A6, 1:1,000; FLAG, 1:1,000; and GAPDH, 1:4,000) at 4°C overnight, and then with secondary antibody (1:5,000) at room temperature for 1 hour. Finally, the signal was developed with ECL and detected with VersaDoc Imaging system (Bio-Rad). The quantification of Western blotting was performed by using Quantity One 4.0.3 software (Bio-Rad).

Immunofluorescence

Freshly isolated alveolar type II cells were fixed with 4% paraformaldehyde for 20 minutes at room temperature. The cells were cytospun onto glass slides and stored at 4°C until use. In brief, cells were permeabilized with 1% TX-100 and then blocked with 10% FBS in 50 mM PBS. The slides were then incubated overnight at 4°C with monoclonal anti-annexin A2 antibodies (1:50 dilution; BD Biosciences) and polyclonal anti-SNAP-23 antibodies (1:100 dilution). Subsequently, they were washed and incubated with Alexa Fluor 488 goat anti-mouse and Alexa Fluor 568 goat anti-rabbit antibodies at 1:250 dilutions for 1 hour at room temperature. Finally, the slides were washed and mounted for fluorescence microscopy (Nikon Inc., Lewisville, TX).

Isolation of Lamellar Bodies

Lamellar bodies were isolated from rat lungs by upward flotation on a discontinuous sucrose gradient, as described by Chattopadhyay and coworkers (28). A perfused rat lung was briefly homogenized in 1 M sucrose and loaded at the bottom of a sucrose gradient (0.2, 0.3, 0.4, 0.5, 0.6, 0.7, and 0.8 M). After centrifugation at $80{,}000 \times g$ for 3 hours, the lamellar body fraction was collected at the 0.4- and 0.5-M interface, and diluted to 0.24 M with cold water. Lamellar bodies were then spun down at $20{,}000 \times g$ and resuspended in 0.24 M sucrose containing 10 mM Tris and 50 mM Hepes (pH 7.0). The protein concentration of lamellar bodies was determined by Bio-Rad protein assay.

Preparation of Plasma Membrane

The preparation of plasma membrane from rat lung tissue was performed, as described previously (28). A Sprague-Dawley rat lung was perfused with saline and homogenized in buffer B (10 mM Na-Pi, pH 7.4, 30 mM NaCl, 1 mM $MgCl_2$, 5 μM PMSF, and 0.32 M sucrose). After a discontinuous sucrose gradient (0.5, 0.7, 0.9, and 1.2 M) centrifugation at $95{,}000 \times g$ for 60 minutes, the plasma membrane fraction was collected at the 0.9- and 1.2-M interface and diluted to 0.32 M sucrose with cold buffer A (buffer B without sucrose). The plasma membrane was spun down at $120{,}000 \times g$ and resuspended in buffer B. To remove the outside-out plasma membrane vesicles, the plasma membrane was incubated with *Maclura pomifera* agglutinin-conjugated beads. The percentage of the protein recovered after the *M. pomifera* agglutinin treatment was approximately 30 to 40%. To incorporate R-18 into the inside-out plasma membrane vesicles, 1 μl of a 20-mM stock solution (in ethanol) of R-18 was injected by using a 10-μl syringe to 300 μg protein of plasma membrane in 300 μl of buffer B by vortexing. The mixture was incubated for 30 minutes by end-to-end rotation at room temperature, and excessive R-18 was removed by dialysis against 3 L of buffer B.

Annexin A2 Tetramer-Mediated Fusion Assay

The fusion between lamellar bodies and the plasma membrane was measured by monitoring the fluorescence changes of a fluorescent dye, R-18, as previously described (28). R-18 was incorporated into the plasma membrane at a self-quenching concentration. Fusion of the plasma membrane with unlabeled lamellar bodies resulted in the dilution of R-18, due to the increased surface area. The relief of the self-quenching of R-18 led to the increase in fluorescence. In our standard assay, 5 μg protein of plasma membrane was mixed with 10 μg protein of lamellar bodies in 1 ml of Ca^{2+}-EGTA buffer (40 mM Hepes, pH 7.0, 100 mM KCl, 1 mM EGTA, 2 mM $MgCl_2$, and 1 mM Ca^{2+}) at 37°C with a continuous stirring. A basal reading was recorded for 2 minutes, and 10 μg of annexin A2 tetramer was added to initiate the fusion. After an additional 6 minutes of recording, 0.1% (vol/vol) Triton X-100 was added to achieve the maximal fluorescence. Fusion was calculated as a percentage of the maximal fluorescence. To investigate the functional relationship between SNAREs and annexin A2, the plasma membrane was incubated with anti-SNAP-23 antibodies at room temperature. It was then mixed with lamellar bodies and annexin A2 tetramer was added to initiate the fusion.

RESULTS

SNAP-23 Binds with Annexin A2 *In Vitro*

To explore the relationship between SNARE proteins and annexin A2, we investigated their physical interactions by using GST fusion protein pulldown assay. SNAP-23, syntaxin 2, and VAMP-2 are the major SNARE isoforms in alveolar type II cells (31, 35) and were tested. We used the same amount (0.2 nmoles) of GST-SNARE fusion proteins and varying doses of purified AIIt in the presence of 1 mM Ca^{2+}. As shown in Figures 1A and 1B, SNAP-23 had a strong binding with purified AIIt. This binding was AIIt dose dependent. The increase in binding was unlikely due to variation of loading, pulldown, or transfer of GST-SNAP-23, since Ponceau S staining showed the same amount of GST-SNARE fusion proteins after electrophoretic transferring. In contrast to SNAP-23, syntaxin 2, VAMP-2, or GST control did not show binding with purified AIIt except that a weak binding was noted at very high doses of AIIt. We also tested the ability of other SNARE isoforms to bind with AIIt under the same conditions as above with 8 μg of purified AIIt. As shown in Figure 1C, SNAP-25, the neuronal isoform of SNAP-23, also had a very strong binding, while syntaxin 1A, 2, 3, 4, and VAMP-2, 8 did not have significant binding.

Most of the activities of annexin A2, including binding to phospholipids and mediating membrane fusion, are Ca^{2+} dependent. We further tested the Ca^{2+} requirement for the binding of annexin A2 and SNAP-23. As shown in Figures 1D and 1E, AIIt did not exhibit any binding to SNAP-23 at ≤ 1 μM Ca^{2+}. There was weak binding at 10 to 100 μM Ca^{2+}. The binding was increased significantly when the Ca^{2+} concentration reached 500 μM.

SNAP-23 Does Not Bind with Other Annexin Isoforms

The experiments above were performed by using purified bovine annexin A2 tetramer. Annexin A2 exists as a monomer without partner p11 as well. To find out whether p11 is required

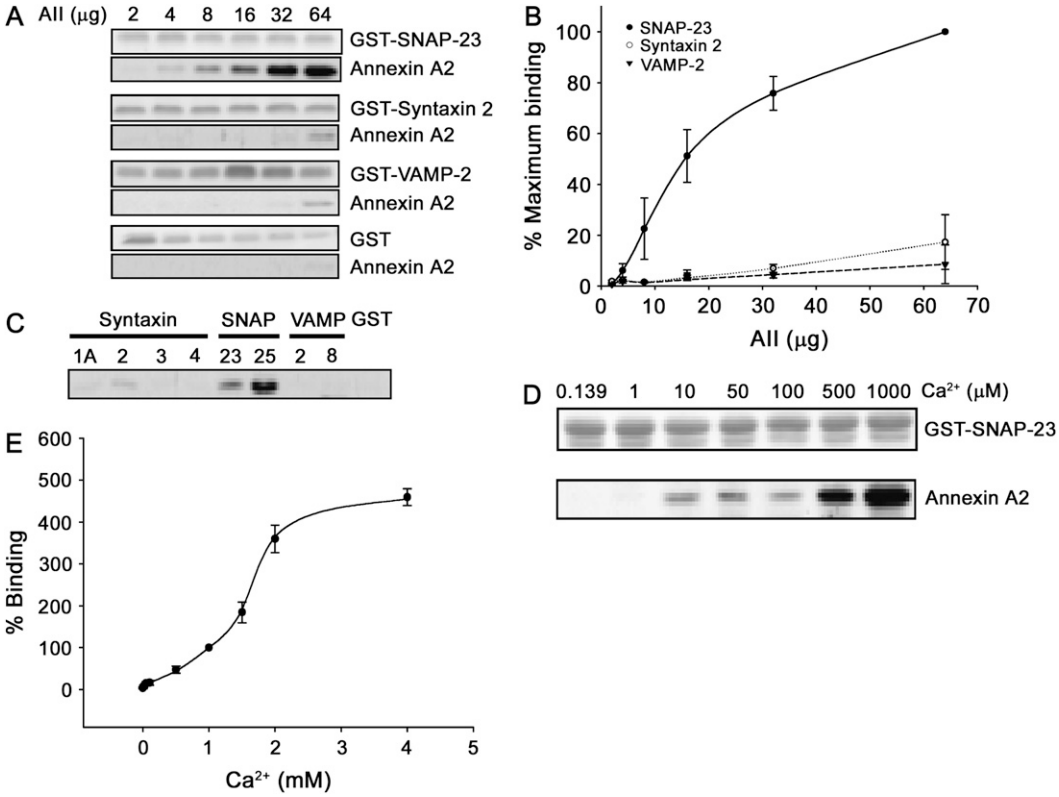

Figure 1. The binding of SNAP-23 with purified annexin A2. (*A*) Glutathione sepharose beads were incubated with 0.2 nmole of GST-SNAP-23, GST-syntaxin 2, GST-VAMP-2, or GST, followed by the incubation with various amounts of purified annexin A2 tetramer (AIIt, 2–64 μg) in the presence of 1 mM Ca^{2+}. The bound annexin A2 was detected with Western blotting (*lower panels*). GST-SNARE fusion proteins were stained with Ponceau S (*upper panels*). (*B*) Quantitation of binding. Each band was quantitated by densitometry. A linear range was determined by using purified AIIt. The background binding with GST was subtracted from those of GST-SNARE proteins. The results are expressed as a percentage of the maximal binding (the binding of GST-SNAP-23 with 64 μg of AIIt). Data shown are means ± SE ($n = 3$). (*C*) The binding of AIIt with other SNAREs. AIIt (8 μg) was incubated with glutathione sepharose beads pre-incubated with 0.2 nmole of GST-syntaxin 1A, 2, 3, 4, GST-SNAP-23, 25, or VAMP-2, 8. The bound annexin A2 was detected by Western blot. (*D–E*) Ca^{2+}-dependence. GST-SNAP-23 (0.2 nmole) pre-bound to glutathione sepharose beads was incubated with 8 μg of AIIt at various concentrations of Ca^{2+}. GST-SNAP-23 was stained with Ponceau S (*D*, *upper panel*) and annexin A2 was detected by Western blot (*D*, *lower panel*). In *E*, the binding was quantitated by densitometry, and the results were expressed as a percentage of the binding at 1 mM Ca^{2+}. Data shown are means ± SE ($n = 4$).

for the binding, we repeated the pulldown experiments with purified annexin A2 monomer. The results showed that annexin A2 monomer bound with GST-SNAP-23 in the presence of 1 mM Ca^{2+}, while GST had no binding (Figure 2A). To investigate the binding specificity of SNAP-23, other members of the annexin family were tested for the binding with GST-SNAP-23. Same amounts (0.2 μmole) of purified annexin A1, A4, A5, and A6 were incubated with GST-SNAP-23 in the presence of 1 mM Ca^{2+}. As shown in Figure 2A, none of these annexins had binding with GST-SNAP-23. Furthermore, we performed the pulldown experiments with rat alveolar type II cell lysate. The results showed that endogenous annexin A2 also bound with GST-SNAP-23 in a Ca^{2+}-dependent manner. The GST control did not bind with annexin A2. Neither annexin A3 nor GAPDH was detected in the GST-SNAP-23 pulldown samples (Figure 2B).

Chemical Modification of Annexin A2 Inhibits its Binding with SNAP-23

We have previously shown that the modification of its cysteine residues by NEM or GSNO reduced AIIt-mediated fusion (28). As shown in Figure 3, the binding between AIIt and SNAP23 was inhibited after the treatment of AIIt with 1 mM NEM (16.1 ± 4.9%) or GSNO (34.8 ± 3.8%).

Cysteine-Rich Region of SNAP-23 Is its Binding Site with Annexin A2

SNAP-23 has two SNARE motifs at its N- and C-termini and a cysteine-rich region (CRR) between them. To identify the domains of SNAP-23 essential for its binding with annexin A2, we constructed various SNAP-23 deletion mutants (Figure 4A). The recombinant GST fusion proteins were expressed in *E. coli* and purified. The purity of these recombinant proteins was shown in Figure 4B. The N76 mutant has a deletion of the C-terminal 77–211 amino acids (CRR and the C-terminal SNARE motif). The ability of the N76 mutant to bind with annexin A2 tetramer was almost abolished in comparison with that of full-length SNAP-23 (7.5 ± 5.9%). However, the mutant N88, which retains the additional 12–amino acid CRR, had a binding of 95.4 ± 16.8% (Figures 4C and 4D). The deletion of C-terminal SNARE motif (ΔC71) had no significant effect on the binding. The ΔC23 mutant, which is equivalent to the botulinum neurotoxin (BoNT) E cleavage, also did not affect the binding. However, the ΔC9 mutant, which is equivalent to the BoNT A cleavage, showed a decreased binding (37.9 ± 13.0%). A similar result was obtained with an independent preparation of the ΔC9 mutant. The deletion of N-terminal 18, 32 amino acids or the N-terminal SNARE motif (1–76 amino acids) renders the recombinant proteins prone to degradation. However, these mixtures containing intact and partially-degraded recombinant proteins still bound to annexin A2 (data not shown). We also tested the ability of annexin A2 monomer to bind with these mutants as well and the same binding pattern as annexin A2 tetramer was observed (data not shown). To further confirm the importance of the cysteine-rich region, we deleted the CRR domain from full-length SNAP-23 (ΔCRR) (Figure 5A). Similar to N76 mutant, the ΔCRR mutant failed to bind with annexin A2 (6.1 ± 2.8%) (Figures 5C and 5D). There are five cysteine residues within the CRR domain. Using site-directed mutagenesis, we substituted one (C83S), two (C85, 87S), and three cysteine residues (C83, 85, 87S) with serine in the N88 mutant. The substitution of cysteine residues in the mutant N88 dramatically decreased its binding with annexin A2

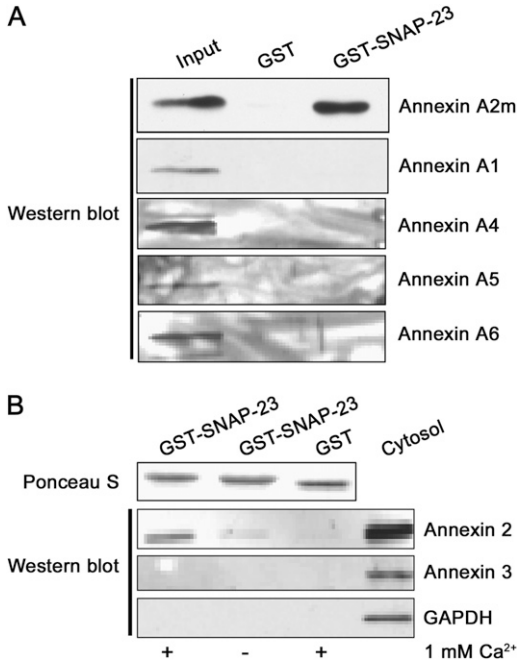

Figure 2. Specificity of SNAP-23 binding with annexins. (*A*) Glutathione sepharose beads were incubated with 0.2 nmole of GST-SNAP-23 or GST, followed by the incubation with 0.2 nmole of various purified annexins (annexin A1, A2 monomer [A2m], A4, A5, and A6) in the presence of 1 mM Ca^{2+}. The bound annexins were probed with the corresponding antibodies. A quantity of 100 ng of each annexin protein was used as a positive control for Western blotting. (*B*) Freshly isolated rat alveolar type II cells were lysed and centrifuged at 100,000 $\times$ *g* for 1 hour. Glutathione sepharose beads bound with GST-SNAP-23 or GST (0.2 nmole) were incubated with the cytosol (500 μg of total protein) in the presence or absence of 1 mM Ca^{2+}. GST-SNAP-23 and GST were stained with Ponceau S. Annexin A2, annexin A3, and GAPDH were detected by Western blot. Type II cell cytosol (10 μg of protein) was used as a positive control for the Western blot.

from 95.4 ± 16.8% (N88) to 39.9 ± 5.1% (C83S), 13.6 ± 3.1% (C85,87S) and 7.6 ± 3.7% (C83,85,87S) (Figures 5C and 5D). We also made the GST-CRR fusion protein and tested its binding with annexin A2. We did not observe binding (data not shown). This is probably due to the sterical hindrance that prevents the binding of annexin A2 to the CCR domain since the CCR domain is too short (only 12 amino acids).

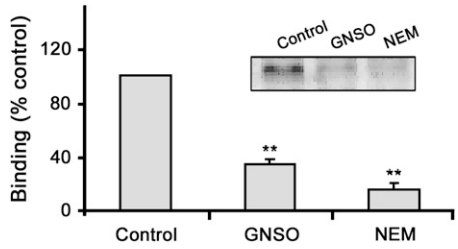

Figure 3. Modification of annexin A2 inhibits its binding with SNAP-23. AIIt (8 μg) was treated with 1 mM N-ethylmaleimide (NEM) or S-nitroso-L-glutathione (GSNO) for 15 minutes in 100 μl of 0.1 M phosphate buffer, pH 7.0. After the incubation, the mixture was dialyzed against binding buffer overnight to remove NEM or GSNO. The binding assay was performed with 8 μg AIIt, 0.2 nmole of GST-SNAP-23, and 1 mM Ca^{2+}. The results are expressed as a percentage of the untreated control. Data shown are means ± SE. $^{**}P < 0.01$ versus control (Student's *t* test, $n = 3$).

SNAP-23 Is Co-Immunoprecipitated with Annexin A2

To investigate the interaction of annexin A2 and SNAP-23 in cells, we co-expressed annexin A2-GFP fusion protein with FLAG-tagged SNAP-23 wild-type or SNAP-23 ΔCRR mutant in 293A cells. Immunoprecipitation was performed by using anti-GFP antibody. As shown in Figure 6A, a similar amount of annexin A2–GFP fusion protein was immunoprecipitated from SNAP-23 wild-type– and SNAP-23 ΔCRR–expressed cells. However, only SNAP-23 wild-type, not the CRR deletion mutant, co-precipitated with annexin A2. The control pre-immune serum did not pull down annexin A2, SNAP-23, or SNAP-23 ΔCRR. These results are consistent with our *in vitro* study.

Interaction of SNAP-23 and Annexin A2 by a Mammalian Two-Hybrid Assay

To investigate the interaction of annexin A2 and SNAP-23 *in vivo*, we used a mammalian two-hybrid system. Annexin A2 and SNAP-23 genes were inserted into plasmids containing an NF-κB activation domain (AD) and a GAL4 DNA binding domain (BD), respectively, resulting in the plasmids, pCMV-AD-AII and pCMV-BD-SNAP-23. While we co-transfected these plasmids into 293A cells, we can detect BD-SNAP-23, but not AD-annexin A2 fusion proteins by Western blots. This is likely due to the fusion of the AD to the N-terminus of annexin A2. We therefore fused the AD to the C-terminus of annexin A2 by sub-cloning the DNA fragment containing a SV40 nuclear localization signal (NLS) and the NF-κB activation domain into the annexin-GFP expression vector, pE/CMV-AII-GFP, resulting in a new vector, pE/CMV-AII-NL-AD. When this vector was transfected into 293A cells, we were able to detect the annexin A2-NLS-AD fusion protein. Using this modified vector, we determined whether annexin A2 interacts with SNAP-23. P11 is known to interact with annexin A2 and was used as a positive control. Co-transfection of pE/CMV-AII-NLS-AD and pCMV-BD-p11 into human embryonic kidney 293A (not shown) or lung epithelial A549 cells (Figure 6B) resulted in a significant increase in normalized luciferase activity, indicating the interaction between annexin A2 and p11 occurred and this modified two-hybrid system can be used to study the interaction of annexin A2 with its binding partners in mammalian cells. Transfection of pE/CMV-AII-NLS-AD or pCMV-BD-SNAP-23 alone showed only negligible activity (0.051 ± 0.007 and 0.070 ± 0.004, respectively). The luciferase activity was not significantly changed by co-transfection of annexin A2 with Rab14 (0.066 ± 0.006). However, co-transfection of annexin A2 with SNAP-23 led to a significant increase (0.132 ± 0.014, $P < 0.01$), indicating a direct interaction of annexin A2 with SNAP-23. It is interesting that unlike p11, co-transfection of SNAP-23 with annexin A2 into the 293A human embryonic kidney cell line or the PC12 adrenal gland cell line only yielded negligible luciferase activity (data not shown). This suggests that cell-specific factors may be required for the interaction of annexin A2 with SNAP-23.

SNAP-23 Is Co-Localized with Annexin A2 in Alveolar Type II Cells

To examine whether SNAP-23 and annexin A2 are co-localized in intact cells, we performed dual-labeling experiments on alveolar type II cells using anti–SNAP-23 and anti–annexin A2. As shown in Figure 6C, the staining patterns of SNAP-23 and annexin A2 overlapped, indicating that they may interact in the cells.

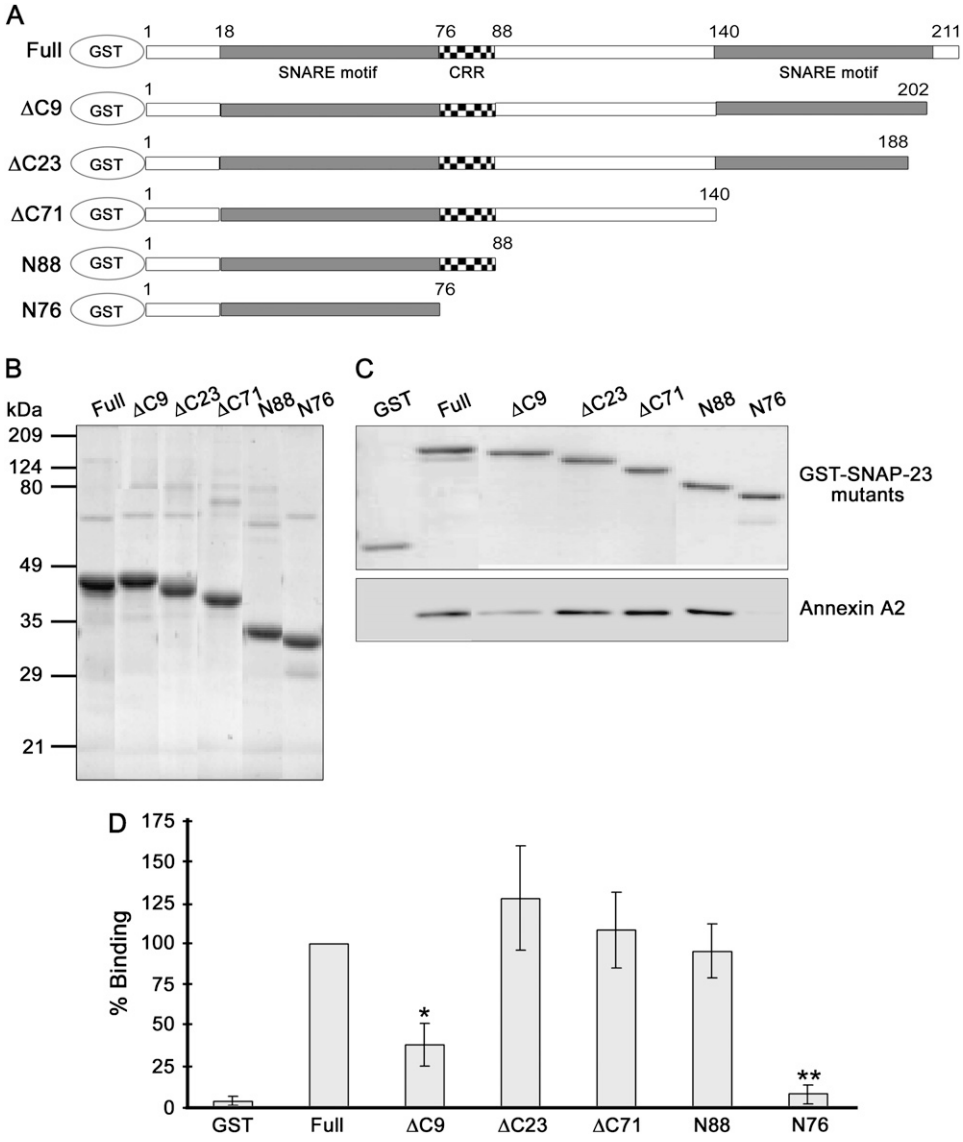

Figure 4. Binding of SNAP-23 deletion mutants with annexin A2. (*A*) Diagrammatic representations of SNAP-23 mutants. The top diagram represents full-length SNAP-23. The cysteine-rich region (CRR) between residues 76 and 88 (*checkerboard pattern*) and two SNARE motifs (*shaded*) are shown. The numbers on the fusion proteins indicate the amino acids of SNAP-23. (*B*) The purity of recombinant proteins as visualized by Coomassie blue R250 staining. (*C*) 0.2 nmole of GST-tagged SNAP-23 mutant protein was bound to glutathione sepharose beads and was incubated with 8 μg of AIIt and 1 mM Ca^{2+}. Recombinant proteins were stained with Ponceau S (*upper panel*) and annexin A2 was detected by Western blot (*lower panel*). (*D*) The bindings were quantitated by VersaDoc Imaging system (Bio-Rad), and the results were expressed as a percentage of full-length SNAP-23 recombinant protein. Data shown are means ± SE. $*P < 0.05$, $**P < 0.01$ versus full-length (Student's *t* test, $n = 3$).

Anti-SNAP-23 Antibody Inhibits AIIt-Mediated Fusion

To investigate the functional interaction of annexin A2 and SNAP-23, we used an *in vitro* biological membrane fusion model, in which the isolated plasma membrane and lamellar bodies were induced to fuse by purified annexin II tetramer (28). Because SNAP-23 showed a direct interaction with annexin A2, we blocked SNAP-23 on the plasma membrane with an affinity-purified antibody and tested AIIt-mediated fusion between lamellar bodies and the plasma membrane. This antibody was raised against C-terminal residues 199–210 of rat SNAP-23. It recognized SNAP-23, but not SNAP-25 or syntaxins 1–4 (31). As shown in Figure 7, anti–SNAP-23 antibody inhibited the fusion in a dose-dependent manner, while rabbit IgG did not show any effects. These results indicated that SNAP-23 was required for AIIt-mediated fusion between lamellar bodies and the plasma membrane.

DISCUSSION

SNARE proteins play an important role in intracellular vesicular trafficking events in eukaryotic cells, and are required for lung surfactant secretion from alveolar type II cells. In this study, we provided evidence for the physical and functional interactions between annexin A2 and SNAP-23. Our results indicated that annexin A2 may interact with SNAP-23, functioning as a Ca^{2+} sensor and/or fusogenic protein.

Many proteins have been reported to interact with SNARE proteins, thus regulating and fine-tuning the processes of intracellular transportation. For example, the interaction of n-Sec1 and syntaxin 1 (36), Snapin and SNAP-23 (37), or synaptosin and VAMP-2 (38) modulates the assembly/disassembly of SNARE complex. Rabphilin directly interacts with SNAP-25 via its C2B domain (39). Expression of the rabphilin mutant lacking the C2B domain in PC12 cells decreases the number of docked vesicle or fusing at the plasma membrane. Synaptotagmin I binds to SNAP-25 in a Ca^{2+}-dependent manner and the cleavage of the C-terminus of SNAP-25 by BoNT E abolished this binding (40). Functionally, synaptotagmin I stimulates SNARE-mediated membrane fusion in the presence of Ca^{2+} (12). Among all of the SNARE partners reported, it appears that only synaptotagmin I requires Ca^{2+} for its interaction. However, synaptotagmin I–depleted neurons can still release neurotransmitter with a different kinetics (13), suggesting that other additional Ca^{2+}-binding proteins behaving as Ca^{2+} sensors may exist.

Annexins are a superfamily of Ca^{2+}-dependent phospholipid binding proteins. Each annexin has a conserved C-terminal core

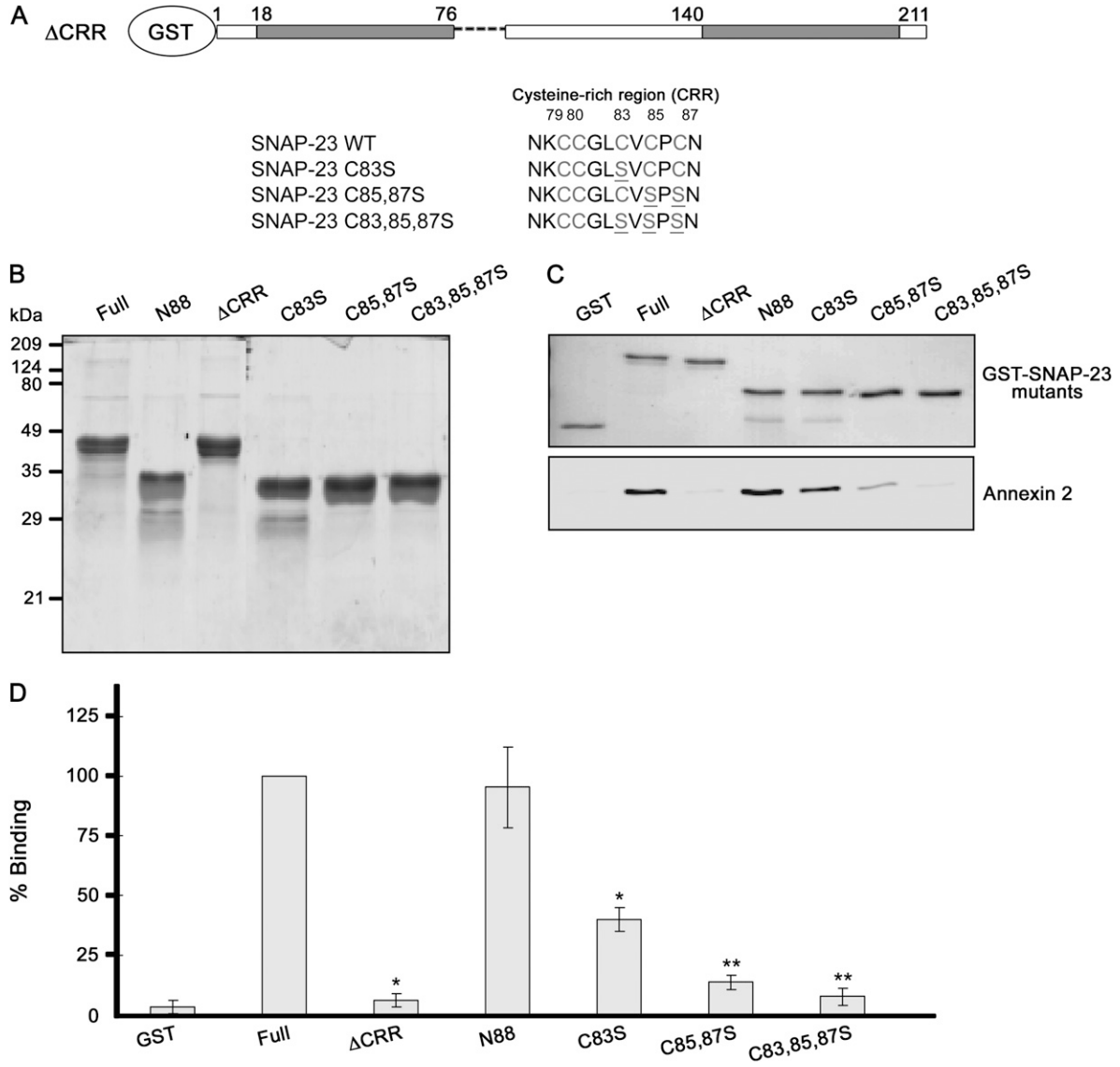

Figure 5. The cysteine residues in the cysteine-rich region of SNAP-23 are required for binding between SNAP-23 and annexin A2. (*A*) The diagrams show full-length SNAP-23 with the CRR deletion (ΔCRR) and various site-directed mutations of cysteine residues in CRR. The sequence in SNAP-23WT represents CRR from wild-type SNAP-23. The *underlined S* represents cysteine residues that were mutated into serine residues in SNAP-23 mutants. (*B*) The purity of mutant proteins as visualized by Coomassie blue staining. (*C*) Pulldown experiments were performed with 0.2 nmole of SNAP-23 mutant proteins incubated with 8 μg of AIIt in the presence of 1 mM Ca^{2+}. Recombinant proteins were stained with Ponceau S (*upper panel*) and annexin A2 was detected by Western blot (*lower panel*). (*D*) The bindings were quantitated by VersaDoc Imaging system (Bio-Rad), and the results were expressed as a percentage of full-length SNAP-23 recombinant protein. Data shown are means ± SE. *$P < 0.05$, **$P < 0.01$ versus full-length or N88 (Student's *t* test, $n = 4$).

domain, which harbors the Ca^{2+}/membrane binding sites. This feature confers annexins as potential Ca^{2+} sensors, monitoring the changes in cytosolic Ca^{2+} concentration. The sequential conformational changes upon the binding with Ca^{2+} may regulate their functions. Annexin A7 has been reported to work as a Ca^{2+} sensor in exocytotic secretion in chromaffin cells (41). The insertion of the N-terminal domain of annexin A1 into its C-terminal core domain in the absence of Ca^{2+} represents the inactive form of the protein, while the conformational switch in the presence of Ca^{2+} may trigger the exposure of some domains for additional interactions (42). Besides the conserved C-terminal domain, annexin A2 contains the phosphorylation sites and p11-binding sites at the N-terminal domain. So far, there are no reports demonstrating the interaction of SNARE proteins with any members of the annexin family. Our data showed that annexin A2 specifically interacted with SNAP-23 in a Ca^{2+}-dependent manner, suggesting that annexin A2 may be a candidate of the Ca^{2+} sensor. This Ca^{2+} requirement may be due to a similar exposure of binding site of annexin A2 induced by its binding with Ca^{2+}. Our previous study has demonstrated that only annexin A2, but not annexin A1, A3, A4, A5, or A6, is able to promote the fusion of lamellar bodies with the plasma membrane (28) and to reconstitute surfactant secretion in the permeabilized type II cells (32). In this study, we also found that only annexin A2, but not other annexins, interacted with SNAP-23. This suggests that the specific interaction between annexin A2 and SNAP-23 may be functionally relevant. Annexin A7 has been shown to be involved in surfactant secretion (43). Whether

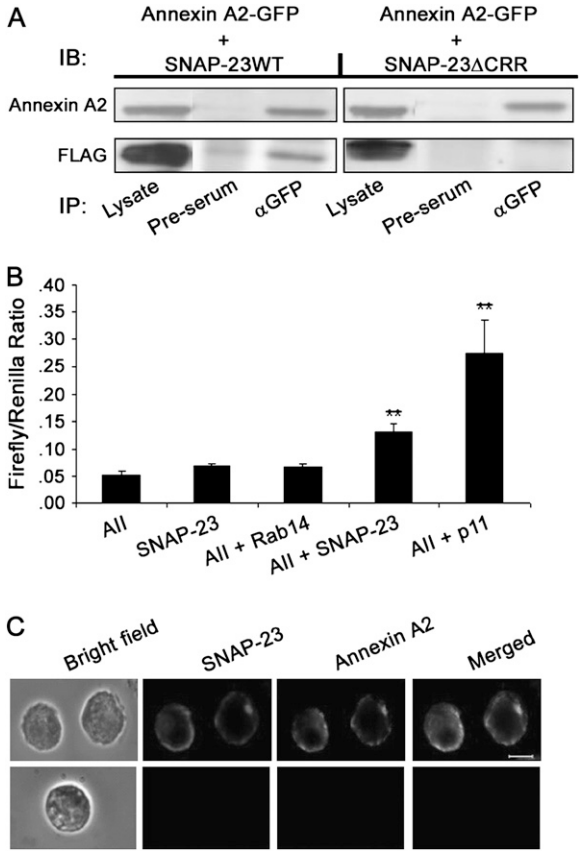

Figure 6. Interaction of SNAP-23 with annexin A2 *in vivo*. (*A*) Co-immunoprecipitation. Annexin A2-GFP and FLAG-tagged SNAP-23WT or SNAP-23ΔCRR fusion proteins were co-expressed in 293A cells, and cells were lysed 48 hours after transfection. Annexin A2-GFP was immunoprecipitated with anti-GFP antibody (α-GFP) or pre-immune serum. The immunoprecipitates were probed with anti-annexin A2 and anti-FLAG antibodies. The cell lysate was used as a positive control for Western blotting. The blot shown is a representative of three independent experiments. (*B*) Mammalian two-hybrid assay. pCMV-BD-SNAP-23, pCMV-BD-Rab14, pCMV-BD-p11, and/or pE/CMV-AII-NLS-AD were co-transfected into A549 cells. The cells were lysed 48 hours later and the dual luciferase activity was measured. Data were expressed as a ratio of Firefly/Renilla luciferase activity and shown as means ± SE. $^{**}P < 0.01$ versus negative control (Student's *t* test, $n = 4$). (*C*) Co-localization of SNAP-23 and annexin A2. Alveolar type II cells were double-labeled with anti-SNAP-23/Alexa Fluor 488 anti-mouse and anti-annexin A2/Alexa Fluor 568 anti-rabbit antibodies (*upper panels*). The controls without primary antibodies are shown in the *lower panels. Scale bar*: 10 μm.

annexin A7 interacts with SNARE proteins remains to be determined.

The *in vitro* binding of annexin A2 with SNAP-23 required a superphysiological concentration of Ca^{2+}. This raises a question whether this interaction occurs *in vivo*. We have previously shown that arachidonic acid significantly reduces the Ca^{2+} requirement for annexin A2–mediated fusion of lamellar bodies with the plasma membrane (28). It is possible that arachidonic acid or an unidentified factor may regulate the interaction of annexin A2 and SNAP-23 and its Ca^{2+} requirement *in vivo*.

In this study, the deletion of various segments of SNAP-23 identified a cysteine-rich region as the binding site of SNAP-23 to annexin A2. There are no reports about direct interactions of SNAP-25/23 with their possible regulatory proteins within this region so far. Although the cysteine-rich region is the site for palmitoylation and may facilitate the association of SNAP-25/23 to the plasma membrane (44, 45), an alternative mechanism for SNAP-25/23 association with membrane via the binding of syntaxin has been proposed (46, 47). Therefore, the cysteine-rich region is still likely available for the interaction with additional proteins *in vivo*. The cysteine residues play a role in fine-tuning the affinities of SNAP-25/23 for the plasma membrane and mutations of these residues only restore the exocytosis to different extents in a permeabilized cell system (48, 49). Another report has shown that the cysteine residue mutant of SNAP-25 still can form a SDS-resistant complex with syntaxin 1A and VAMP-2. However, this complex cannot be disrupted by NSF. Furthermore, these mutants are not able to rescue exocytosis (47). SNAP-23 has five cysteine residues within this region. The mutation of cysteine residue at 83 of SNAP-23 resulted in a significant decrease in its binding to annexin A2, and the mutations of three cysteine residues (83, 85, and 87) almost abolished the binding. These results suggest that the cysteine residues of SNAP-23 may control its interaction with annexin A2 in the process of exocytosis.

BoNT A and BoNT E cleave the C-terminal 9 and 23 amino acids of SNAP-23, respectively. Overexpression of the SNAP-25 mutant ΔC9 decreases the fast component of the exocytotic burst in bovine chromaffin cells, even though the overexpressed mutant still can assemble into SNARE complexes *in vivo* (50). Interestingly, deletion of the C-terminal 9 amino acids, but not the 23 amino acids, markedly reduced its binding with annexin

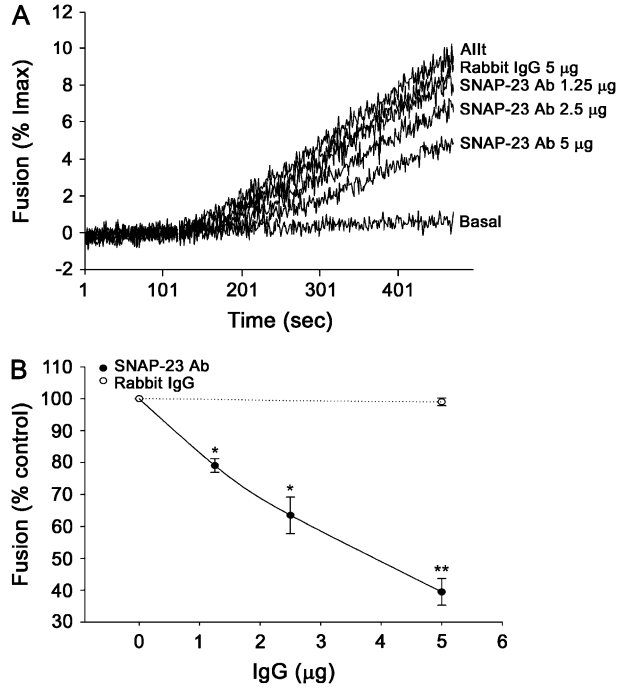

Figure 7. Anti-SNAP-23 antibody inhibits AIIt-mediated fusion between lamellar bodies and the plasma membrane. Five micrograms of protein or R-18–labeled plasma membrane were incubated with various amounts of anti–SNAP-23 antibody or rabbit IgG control at room temperature for 30 minutes and then mixed with 10 μg protein of lamellar bodies in 1 ml of Ca^{2+}-EGTA buffer (1 mM free Ca^{2+}). Ten micrograms of AIIt was added to initiate the fusion. The fusion was expressed as a percentage of the maximal fluorescence (Imax) achieved by adding 0.1% Triton X-100. (*A*) A representative of the fusion curves. (*B*) Data are expressed as a percentage of the control (the fusion content during 6 min incubation) and shown as means ± SE. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control (Student's *t* test, $n = 3$).

A2. The reason for this is unclear, but could be due to the different conformational changes caused by the two deletions. It is also possible that the 14–amino acid motif (C23-C9) is inhibitory to the binding, and that the deletion of the C-terminal 9 amino acids uncovers the inhibitory motif.

To carry out the functional study, we used a specific antibody against the C-terminal last 11 amino acids of rat SNAP-23 to block its interaction with annexin A2. This antibody recognizes SNAP-23 specifically and inhibits surfactant secretion in the permeabilized alveolar type II cells (31). In this study, when the plasma membrane was pre-incubated with the anti–SNAP-23 antibody, AIIt-mediated fusion of lamellar bodies with the plasma membrane was decreased. One possible reason is that the antibody blocks the direct interaction of SNAP-23 and annexin A2. However, we cannot rule out the possibility that the spatial hindrance from the binding of antibody interferes with the formation of functional SNARE complexes between lamellar bodies and the plasma membrane.

In summary, annexin A2 may function as a Ca^{2+} sensor and/or fusogenic proteins via its interaction with SNAP-23 in the surfactant secretion of alveolar type II cells. Annexin A2 has been reported to be involved into the Ca^{2+}-evoked exocytosis in chromaffin cells (22, 23, 32). Since annexin A2 also binds with SNAP-25, this interaction may not be unique to alveolar type II cells and annexin A2 may function in a similar way in the exocytotic secretion in some endocrine cells, such as chromaffin cells.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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