Degradation of Annexin I in Bronchoalveolar Lavage Fluid from Patients with Cystic Fibrosis

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Annexin I is a 36 kilodalton (kD) calcium-dependent phospholipid-binding protein which may have anti-inflammatory properties. Previous investigations which sampled lower respiratory tract epithelial lining fluid (ELF) via bronchoalveolar lavage (BAL) have demonstrated that annexin I can be degraded in inflammatory lung disease. We analyzed BAL fluid from patients with cystic fibrosis (CF) to determine the effects of lung inflammation on the structure and activity of annexin I. Intact annexin I was absent in 17 out of 20 BAL fluid samples from patients with CF, due largely to degradation to a 33 kD protein. The three CF BAL fluids in which annexin I was detectable had very little or no unopposed neutrophil elastase activity in contrast to the 17 in which no annexin I was detectable. Annexin I was present in all BAL fluid samples from 10 normal volunteer (NV) subjects and 12 patients with interstitial lung disease (ILD). The 33 kD annexin I breakdown product was not detectable in samples from NV, but was detectable only in ILD patients with relatively high percentages of neutrophils on BAL differential cell counts. Annexin I appeared to be cleaved by neutrophil elastase at the N-terminal portion between Val-36 and Ser-37 to yield the 33 kD protein. Cleavage of the N-terminal portion of annexin I was accompanied by a marked change in the annexin I isoelectric point (pI) value (from 6.0 to 8.5–9.0) and greatly diminished annexin I functional activity. Our findings demonstrate that annexin I degradation in epithelial lining fluid is closely related to lung inflammation. **Tsao, F. H. C., K. C. Meyer, X. Chen, N. S. Rosenthal, and J. Hu. 1998. Degradation of annexin I in bronchoalveolar lavage fluid from patients with cystic fibrosis. Am. J. Respir. Cell Mol. Biol. 18:120–128.**

Annexins are a group of calcium-dependent phospholipid-binding proteins (1). These proteins are widely distributed in eucaryotes with at least nine members of the annexin family of proteins having been identified in mammalian tissues. The calcium- and phospholipid-binding sites of most annexins are located in the four repeated and highly conserved regions each of which contains about 70 amino acids with the exception of annexin VI, which has a molecular weight of 68 kD and contains eight repeated regions. The amino terminal segment is unique for each member of the annexin family of proteins and may be related to each annexin's specific biological function (1). Although multiple cellular functions of annexins have been suggested, including activities in exocytosis (2–4), signal transduction (3, 5), anti-inflammation (6, 7), anticoagulation (8), and calcium-channel regulation (9), it is possible that each of these annexin functions is tissue-specific.

The lung is rich in annexins. Several members of the annexin family of proteins with apparent molecular weights ranging from 32 to 40 kD have been isolated from bovine (10–13), mouse (14), porcine (15), and rabbit (16) lungs. Annexin I, a 36 kD protein also widely known as lipocortin 1, appears to be the most abundant protein among the annexin family of proteins in the mammalian lung. This member of the annexin family of proteins was first isolated from rat peritoneal exudate (17). Annexin I has been alleged to participate in anti-inflammation associated with glucocorticoid administration (18–22) and to antagonize pyrogenic activity of cytokines (23–25). In the lung, annexin I has been found in alveolar epithelial type II cells which synthesize, store, and secrete pulmonary surfactant complex (26–28) and in alveolar macrophages (29). The abundance of annexin I in type II cells and the increase in annexin I in fetal rabbit lungs at late gestational ages suggest a role for annexin I in lung development (28). In addition to the intracellular localization of annexin I in alveo-

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Abbreviations: bronchoalveolar lavage, BAL; cystic fibrosis, CF; dimethyl sulfoxide, DMSO; ethylenediaminetetraacetic acid, EDTA; epithelial lining fluid, ELF; N-2-hydroxyethylpiperazine-N′-ethane sulfonic acid, Hepes; high-performance liquid chromatography, HPLC; isoelectric focusing, IEF; interstitial lung disease, ILD; kilodalton, kD; normal volunteer, NV; phenylmethyl sulfonyl fluoride, PMSF; sodium dodecyl sulfate polyacrylamide gel electrophoresis, SDS-PAGE.

lar type II cells and macrophages, this protein has been found in bronchoalveolar lavage (BAL) fluids from humans (30, 31) and animals (27, 28). Annexin I levels in BAL fluid from humans increased in response to corticosteroids, suggesting a role for this protein in inflammation suppression (30). Annexin I degradation has often been observed in some of the BAL fluid samples from patients with various lung diseases (30, 31). This degradation has been suggested to be mediated by neutrophil elastase in epithelial lining fluid, demonstrating the linkage between annexin I degradation and lung inflammation (31). Lung disease in cystic fibrosis (CF) is characterized by intense, neutrophil-dominated inflammation. Lower respiratory tract secretions contain high amounts of proteases, particularly the elastase from neutrophils and from the *Pseudomonas aeruginosa* which infect the respiratory tract of most CF patients (32). These proteases may degrade proteins including annexins in bronchi and bronchioles. In view of the role of annexin I in anti-inflammation, we conducted a thorough biochemical analysis of the effects of lung inflammation on the properties of annexin I structure and activity in BAL fluid from patients with CF.

Materials and Methods

Patient Population

Bronchoalveolar lavage fluid was obtained from normal volunteer (NV) subjects, patients with CF, and patients with interstitial lung disease (ILD). All normal volunteer subjects had unremarkable medical histories, normal physical examination, normal spirometry, and no symptoms of an upper respiratory infection in the 4-wk period prior to participation in the study. Patients with interstitial lung disease had specific diagnoses made by clinical and histopathologic criteria. No study subjects had a history of tobacco smoking. All study protocols were approved by the University of Wisconsin Human Subjects Committee.

The patients with CF were hospitalized at the University of Wisconsin Hospital for subacute exacerbations of their lung disease or seen in the Adult Cystic Fibrosis Center Outpatient Clinic with stable chest symptoms and objective findings. The diagnosis of CF was established by typical clinical manifestations of the disease and confirmed by positive sweat tests in all CF patients. The CF patients ranged in age from 17 to 42 yr and were chronically infected with *Pseudomonas aeruginosa.*

Bronchoalveolar Lavage

Normal volunteers and patients were subjected to bronchoscopy and BAL as previously described (33). Intravenous access was maintained throughout the procedure in patients with CF and ILD, and oxygen was given as indicated by continuous oximetric monitoring. Atropine (0.6 mg) or glycopyrrolate (0.2 mg) was given intravenously or intramuscularly, and midazolam was administered intravenously or intramuscularly at the beginning of and during the procedure as needed. Upper-airway anesthesia was obtained with 4% aerosolized lidocaine to the upper airways and posterior pharynx. The fiberoptic bronchoscope (Olympus BR 4B; Olympus Corp. of America, New Hyde Park, NY) was passed nasally unless anatomical problems necessitated passage via the oropharynx, lidocaine (1%) was delivered via the bronchoscope onto the epiglottis and vocal cords prior to passage into the trachea, and 1 to 3 ml of lidocaine (1%) was administered to the tracheobronchial tree just prior to obtaining the wedge position. Bronchoalveolar lavage was performed in all subjects by wedging the bronchoscope into a segmental bronchus of the right middle lobe or lingula. Four 60-ml aliquots of sterile normal saline were injected and gently aspirated via hand-held syringe. BAL fluid was filtered through one layer of a loose, sterile gauze, then centrifuged at 400 $\times$ *g* for 10 min at 22°C. Aliquots of pooled BAL supernatant fluid were immediately frozen at −70°C until assayed. An additional aliquot of BAL fluid from CF patients was set aside prior to filtration and treated with N-acetyl-cysteine to disperse mucus for subsequent quantitative aerobic bacterial culture. Air-space cells were resuspended in Hanks' balanced salt solution (HBSS). A hemocytometer and cytocentrifuge preparations of the cell suspensions were used to obtain total and differential cell counts.

Bronchoalveolar lavage fluids were concentrated 5- to 10-fold by centrifugation using AmiconCentricon 10 filters (molecular weight cut-off of 10 kD) (Amicon, Beverly, MA). Two-ml aliquots of BAL fluid were placed in the filter and centrifuged at 3,000 $\times$ *g* at 4°C. Fluid retained by the filter was saved for further analysis.

Determination of Neutrophil Elastase Activity

Neutrophil elastase activity was determined spectrophotometrically at 410 nm using a specific substrate, MeO-Suc-Ala-Ala-Pro-Val-pNA, and inhibitor, MeO-Suc-Ala-Ala-Pro-Val-CH_2Cl as previously described (33). Initial velocity assays were performed in 1 ml of 0.10 M N-2-hydroxyethylpiperazine-N′-ethane sulfonic acid (Hepes), 0.5 M NaCl, 5% DMSO, 0.5 mM substrate with or without 0.10 mM inhibitor, pH 7.5 at 37°C. Optical density changes were converted to molar concentrations using an extinction coefficient of 8,800 $L\text{-}mol^{-1}cm^{-1}$. Positive and negative controls were analyzed with each set of assays. Specific activity is expressed as nanomoles of peptide hydrolyzed/min/ml BAL fluid.

Isolation of Lung Surfactant and Annexin I

Lung surfactant and annexin I were isolated from two adult New Zealand white rabbits. Each rabbit was anesthetized with intravenous injection of sodium pentobarbital/heparin (20 mg/1,000 units per kg body weight). Tracheotomy was performed and the lung of each animal was lavaged with 20 ml of 0.9% NaCl three times through the trachea. The lavage was centrifuged at 1,000 rpm for 10 min. The pellets were lysed and the lysate was referred to as macrophage lysate. The volume of the pool of the supernatant was reduced to about 5 ml by ultrafiltration (molecular weight cut-off of 10 kD) followed by centrifugation at 46,000 $\times$ *g* for 60 min. The pellets were resuspended in 0.01 M Tris-HCl, pH 7.4, and the suspension was referred to as surfactant fraction; the 46,000 $\times$ *g* supernatant was referred to as nonsurfactant fraction.

After lavage, the lung was ventilated and the chest opened. The lung was perfused with saline through the pulmonary artery and removed for homogenization. Annexin I was isolated from the cytosolic fraction of the lungs

from the two rabbits by the methods described previously (16). Human lung annexin I was isolated from the postmortem lung tissue (about 30 g) by the same procedures.

Analysis of Annexin I

Proteins (50–100 μg) in the samples as specified were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) method under denaturing conditions, and then electrophoretically transferred onto a nitrocellulose membrane (28). Annexin I on the membrane was immunoblotted by using the polyclonal antibody raised in guinea pig against rabbit lung annexin I as first antibody, and alkaline phosphatase-conjugated goat anti-guinea pig IgG as second antibody (28). Surfactant-associated protein A (SP-A) was immunoblotted by the use of polyclonal antibody raised in rabbit against human SP-A as first antibody (kindly provided by Dr. Jeffrey A. Whitsett, Department of Pediatrics, University of Cincinnati) and alkaline phosphatase-conjugated goat anti-rabbit IgG as second antibody.

The isoelectric point (pI) values of annexin I was determined by the isoelectric focusing (IEF) method using a thin-layer IEF agarose gel with pH range between 3 and 10 (10 × 12.5 cm; FMC, Rockland, ME). Proteins on the IEF gel were transferred to a nitrocellulose membrane by capillary force according to the protocol of the manufacturer, and annexin I on the membrane was analyzed by Western blot as described earlier. As specified, in some studies proteins on the IEF gel were visualized by Coomassie brilliant blue staining (16).

Effects of BAL Fluid from CF Patients on the Activity and Structure of Annexin I

An amount of purified rabbit lung annexin I was incubated with CF BAL fluid samples in a ratio of 1 μg annexin I/20 μg BAL protein in 10 μl of 0.01 M Tris-HCl, pH 7.4 (for Western blot as described previously), or in a ratio of 10 μg annexin I/100 μg BAL protein in 50 μl of 0.01 M Tris-HCl, pH 7.4 (for annexin I activity measurement), at 37°C for 1 h. The annexin I activity was determined by measuring the aggregation of ^{14}C-labeled phosphatidylcholine (PC) unilamellar liposomes and negatively charged multilamellar liposomes made of PC, cholesterol, and dicetyl phosphate (16). Also, effect of annexin I degradation on phospholipase A_2 activity was studied. The phospholipase A_2 reaction mixture in 0.1 ml of 0.01 M Tris-HCl buffer contained 10 mM $CaCl_2$, 5 nmol unilamellar liposomes made of dioleoyl PC and phosphatidylglycerol (PG) (50%-50%, by wt) labeled with L-α-[1-^{14}C]dioleoyl PC (Du Pont-NEN, Wilmington, DE) in the absence or presence of 10 μg annexin I. An amount of 0.5 μg porcine pancreatic phospholipase A_2 (Sigma) was introduced to the reaction mixture, and the reaction was carried out at room temperature for 30 s (34). Lipids were extracted, phospholipase A_2 activity was expressed as either decrease in PC radioactivity or increase in fatty acid or lysoPC radioactivity as described previously (34). In some of the studies, annexin I was preincubated in saline or CF BAL fluid containing 100 μg protein prior to its addition to the phospholipase A_2 reaction mixture.

In a separate experiment, 0.2 mg of purified rabbit lung annexin I was incubated with BAL fluid containing 0.42 mg total proteins in 0.3 ml of 0.01 M Tris-HCl, pH 7.4, at 37°C for 2 h. After reaction, the reaction mixture was centrifuged at 100,000 × *g* for 10 min. Annexin I in the supernatant was isolated by HPLC C4 Vydac reverse phase (34). The purity and molecular weight of annexin I obtained from HPLC reverse phase column were examined by SDS-PAGE as described above. The N-terminal sequence of annexin I from HPLC reverse phase was determined using an automated Model 477A Liquid Pulse Sequencer and Model 475A Gas Phase Sequencer with on-line Model 120A PTH Analyzer and 610A Data Analysis System (Applied Biosystems, Foster City, CA).

Results

Western Blot Analysis of Annexin I and SP-A in Lung Lavage Fractions

The distribution of annexin I in surfactant and non-surfactant fractions in lung lavage fluid was analyzed. After the lung lavage fluid was fractionated into surfactant and nonsurfactant fractions, annexin I was predominately found in the nonsurfactant fraction (Figure 1). Only a trace amount of annexin I was in the surfactant fraction, which was distinguished from nonsurfactant fraction by the immunoblot of SP-A, a surfactant-associated protein (Figure 1). The rab-

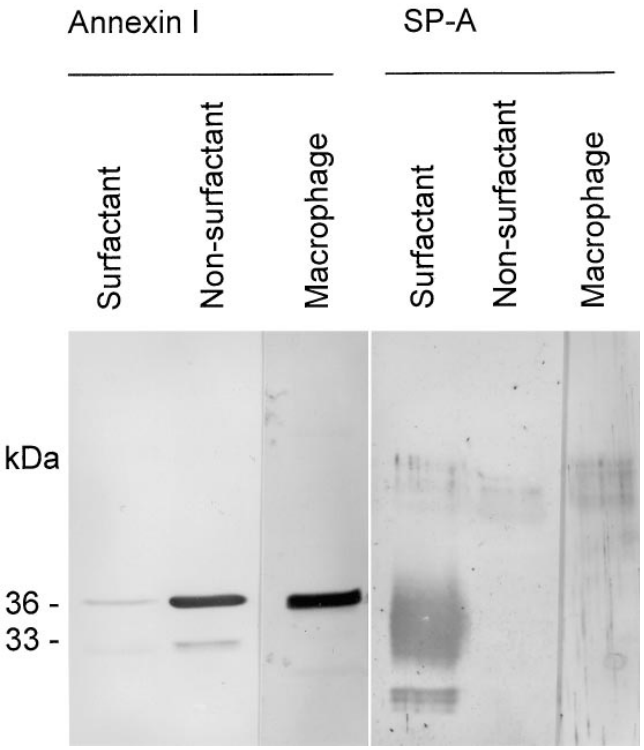

Figure 1. Western blot of annexin I and SP-A in lung lavage fluid from rabbits. Annexin I (*left panel*) represents immunoblot by antibodies against annexin I; Sp-A (*right panel*) represents immunoblot by antibodies against SP-A. Surfactant and nonsurfactant represent the fractions of the 46 × *g* pellet and supernatant, respectively, isolated from BAL fluid. The fraction of macrophage lysate was used in this analysis. The amount of protein in each sample was 50 μg.

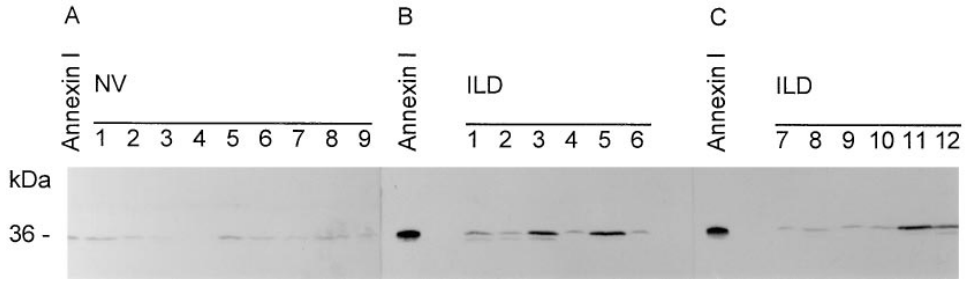

Figure 2. Western blot analysis of annexin I in BAL fluid from normal volunteers (NV) and patients with interstitial lung disease (ILD). Each lane represents BAL fluid sample from individual subject. The ILD lane number corresponds to the Subject number in Table 1. An amount of 0.1 mg of total protein in each BAL fluid sample was employed for analysis. Purified rabbit lung annexin I (0.1 or 0.5 μg) was used as reference.

bit alveolar macrophage lysate also contained annexin I, similar to that found in the human alveolar macrophages (29).

Western Blot Analysis of Annexin I in Human BAL Fluid

With the use of 100 μg total BAL fluid proteins, annexin I was detected in BAL fluid samples from normal volunteers by Western blot (Figure 2A). Annexin I in one of the 9 normal volunteer BAL samples was only barely detected (Figure 2A, NV lane 4). Annexin I also was present in all 12 BAL fluid samples from patients with interstitial lung diseases. Small amounts of an immunoreacted protein with molecular weight around 33 kD also were observed in some of the samples (Figures 2B and 2C). It appeared that the BAL fluid samples of patients with interstitial lung disease, which had about 20% or more neutrophils on the differential cell count (Table 1; Subjects 1, 2, 3, and 12), also contained small amounts of the 33 kD protein (Figures 2B; ILD lanes 1, 2, 3, and 2C; ILD lane 12, respectively). In contrast, in 20 BAL fluid samples from CF patients, 17 samples had annexin I absent (Figure 3). In 11 of the 17 samples with annexin I absent, the only immunoreactive protein was the 33 kD protein. The other 6 among the 17 BAL fluid samples had no detectable immunoreactive proteins at all (Figure 3; CF BAL C lanes 3, 4, 6, and 7; CF BAL D lanes 2 and 3). Among the 20 CF BAL specimens, only three samples had annexin I, but two of these three samples also contained the immunoreactive 33 kD protein (Figure 3; CF BAL B lane 5 and CF BAL C lanes 2 and 5). Two of the three CF BAL samples that contained annexin I also had lower neutrophil elastase activity (Table 2; CF Subjects 7 and 1‡). All the BAL fluid of CF patients contained neutrophils over 10^5 cells/ml (Table 2), compared with the very low numbers of neutrophils in normal volunteers and patients with interstitial lung diseases (Table 1). One additional BAL fluid from a normal volunteer contained annexin I (Figure 3; CF BAL D lane 5).

Conversion of Annexin I to 33 kD Protein by CF BAL Fluid and Elastase

The incubation of purified rabbit lung annexin I (1 μg) with four different CF BAL fluid samples (20 μg protein) yielded a 33 kD protein which was immuno-recognized by the antibody (Figure 4A, lanes 2–5). The 33 kD protein was solely derived from substrate of rabbit lung annexin I since the four CF BAL samples employed in the tests contained only 20 μg of total proteins in which little annexin I and 33 kD protein could be detected (Figure 4A, lanes 6–9). Under the reaction conditions, three BAL fluid samples converted most annexin I to the 33 kD protein (Figure 4A,

TABLE 1

Demographic and bronchoalveolar lavage analytical data for patients with interstitial lung disease

Subject		Disease Type	Cells/ml	% Mac	% Lymph	% Neut	% Eos
	Figure 2B						
1	ILD lane 1	IPF	180	70	9.3	19	1.3
2	ILD lane 2	IPF	90	61	6	32	1.0
3	ILD lane 3	IPF	80	52	8	22	18
4	ILD lane 4	IPF	160	84.7	3.3	9	3.0
5	ILD lane 5	IPF	460	89	4.3	0.3	6.3
6	ILD lane 6	IPF	390	89.7	0.3	4	6.0
	Figure 2C						
7	ILD lane 7	SC	670	81.3	17.7	0.7	0.3
8	ILD lane 8	SC	310	74.6	20.9	3.9	0.6
9	ILD lane 9	IPF	420	50	22.7	14.3	12.0
10	ILD lane 10	SC	403	88	11.7	0.3	0
11	ILD lane 11	IPF	150	89	3.7	2.7	0
12	ILD lane 12	SC	250	54.7	23	21	0.3
NV			105 ± 12	85 ± 1.6	12.4 ± 1.6	2.4 ± 0.5	0.3 ± 0.2

Mac = macrophage; Lymph = lymphocyte; Neut = neutrophil; NV = normal volunteer; Eos = eosinophil.

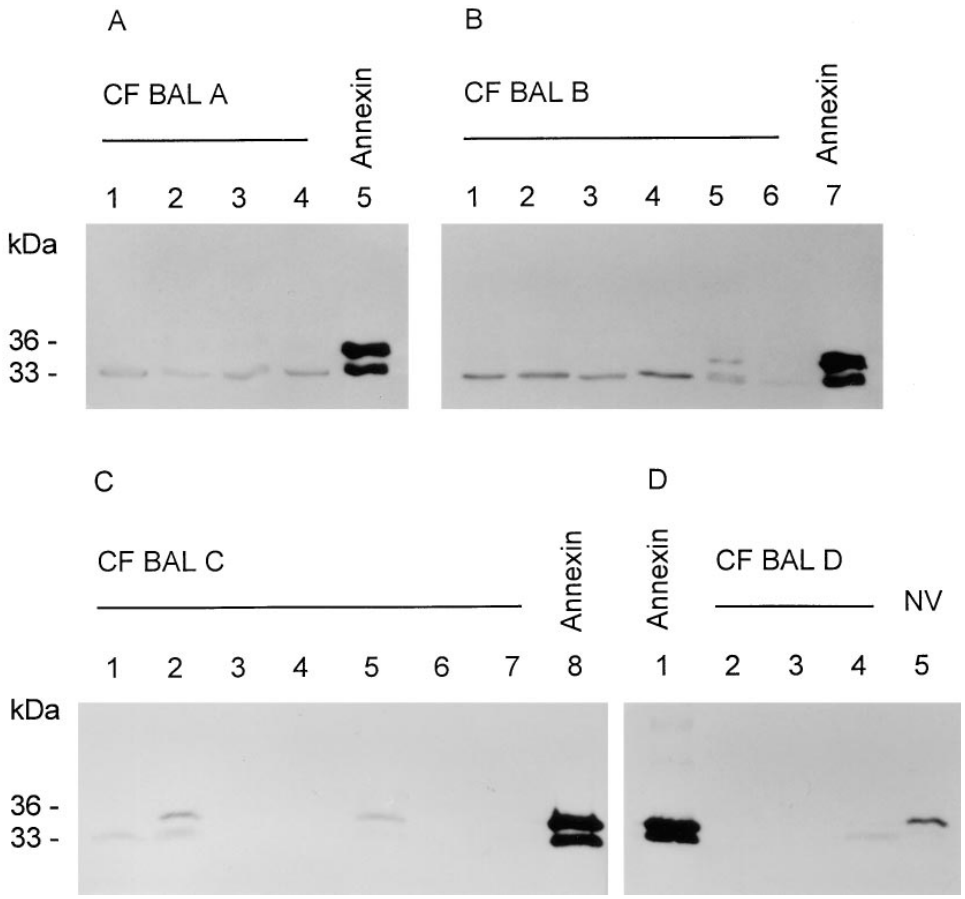

Figure 3. Western blot analysis of annexin I in BAL fluid from patients with CF. Each CF BAL lane corresponds to a Subject number in Table 2. An amount of 0.1 mg of total protein in each BAL fluid sample was used for analysis; 0.5 μg rabbit lung annexin I and its breakdown product were used as references.

lanes 2, 3, and 5), whereas one BAL fluid sample degraded less annexin I to the 33 kD protein (Figure 4A, lane 4). Interestingly, this one CF BAL fluid sample also contained both endogenous annexin I and 33 kD protein (CF 7 in Figure 3, CF BAL B lane 5), whereas the other three CF BAL samples used in the reactions (Figure 4A, lanes 2 [CF1^e], 3 [CF6], and 5 [CF5]) had no annexin I but only the 33 kD protein (Figure 3; CF BAL B lanes 1, 3, and 4, respectively).

The porcine pancreatic elastase (Sigma) also degraded rabbit lung annexin I to 33 kD protein which was immunorecognized by the antibody to annexin I (Figure 4B). Further degradation of the 33 kD annexin I breakdown product by elastase appeared to continue, which might result in a weak band intensity (Figure 4B, lane Annexin I + elastase). The presence of phenylmethyl sulfonyl fluoride (PMSF) in the reaction solution totally inhibited the proteolytic hydrolysis of annexin I catalyzed by elastase (Figure 4B). To determine whether the degradation of annexin I in CF BAL fluid was caused by metalloelastase, EDTA was added to the reaction mixture in a final concentration of 2 mM. EDTA did not inhibit the degradation of annexin I induced by CF BAL fluid (Figure 4C). Under the same incubation conditions, normal volunteer BAL fluid did not degrade annexin I (Figure 4C).

The rabbit lung annexin I breakdown product, the 33 kD protein, generated by CF BAL fluid sample (CF6) (Figure 3, CF BAL B lane 4) had a basic isoelectric point value with pI at around 8.5 (Figure 5, *left panel* lane 2), markedly different from pI 6.0 of annexin I (Figure 5, *left panel* lane 1). Similarly, the endogenous annexin I breakdown product in the CF BAL fluid samples (CF5 and CF6) had pI around 9 (Figure 5). The crescents shown in Figure 5 *left panel* lane 3 (CF5 BAL fluid) and lane 4 (CF6 BAL fluid) at pI 6.0 were probably caused by denatured proteins precipitated at the spots where samples were applied onto the agarose gel. The 33 kD annexin I breakdown product produced from annexin I degradation by elastase also had a pI value of 8.5 (Figure 5, *right panel*).

The incubation of purified rabbit lung annexin I with CF BAL fluid samples in which annexin I was absent (Figure 3; CF BAL B lanes 1, 3, and 4) and elastase activity was high (Table 2; CF subjects 1*, 5, and 6, respectively) decreased annexin I activity in liposome aggregation (Table 3). In contrast, the incubation of rabbit lung annexin I with a CF BAL sample in which endogenous annexin I was present (Figure 3; CF BAL B lane 5) and elastase activity was low (Table 2; CF subject 7) did not affect annexin I activity (Table 3). Annexin I has been suggested as a phospholipase A_2 inhibitor (17). Degradation of annexin I induced by the CF BAL fluid also diminished annexin I inhibitory action on phospholipase A_2 activity (Table 4).

Isolation and Characterization of Annexin I Breakdown Product

Further purification of the isolated rabbit lung annexin I, annexin I breakdown product generated by incubation of

TABLE 2

Clinical characteristics and bronchoalveolar lavage data for patients with cystic fibrosis

CF Subject		Age	Sex	FEV_1%	Neut/ml	CFU	NE
	Figure 3						
1	CF BAL A lane 1	20	M	65	1.0	0	24
2	CF BAL A lane 2	18	M	40	10.0	14.0	70
3	CF BAL A lane 3	25	M	33	7.8	3.0	185
4	CF BAL A lane 4	20	F	45	8.0	0	85
1*	CF BAL B lane 1	19	M	68	21.6	20.3	98
1*	CF BAL B lane 2	19	M	70	3.6	1.0	249
5	CF BAL B lane 3	22	M	36	9.2	0	109
6	CF BAL B lane 4	35	F	34	12.6	1.0	341
7	CF BAL B lane 5	17	M	34	9.3	0.1	0.9
5†	CF BAL B lane 6	22	M	40	9.2	0	109
1‡	CF BAL C lane 1	22	M	61	0.8	1.5	23
1‡	CF BAL C lane 2	22	M	64	2.5	0.8	0.9
8§	CF BAL C lane 3	42	M	30	2.4	3.1	75
8§	CF BAL C lane 4	42	M	29	47.0	20	895
1‖	CF BAL C lane 5	23	M	62	0.03	7	1.1
1‖	CF BAL C lane 6				0.4	80	50
1‖	CF BAL C lane 7				2.5	80	279
2¶	CF BAL D lane 2	20	M	46	8.4	2.6	1,163
2¶	CF BAL D lane 3				3.4	10.5	522
2¶	CF BAL D lane 4				0.3	0.01	14

FEV_1% = forced expiratory volume in one second as percent of normal predicted value; Neut/ml = total neutrophils $\times 10^5$/ml BAL fluid; CFU/ml = colony forming units of aerobic bacteria $\times 10^5$/ml BAL fluid; isolates were predominantly *P. aeruginosa*; NE = neutrophil elastase activity as nmol peptide hydrolyzed/min/ml BAL fluid.

* Subject 1 before and after (Figure 3; CF BAL B lanes 1 and 2, respectively) a 2-wk course of anti-pseudomonal antibiotics.

† Subject 5 (Figure 3; CF BAL B lane 6) on when undergoing a second BAL 7 months removed from the first BAL.

‡ Subject 1 before and after (Figure 3; CF BAL C lanes 1 and 2, respectively) receiving a specific inhibitor of neutrophil elastase for 7 days.

§ Subject 8 sampled as a stable outpatient and 7 days later (Figure 3; CF BAL C lanes 3 and 4, respectively) without receiving any specific therapy.

‖ Subject 1 undergoing BAL of multiple lung regions simultaneously; right upper lobe, middle lobe, and lower lobe (Figure 3; CF BAL C lanes 5, 6, and 7, respectively).

¶ Subject 2 undergoing BAL of multiple lung regions simultaneously; right upper lobe, middle lobe, and lower lobe (Figure 3; CF BAL D lanes 2, 3, and 4, respectively).

rabbit lung annexin I and CF BAL fluid, and the isolated human lung annexin I each had a single major peak eluted at 35 min from HPLC reverse phase column. Each annexin product showed a single polypeptide on the SDS gel with an apparent molecular weight of 36 kD (rabbit lung annexin I) and 33 kD (rabbit lung annexin I breakdown product and human lung annexin I) (data not shown). The 15 amino acid residues determined for the N-terminal sequence of the 33 kD protein derived from rabbit lung annexin I matched the amino acid sequence between Ser-37 and Leu-51 of human annexin I whose entire amino acid sequence had been deduced from cDNA (6) (Table 5). Among the 15 amino acids, Thr-41 in human annexin I sequence was replaced with Ala in the 33 kD protein and Asp-47 in human annexin I sequence could not be determined for the 33 kD annexin I breakdown product by protein sequencing. The determined partial amino acid sequence of the 33 kD annexin I breakdown product was identical to that of rabbit lung annexin I deduced from cDNA (GenBank, accession No. U24656). Eleven amino acid residues had been determined for the human annexin I isolated by HPLC reverse phase. These 11 amino acid residues were identical to the known human annexin I sequence between Pro-38 and Ala-49, except Asp-47 was not able to be determined via protein sequencing (Table 5). These results indicate that 37 amino acid residues of the human annexin I N-terminus were depleted during protein isolation.

Discussion

Although annexin I is a phospholipid-binding protein and lung lavage fluid contains large amounts of surfactant phospholipids, the results of this study clearly showed that annexin I in lung lavage fluid was not associated with surfactant phospholipids. The source(s) of annexin I in BAL fluid is not known. It might be derived from alveolar type II cells (26–28) or macrophages (29). Annexin I has also been found on the cell surface of BAL cells (35). In inflamed lung, annexin I in neutrophils (36) may also contribute to a certain extent of this protein in the BAL fluid.

The molecular weight of human annexin I calculated from the amino acid sequence deduced from cDNA is 38,712.16 (6). Recently, we have cloned and sequenced the rabbit annexin I cDNA; the deduced protein sequence has 346 amino acids with a calculated molecular weight of 38,831.28 (unpublished data; sequence has been deposited in GenBank; accession No. U24656), similar to the annexin I of humans (6). The apparent molecular weight of rabbit lung annexin I was 36 kD as vigorously examined by SDS

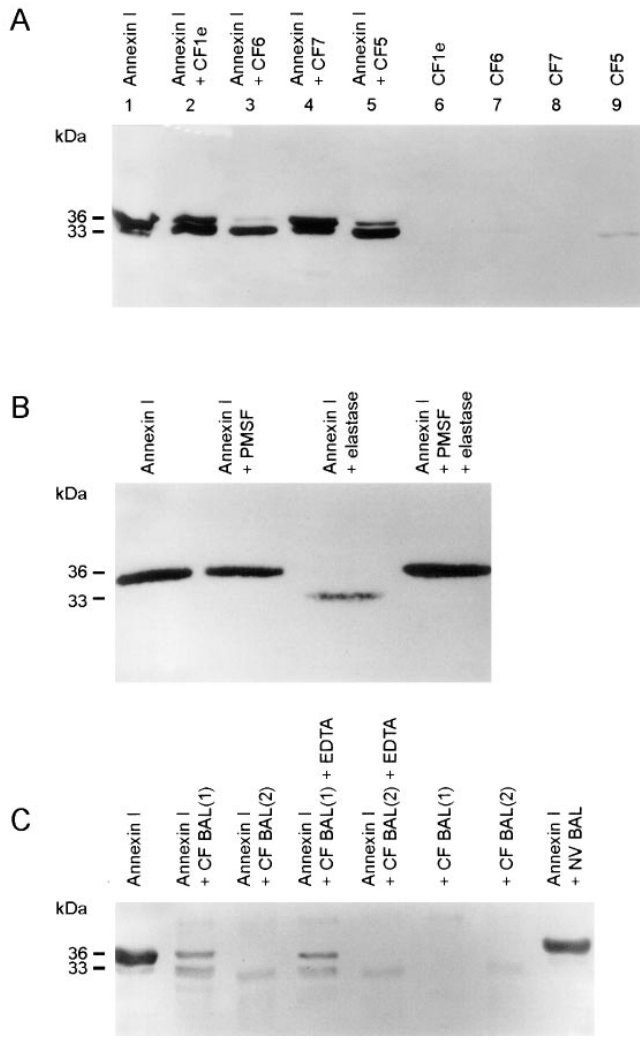

Figure 4. Western blot analysis of the degradation of rabbit lung annexin I by BAL fluid from CF patients (*A*) and (*C*) or elastase (*B*). (*A*) An amount of 1 μg purified rabbit lung annexin I was incubated with CF BAL fluid which contained 20 μg total protein in 10 μl of 0.01 M Tris-HCl, pH 7.4, at 37°C for 1 h. Proteins in the reaction mixture were analyzed using SDS-PAGE and Western blot. Lane 1 is the control reaction mixture which contained rabbit lung annexin I but no BAL fluid; lanes 2–5 represent four different reaction mixtures; each contained rabbit lung annexin I and different CF BAL fluid samples. Lanes 6–9 represent the four reaction mixtures which contained the corresponding BAL fluid samples as Lanes 2–5 but no rabbit lung annexin I. The CF BAL fluid samples were those shown in Figure 3, CF BALB lanes 1 (CF1*), 4 (CF6), 5 (CF7), and 3 (CF5). (*B*) An amount of 0.1 μg of rabbit lung annexin I was incubated in 13 μl of 0.01 M Tris-HCl, pH 7.4 in the absence or presence of 50 ng of elastase of 7.7 mM PMSF at 37°C for 1 h. (*C*) An amount of 0.5 μg or rabbit lung annexin I was incubated in buffer in the absence or presence of CF BAL fluid or NV BAL fluid containing 10 μg of protein or 2 mM EDTA under similar conditions as described in Figure 4 (*A*). The number shown in parentheses of CF BAL(1) and CF BAL(2) was arbitrarily assigned for two different BAL fluid samples.

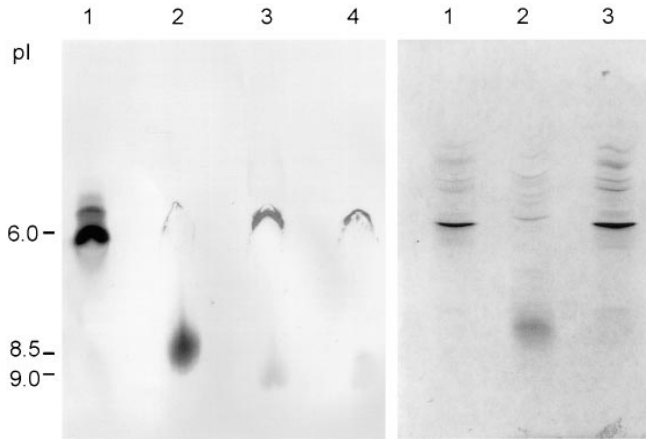

Figure 5. Isoelectric focusing of annexin I and annexin I breakdown products. *Left panel:* Annexin I and annexin I breakdown products were visualized by Western blot after isoelectric focusing as described in the text. Lanes 1–4 represent the purified rabbit lung annexin I (1 μg), annexin I (1 μg) + CF6 BAL fluid (20 μg proteins) at 37°C for 1 h, CF5 BAL fluid (50 μg protein), and CF6 BAL fluid (50 μg protein), respectively. *Right panel:* Proteins were visualized by Coomassie brilliant blue staining after isoelectric focusing. Lanes 1–3 represent rabbit lung annexin I (5 μg), annexin I (5 μg) + elastase (50 ng) in 0.01 M Tris-HCl, pH 7.4 buffer at 37°C 1 h, and annexin I (5 μg) in buffer at 37°C 1 h, respectively. Minor bands shown above annexin I were probably annexin I isoforms (28).

gel (16, 28). The observed molecular weights of human annexin I have also been reported to be around 35–37 kD (30, 31). The difference in molecular weights between the calculated and experimentally determined values might be caused by protein charge effect on the migration of the protein on SDS gel (37). To be consistent with the observed molecular weight, we refer to the lung annexin I as a 36 kD protein in this study, because annexin I was mostly analyzed by SDS-PAGE and Western blot.

Annexin I was present in all BAL fluid samples from normal volunteers. A few of these samples had only trace

TABLE 3

Inhibition of rabbit lung annexin I activity by bronchoalveolar lavage (BAL) fluid from patients with cystic fibrosis

Reactions*	Annexin I Activity (%)†
1. Annexin I (control)	100
2. Annexin I + CF subject 1* BAL fluid	50.3
3. Annexin I + CF subject 5 BAL fluid	26.9
4. Annexin I + CF subject 6 BAL fluid	24.6
5. Annexin I + CF subject 7 BAL fluid	104

*Annexin I and BAL fluid were incubated at 37°C for 1 h prior to the annexin I activity assay as described in the text.

†Annexin I activity was determined by the aggregation of radiolabeled unilamellar liposomes to multilamellar liposomes. The results are average of duplicate. Reaction 1 (control) contained no CF BAL fluid. The clinical characteristics and BAL fluid data from CF subject 1*, 5, 6, and 7 are listed in Table 2, and the annexin I results of these fluids are shown in Figure 3; CF BAL B lanes 1, 3, 4, and 5, respectively.

TABLE 4

Effect of annexin I in bronchoalveolar lavage fluid from patients with cystic fibrosis on inhibition of phospholipase A_2 activity

Reaction	Phospholipase A_2 Activity (*% PC hydrolysis*)
1. Phospholipase A_2	33.1 ± 3.3
2. Phospholipase A_2 + annexin I	14.5 ± 7.3*
3. Phospholipase A_2 + (annexin I + saline)	14.3 ± 6.8†
4. Phospholipase A_2 + (annexin I + CF BAL fluid 1)	35.6 ± 5.1
5. Phospholipase A_2 + (annexin I + CF BAL fluid 2)	37.4 ± 2.0

Phospholipase A_2 activity was determined by the percentage hydrolysis of L-α-[1-^{14}C]dioleoyl PC per 30 s as described in the text. In reactions where annexin I was present, annexin I was added to the reaction mixture prior to the addition of phospholipase A_2. The mixture of (annexin I + saline) or (annexin I + BAL fluid) was preincubated at 37°C for 1 h prior to addition to the phospholipase A_2 reaction mixture.

Results are means ± SD from 4 tests. The significant differences between Reactions 1 and 2, and Reactions 1 and 3 determined by unpaired *t* test are: $*P = 0.013$ and $^{\dagger}P = 0.0074$, respectively.

amounts of annexin I, perhaps due to variations in the concentration of proteins in BAL preparations. The finding of annexin I in BAL fluid from normal volunteers observed in this study is consistent with the previous reports that annexin I was present in lung lavage fluid from animals (27, 28) and humans (30, 31). We found little degradation of annexin I in BAL fluid samples from normal volunteers. However, degradation of annexin I was found almost uniformly in all BAL fluid samples from CF patients. In most CF BAL fluid samples, annexin I was completely degraded to a protein which had a molecular weight of 33 kD as compared with the 36 kD annexin I. Among the CF BAL fluid samples only a few contained annexin I, but these samples also had the 33 kD protein. Although annexin I was present in all the BAL fluid samples from patients with interstitial lung diseases, some of the samples contained the 33 kD protein. It is interesting to note that among the BAL fluid samples from patients with interstitial lung diseases, the appearance of the 33 kD protein was closely associated with a relatively higher percentage of neutrophils in these samples. Since all the BAL fluid samples from CF patients contained abundant neutrophils, we concluded that the degradation of 36 kD annexin I to 33 kD protein was associated with the presence of excessive numbers of neutrophils. Likely, the higher the neutrophil elastase in the BAL fluid, the more degradation of annexin I took place. For those CF BAL fluid samples which had low elastase activity (e.g., CF7, CF1‡, and CF1‖) (Figure 3; CF BAL B lane 5 and CF BAL C lanes 2 and 5, respectively), annexin I was present in the BAL fluid. Thus, the breakdown of annexin I in BAL fluid is associated closely with lung inflammation, particularly with the presence of unopposed neutrophil elastase proteolytic activity in BAL samples from CF patients.

In addition to the correlation between neutrophil elastase activity in BAL fluid and annexin I breakdown, the proteolytic degradation of annexin I was further demonstrated by incubation of purified rabbit lung annexin I and CF BAL fluid. The degradation was likely caused by neutrophil elastase rather than by the *P. aeruginosa* metalloelastase (38, 39). This conclusion was made based on two observations—first, that CF BAL fluid contained a high level of neutrophil elastase, and second, that EDTA did not inhibit the degradation of annexin I in the annexin I-CF BAL fluid reaction mixture.

The structure of the rabbit lung annexin I breakdown product catalyzed by CF BAL fluid or elastase was similar to the human lung annexin I breakdown product in the CF BAL fluid samples, i.e., same molecular weight around 33 kD and pI between 8.5–9.0. Although the cleavage site of annexin I was determined by using rabbit lung annexin I as the substrate, both rabbit lung annexin I and human lung annexin I have nearly identical amino acid sequences in this region. Additionally, we also determined that human lung annexin I could be cleaved at Ser-37. Our findings suggest that degradation of annexin I could occur at more than one position at the N-terminus. It has been shown that the N-terminus of annexin I can be cleaved at several positions by different proteases; cathespin D, calpain, or plasmin have been demonstrated to cleave human annexin I at Trp-12, Lys-26, or Lys-29, respectively (15). The N-terminus truncated annexin I with less than 26 amino acids actually increases its binding affinity with calcium and phospholipid (40, 41), whereas a 29 amino acid-truncated annexin I decreases its liposome aggregation activity (41). Here, we also demonstrated that depletion of 36 amino acids from the N-terminus of annexin I by BAL fluid from patients with

TABLE 5

Partial amino acid sequence of annexin I and annexin I breakdown product

Rabbit lung annexin I 33 kD breakdown product*

S-P-Y-P-A-F-N-P-S-S-X-V-A-A-L-

Rabbit lung annexin I deduced from cDNA†

36 40 45 50

-V-S-P-Y-P-A-F-N-P-S-S-D-V-A-A-L-

Human lung annexin I‡

P-Y-P-T-F-N-P-S-S-X-V-A-

Human annexin I deduced from cDNA§

36 40 45 50

-V-S-P-Y-P-T-F-N-P-S-S-D-V-A-A-L-

* From rabbit lung annexin I hydrolyzed by CF BAL fluid.

† From GenBank/NCBI accession no. U24656.

‡ The N-terminus of human annexin I was probably depleted by proteolytic hydrolysis during protein isolation.

§ From reference 6.

CF markedly decreased annexin I activity in liposome aggregation and phospholipase A_2 inhibition. The diminution of annexin I inhibitory action on phospholipase A_2 activity might reduce the ability of annexin I to suppress inflammation.

Several members of the annexin family of proteins have been found in lung tissue (13). However, to date, only annexin I has been identified in BAL fluid. The unstable N-terminus of annexin I that can be readily depleted by neutrophil elastase in BAL fluid suggests that the annexin I low molecular weight breakdown product might be a possible marker of proteolytic activity in the lung.

In conclusion, annexin I in BAL fluid from patients with CF is largely degraded. The degradation is mostly due to the removal of more than 36 amino acids at the N-terminus of annexin I by neutrophil elastase to form functionally inactive 33 kD breakdown products which were eventually depleted in the BAL fluid. Clearly, the annexin I degradation in BAL fluid is closely associated with lung inflammation. In view of annexin I anti-inflammatory properties, the degradation and depletion of this protein in the airway might further enhance susceptibility to lung inflammation.

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References

1. Moss, S. E. 1992. The annexins. *In* The Annexin. S. E. Moss, editor. Portland Press, London and Chapel Hill. 1–9.
2. Creutz, C. E. 1992. The annexins and exocytosis. *Science* 258:924–931.
3. Haigler, H. T., and D. D. Schlaepfer. 1992. Annexin I phosphorylation and secretion. *In* The Annexin. S. E. Moss, editor. Portland Press, London and Chapel Hill. 11–22.
4. Creutz, C. E., C. Comera, M. Junker, N. G. Kambouris, J. R. Klein, M. R. Nelson, P. Rock, S. L. Snyder, and W. Wang. 1992. The annexins and exocytosis: some close approaches. *In* The Annexins. S. E. Moss, editor. Portland Press, London and Chapel Hill. 77–88.
5. Pepinsky, R. B. 1991. Phosphorylation of lipocortin-1 by the epidermal growth factor receptor. *Methods Enzymol.* 198:260–272.
6. Wallner, B. P., R. J. Mattaliano, C. Hession, R. L. Cate, R. Tizard, K. Sinclair, C. Foeller, E. P. Chow, J. L. Browning, K. L. Ramachandran, and R. B. Pepinsky. 1986. Cloning and expression of human lipocortin, a phospholipase A2 inhibitor with potential anti-inflammatory activity. *Nature* 320:77–81.
7. Peers, S. H., and R. J. Flower. 1990. The role of lipocortin in corticosteroid actions. *Am. Rev. Respir. Dis.* 141:S18–S21.
8. Romisch, J., and E. P. Paques. 1991. Annexins: calcium-binding proteins of multi-functional importance? *Med. Microbiol. Immunol.* 180:109–126.
9. Rojas, E., N. Arispe, H. T. Haigler, A. L. Burns, and H. B. Pollard. 1992. Identification of annexins as calcium channels in biological membranes. *Bone and Mineral.* 17:214–218.
10. Glenney, J. R., Jr., B. Tack, and M. A. Powell. 1987. Calpactins: two distinct Ca^{++}-regulated phospholipid- and actin-binding proteins isolated from lung and placenta. *J. Cell Biol.* 104:503–511.
11. Boustead, C. M., J. H. Walker, and M. J. Geisow. 1988. Isolation and characterization of two novel calcium-dependent phospholipid-binding proteins from bovine lung. *FEBS Lett.* 233:233–238.
12. Khanna, N. C., E. D. Helwig, N. W. Ikebuchi, S. Fitzpatrick, R. Bajwa, and D. M. Waisman. 1990. Purification and characterization of annexin proteins from bovine lung. *Biochemistry* 29:4852–4862.
13. Liu, L., M. Wang, A. B. Fisher, and U. J. P. Zimmerman. 1996. Involvement of annexin II in exocytosis of lamellar bodies from alveolar epithelial type II cells. *Am. J. Physiol.* 270 (*Lung Cell. Mol. Physiol.*) 14:L668–L676.
14. Errasfa, M., and F. Russo-Marie. 1988. Rapid purification of two lipocortin-like proteins from mice lung. *Biochem. Biophys. Res. Commun.* 153:1271–1275.
15. Ando, Y., S. Imamura, Y. M. Hong, K. Owada, T. Kakunaga, and R. Kannagi. 1989. Enhancement of calcium sensitivity of lipocortin I in phospholipid binding induced by limited proteolysis and phosphorylation at the amino terminus as analyzed by phospholipid affinity column chromatography. *J. Biol. Chem.* 264:6948–6955.
16. Tsao, F. H. C. 1990. Purification and characterization of two rabbit lung Ca^{2+}-dependent phospholipid-binding proteins. *Biochim. Biophys. Acta* 1045: 29–39.
17. Pepinsky, R. B., L. K. Sinclair, J. L. Browning, R. J. Mattaliano, J. E. Smart, E. P. Chow, T. Falbel, A. Ribolini, J. L. Garwin, and B. P. Wallner. 1986. Purification and partial sequence analysis of a 37-kDa protein that inhibits phospholipase A_2 activity from rat peritoneal exudates. *J. Biol. Chem.* 261: 4239–4246.
18. Flower, R. 1989. Lipocortin. *Biochem. Soc. Trans.* 17:276–278.
19. Errasfa, M., and F. Russo-Marie. 1989. A purified lipocortin shares the anti-inflammatory effect of glucocorticosteroids in vivo in mice. *Br. J. Pharmacol.* 97:1051–1058.
20. Cirino, G., S. H. Peers, R. J. Flower, J. L. Browning, and R. B. Pepinski. 1989. Human recombinant lipocortin-1 has acute local anti-inflammatory properties in the rat paw edema test. *Proc. Natl. Acad. Sci. USA* 86:3428–3432.
21. Perretti, M., and R. J. Flower. 1993. Modulation of IL-1-induced neutrophil migration by dexamethasone and lipocortin 1. *J. Immunol.* 150:992–999.
22. Duncan, G. S., S. H. Peers, F. Carey, R. Forder, and R. J. Flower. 1993. The local anti-inflammatory action of dexamethasone in the rat carrageenin oedema model is reversed by an antiserum to lipocortin-1. *Br. J. Pharmacol.* 108:62–65.
23. Carey, F., R. Forder, M. D. Edge, A. R. Greene, M. A. Horan, P. J. Strijbos, and N. J. Rothwell. 1993. Lipocortin 1 fragment modifies pyrogenic action of cytokines in rats. *Am. J. Physiol.* (*Regulatory Integrative Comp. Physiol.* 28) 259:R266–R269.
24. Davidson, J., R. J. Flower, A. S. Milton, S. H. Peers, and D. Rotondo. 1991. Antipyretic actions of human recombinant lipocortin-1. *Br. J. Pharmacol.* 102:7–9.
25. Strijbos, P. J., A. J. Hardwick, J. K. Relton, F. Carey, and J. Rothwell. 1993. Inhibition of central action of cytokines on fever and thermogenesis by lipocortin-1 involves CRF. *Am. J. Physiol.* (*Endocrinol. Metab.* 26) 263: E632–E636.
26. Ambrose, M. P., and G. W. Hunninghake. 1990. Corticosteroids increase lipocortin I in alveolar epithelial cells. *Am. J. Respir. Cell Mol. Biol.* 3:349–353.
27. Das, S. K., P. Chakrabarti, F. H. C. Tsao, T. Nayyar, and S. Mukherjee. 1992. Identification of calcium-dependent phospholipid-binding proteins (annexins) from guinea pig alveolar type II cells. *Mol. Cell. Biochem.* 115: 79–84.
28. Tsao, F. H. C., X. R. Chen, X. M. Chen, and V. X. Vu. 1994. Immunocharacterization and developmental regulation of rabbit lung calcium-dependent phospholipid-binding proteins. *Biochim. Biophys. Acta* 1213:91–99.
29. Ambrose, M. P., C. L. C. Bahns, and G. W. Hunninghake. 1992. Lipocortin I production by human alveolar macrophages. *Am. J. Respir. Cell Mol. Biol.* 6:17–21.
30. Ambrose, M. P., and G. W. Hunninghake. 1990. Corticosteroids increase lipocortin I in BAL fluid from normal individuals and patients with lung disease. *J. Appl. Physiol.* 68(4):1668–1671.
31. Smith, S. F., T. D. Tetley, A. Gus, and R. J. Flower. 1990. Detection of lipocortin 1 in human lung lavage fluid: lipocortin degradation as a possible proteolytic mechanism in the control of inflammatory mediators and inflammation. *Enviro. Health Perspec.* 85:135–144.
32. Berger, M. 1991. Inflammation in the lung in cystic fibrosis: a vicious cycle that does more harm than good? *Clin. Rev. Allergy* 9:119–142.
33. Meyer, K. C., J. R. Lewandoski, J. J. Zimmerman, D. Nunley, W. J. Calhoun, and G. A. doPico. 1991. Human neutrophil elastase and elastase/$alpha_1$-antiprotease complex in cystic fibrosis: comparison with interstitial lung disease and evaluation of the effect of intravenously administered antibiotic therapy. *Am. Rev. Respir. Dis.* 144:580–585.
34. Tsao, F. H. C., W. M. Hull, M. S. Strickland, J. A. Whitsett, T. K. F. Foo, G. Zografi, and P. M. DeLuca, Jr. 1991. Lung calcium-dependent phospholipid-binding proteins: structure and function. *Biochim. Biophys. Acta* 1081:141–150.
35. Smith, S. F., T. D. Tetley, A. K. Datta, T. Smith, A. Guz, and F. J. Flower. 1995. Lipocortin-1 distribution in bronchoalveolar lavage from healthy human lung: effect of prednisolone. *J. Appl. Physiol.* 79(1):121–128.
36. Meers, P., T. Mealy, N. Pavlotsky, and A. I. Tauber. 1992. Annexin I-mediated vesicular aggregation: mechanism and role in human neutrophils. *Biochemistry* 31:6372–6382.
37. Learmonth, M. P., S. A. Howell, A. C. M. Harris, B. Amess, Y. Patel, I. Giambanco, R. Bianchi, G. Pula, P. Ceccarelli, R. Donato, B. N. Green, and A. Aitken. 1992. Novel isoforms of CaBP 33/37 (annexin V) from mammalian brain: structural and phosphorylation differences that suggest distinct biological roles. *Biochim. Biophys. Acta* 1160:76–83.
38. Morihara, K., H. Tsuzuki, T. Oka, H. Inouie, and M. Ebata. 1965. *Pseudomonas aeruginosa* elastase: isolation, crystallization and preliminary characterization. *J. Biol. Chem.* 240:295–304.
39. Morihara, K., and H. Tsuzuki. 1975. *Pseudomonas aeruginosa* elastase: affinity chromatography and some properties as a metallo neutral proteinase. *Agric. Biol. Chem.* 39:1123–1128.
40. Wang, W., and C. E. Creutz. 1994. Role of the amino-terminal domain in regulating interactions of annexin I with membranes: effects of amino-terminal truncation and mutagenesis of the phosphorylation sites. *Biochemistry* 33:275–282.
41. Liu, L., A. B. Fisher, and U. J. P. Zimmerman. 1995. Regulation of annexin I by proteolysis in rat alveolar epithelial type II cells. *Biochem. Mol. Biol. Internat.* 36:373–381.