

Degradation of Pulmonary Surfactant Protein D by *Pseudomonas aeruginosa* Elastase Abrogates Innate Immune Function*

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The alveolar epithelium is lined by surfactant, a lipoprotein complex that both reduces surface tension and mediates several innate immune functions including bacterial aggregation, alteration of alveolar macrophage function, and regulation of bacterial clearance. Surfactant protein-D (SP-D) participates in several of these immune functions, and specifically it enhances the clearance of the pulmonary pathogen *Pseudomonas aeruginosa*, a common cause of morbidity and mortality in cystic fibrosis (CF) patients. *P. aeruginosa* secretes a variety of virulence factors including elastase, a zinc-metalloprotease, which degrades both SP-A and SP-D. Here we show that SP-D is cleaved by elastase to produce a stable 35-kDa fragment in a time-, temperature-, and dose-dependent manner. Degradation is inhibited by divalent metal cations, a metal chelator, and the elastase inhibitor, phosphoramidon. Sequencing the SP-D degradation products localized the major cleavage sites to the C-terminal lectin domain. The SP-D fragment fails to bind or aggregate bacteria that are aggregated by intact SP-D. SP-D fragment is observed when normal rat bronchoalveolar lavage (BAL) is treated with *Pseudomonas aeruginosa* elastase, and SP-D fragments are present in the BAL of CF lung allograft patients. These data show that degradation of SP-D occurs in the BAL environment and that degradation eliminates many normal immune functions of SP-D.

Surfactant protein D (SP-D)¹ is one of the four surfactant proteins that is synthesized by alveolar type II epithelial cells as a component of the lipoprotein complex known as pulmonary surfactant. Although SP-D has not been shown to participate in the surface tension-reducing properties of surfactant, it has been demonstrated to participate in the host defense functions of surfactant (1). SP-D as well as SP-A belong to the collectin family of proteins, named for their N-terminal collagen region and C-terminal lectin domain. Intact SP-D consists of four trimers, which interact in their N-terminal region to form a cruciform structure (2). The collectins are pattern recognition molecules that bind, in a calcium-dependent manner, to non-self oligosaccharides presented on the surface of many bacteria and viruses.

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¹ The abbreviations used are: SP, surfactant protein; CF, cystic fibrosis; BAL, bronchoalveolar lavage; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; cfu, colony-forming units; FITC, fluorescein isothiocyanate; FACS, fluorescence-activated cell sorting; CRD, carbohydrate recognition domain.

SP-D binds to several pathogens and in many cases enhances their phagocytosis by innate immune cells. For example, SP-D binds to Gram-negative bacteria such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, rough strains of *Escherichia coli*, and *Salmonella minnesota* (3). Additionally, SP-D has been shown to bind other pathogens including influenza virus, respiratory syncytial virus, and *Pneumocystis carinii* (4–6). Binding is mediated by interaction of the SP-D lectin domain with core oligosaccharides of lipopolysaccharide on Gram-negative bacteria or with surface glycoproteins on fungi and viruses. Binding of SP-D often results in organism aggregation, although SP-D does not aggregate *P. aeruginosa* (7). SP-D increases uptake of *P. aeruginosa* and a variety of other pathogens by alveolar macrophages *in vitro* (7). Furthermore, infection of SP-D null mice with either Group B *Streptococcus* or *Haemophilus influenzae* results in decreased bacterial uptake by alveolar macrophages versus wild-type controls (8). Furthermore, SP-D null mice fail to clear influenza virus as effectively as controls (9).

Pseudomonas aeruginosa is a Gram-negative bacterium that is the predominant cause of morbidity and mortality in patients with cystic fibrosis (CF) (10). *P. aeruginosa* infections are characterized by a mucoid biofilm consisting mainly of secreted oligosaccharides. The lipopolysaccharide expressed by mucoid *P. aeruginosa* is of the rough phenotype and is less cytotoxic than that of other Gram-negative bacteria (11). Virulence is induced by *P. aeruginosa* via secretion of several enzymes, the most cytotoxic of which is exotoxin A, which directly inhibits protein synthesis (12). In addition to this toxin, *P. aeruginosa* secretes several proteases including elastase (Las B), protease IV, Las A protease, and alkaline protease (13, 14).

Surfactant protein composition is altered in cystic fibrosis patients. Several reports have shown that whereas phospholipids levels are largely unchanged, the levels of both intact SP-A and SP-D are decreased (15–17). Surfactant protein levels have also been shown to be altered in a rat model of *P. aeruginosa* infection, although SP-D levels were not measured (18). We have previously shown *P. aeruginosa* elastase degrades both SP-A and SP-D and that SP-A degradation fragments are present in the bronchoalveolar lavage (BAL) of cystic fibrosis patients after lung transplant (19). Detectable levels of *P. aeruginosa* elastase have been found in the BAL and serum of CF patients (20). Furthermore, histologic studies have detected abnormal elastin fibers in lung alveoli of CF patients on autopsy (21), and *P. aeruginosa* elastase activity was detectable in sputum samples. These data suggest that SP-D is probably cleaved by elastase *in vivo* during the progression of CF.

We propose that *P. aeruginosa* elastase cleaves SP-D to a stable 35-kDa fragment, which has impaired immune regulatory function. To test this hypothesis, we purified both *P. aeruginosa* elastase by ion exchange chromatography and its 35-kDa SP-D cleavage product by gel filtration chromatogra-

phy. The cleavage site was mapped by Edman degradation. Purified SP-D fragment failed to aggregate, bind, or enhance the phagocytosis of several Gram-negative bacteria including *P. aeruginosa*, *E. coli*, and *Salmonella typhimurium*. These studies demonstrate that the stable, 35-kDa fragment of SP-D, produced by degradation by *P. aeruginosa* elastase, has altered ability to regulate innate immunity.

EXPERIMENTAL PROCEDURES

Animals and Reagents—All experiments utilizing primary culture cells were conducted with pathogen-free male Sprague-Dawley rats (Taconic, Germantown, NY) ranging from 2 to 4 months of age. *P. aeruginosa* isolated from the sputum of CF patients was obtained from the Clinical Microbiology Laboratories at Duke University (S470); PA01 was kindly provided by Dr. Paul Phibbs (Eastern Carolina University, Greenville, NC); PA01 and PA01-B1 isolates were obtained from Dr. Barbara Iglewski (University of Rochester, Rochester, NY). *S. typhimurium* was obtained from Dr. Ken Sanderson (University of Calgary, Calgary, Alberta). *E. coli* HB101 was obtained from Dr. Clifford Harding (Case Western Reserve University, Cleveland, OH). All chemicals were purchased from Sigma except where noted.

Purification of *P. aeruginosa* Elastase—Proteases secreted by *P. aeruginosa* were isolated following the protocol of Coin *et al.* (22). A mucoid clinical isolate (S470) of *P. aeruginosa* that was found to degrade SP-A was grown in a 2-liter culture in nutrient broth (Difco) for 18 h at 37 °C with shaking at 250 rpm. Alginate was precipitated from the cell-free culture supernatant with 0.25 M CaCl₂ for 2 h at 4 °C and removed by centrifugation. The supernatant was then concentrated by ultrafiltration with tangential flow using the Minitan system (Millipore Corp., Marlborough, MA) with a 10-kDa cut-off membrane to a volume of 150 ml. The culture was then applied to a DEAE-Sepharose CL-6B column (1.6 × 50 cm) in 30 mM Tris-HCl, pH 8.3, and bound proteins were eluted with a continuous linear gradient of NaCl (0–0.5 M) in the same buffer. Pooled fractions were then analyzed by Western blot with a polyclonal anti-elastase antibody. The polyclonal rabbit anti-elastase antibody was kindly provided by Dr. Efrat Kessler (Sheba Medical Center, Tel Aviv University, Tel Aviv, Israel). Pooled fractions containing elastase were then analyzed for purity by Coomassie stain and silver stain (data not shown). The elastase preparations contained only 1 band (38 kDa) by Coomassie staining and three bands (65, 38, and 25 kDa) by silver stain; however, the predominant band was the 38-kDa elastase as determined by densitometry.

Purification of Rat Recombinant SP-D—Recombinant rat SP-D was purified by maltose affinity chromatography from the media of a stably transfected Chinese hamster ovary cell line (23). Briefly, Chinese hamster ovary cells were grown in serum-free medium for 10–12 days, and the culture supernatant was collected and dialyzed to remove glucose against maltose loading buffer containing 50 mM Tris, 150 mM NaCl, and 5 mM CaCl₂, pH 7.8. The dialyzed supernatant was then incubated with maltose-Sepharose beads overnight at 4 °C. The maltose-Sepharose beads were then poured into a column and washed with maltose loading buffer to remove unbound proteins. SP-D was then eluted from the column with maltose elution buffer, 50 mM Tris, 150 mM NaCl, and 2 mM EDTA, pH 7.8, and stored at 4 °C.

Alveolar Macrophage Isolation—Alveolar macrophages were obtained by BAL of pathogen-free rats. Rats were killed by intraperitoneal injection of Nembutal (Abbott) followed by exsanguination; the trachea was cannulated, and the lungs were isolated and removed from the rats. The lungs were then lavaged six times to total lung capacity with phosphate-buffered saline containing 0.2 mM EGTA previously warmed to 37 °C. Cells were then collected by centrifugation at 230 × *g* for 10 min. The cell pellet was then resuspended in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin, bovine serum albumin, low endotoxin (Sigma). Average cell yields were ~6–8 × 10⁶ cells/rat, and greater than 95% of the cells were macrophages.

Degradation of Surfactant Proteins with *P. aeruginosa* Elastase—Rat recombinant SP-D in 50 mM Tris-buffered saline (TBS) plus 2 mM EDTA was incubated with purified elastase in 30 mM TBS at varying concentrations, times, and temperatures as noted in the figure legends. Volumes used in these assays resulted in a 4–15-fold final dilution of the EDTA containing SP-D buffer. The resulting products were resolved by 15% SDS-PAGE gel and Western blot using a well characterized anti-SP-D antibody (24). SP-A was obtained from the lavage of alveolar proteinosis patients as previously reported (25). Detection of SP-A degradation fragments was determined using anti-human SP-A antibody as previously reported (19). Cell-free, normal rat BAL was concentrated

using an Apollo spin concentrator (Orbital Biosciences, Topsfield, MA), with a molecular mass cut-off of 10 kDa. Aliquots of rat BAL were then incubated with purified elastase as indicated, and degradation fragments were detected by Western blot.

Analysis of Lung Transplant BAL—BAL from CF patients who had received bilateral lung allografts were analyzed for the presence of SP-D and possible degradation fragments. Samples from the CF lung transplant recipients were separated under nonreducing conditions by SDS-PAGE and transferred to nitrocellulose. Immunoreactive SP-D was detected using an anti-human SP-D antibody provided by Dr. Erika Crouch (Washington University, St. Louis, MO).

SP-D Fragment Purification—SP-D (227 µg/ml) was incubated with *P. aeruginosa* elastase (150 µg/ml) for at least 24 h at 37 °C. The resulting solution was then centrifuged at 8000 × *g* for 5 min to remove any large debris and applied to a Superose 6 high pressure liquid chromatography column (Amersham Biosciences). The sample volume was 200 µl, and the column was eluted at a flow rate of 0.5 ml/min using TBS containing 2 mM EDTA, pH 7.60. Absorbance at 280 nm was monitored throughout the run. Fractions were collected every minute and were analyzed by Western blot for intact SP-D, SP-D fragment, and elastase. Elastase was shown to separate from SP-D fragment (data not shown). SP-D fragment-containing fractions were then pooled and concentrated using the Apollo spin concentrator (Orbital Biosciences) to a final volume of ~1 ml. Protein concentration was determined by the Nano-orange protein assay (Molecular Probes, Inc., Eugene, OR).

Sequence Analysis of SP-D Fragments—Recombinant rat SP-D (10 µg) was incubated with *P. aeruginosa* elastase (Elastin Products Co., Owensville, MO) (10 µg). The resulting solution was then analyzed via Edman degradation performed by the Harvard Microchemistry Facility (Cambridge, MA).

Aggregation of Bacteria by SP-D and SP-D Fragment—Bacteria were grown overnight on Nutrient Agar plates (Difco) and collected into 1 ml of L-broth. Colony-forming units (cfu) were determined by multiplication of absorbance at 660 nm times the calculated extinction coefficients for each isolate. The extinction coefficients were determined by serial dilution and counting of live bacterial colonies. A volume equal to 10⁸ cfu of bacteria was suspended in 1 ml of TBS containing 2 mM CaCl₂, pH 7.60. SP-D and SP-D fragment were added to the bacteria at the indicated concentrations, and absorbance at 660 nm was measured every 30 min for 180 min. Aggregation is observed as a decrease in absorbance as bacterial aggregates precipitate out of solution.

Binding Assays with Intact SP-D and SP-D Fragment—Bacteria (10⁸ cfu) were pelleted by centrifugation at 8000 × *g* for 5 min and resuspended in 250 µl of TBS containing either 2 mM CaCl₂ or 10 mM CaCl₂, pH 7.60. SP-D and SP-D fragment were added at concentrations of 1 and 2 µg/ml, and binding was allowed to proceed for 1 h at 4 °C. The bacteria were then collected by centrifugation and washed twice with TBS. Fluorescein-5-isothiocyanate (FITC)-labeled anti-rat SP-D IgG was then utilized to measure SP-D binding. Anti-rat SP-D IgG was purified from rabbit serum using a Protein A-Sepharose column (Pierce). Purified IgG was then dialyzed into Na₂CO₃, pH 9.0. FITC labeling of the antibody was then performed by adding 10 µl of FITC (Molecular Probes) at a concentration of 10 mg/ml in dimethylformamide for 6 h at 4 °C. Excess FITC was removed by dialysis *versus* PBS. Labeled anti-rat SP-D was added to bacteria at a volume of 25 µl per 500 µl of bacteria in TBS plus 2 mM CaCl₂ or 10 mM CaCl₂, pH 7.60, for 30 min at 4 °C. Bacteria were then washed twice with TBS, and fluorescence was measured at 495-nm excitation, 530-nm emission. Binding of SP-D was expressed as the relative fluorescence units above background levels.

Phagocytosis of Bacteria as Measured by FACS Analysis—Bacteria were collected from nutrient agar plates and were resuspended in Na₂CO₃, pH 9.0. FITC labeling of the bacteria was then performed by adding 10 µl of FITC (Molecular Probes) at a concentration of 10 mg/ml in dimethylformamide for 1 h at room temperature. Excess dye was then removed by washing of the bacteria three times with PBS. Bacterial concentration was determined by measurement of absorbance at 660 nm, utilizing known extinction coefficients. FITC-labeled bacteria were then frozen in aliquots with 10% glycerol or stored at 4 °C. Labeled bacteria were later thawed and then added to alveolar macrophages at a ratio of 5 × 10⁷ bacteria to 5 × 10⁵ macrophages in 250 µl of PBS plus 2 mM CaCl₂ and 0.1% bovine serum albumin. Assay tubes were pre-coated with 1% bovine serum albumin in PBS for 1 h at 4 °C. Phagocytosis was allowed to occur for 1 h at 37 °C, and then the cells were washed twice with PBS buffer. Finally, macrophages were fixed in 300 µl of 1% formaldehyde in PBS and analyzed for fluorescence by FACS.

Data Analysis—All of the data presented in the figures were subjected to unpaired Student's *t* test analysis assuming unequal vari-

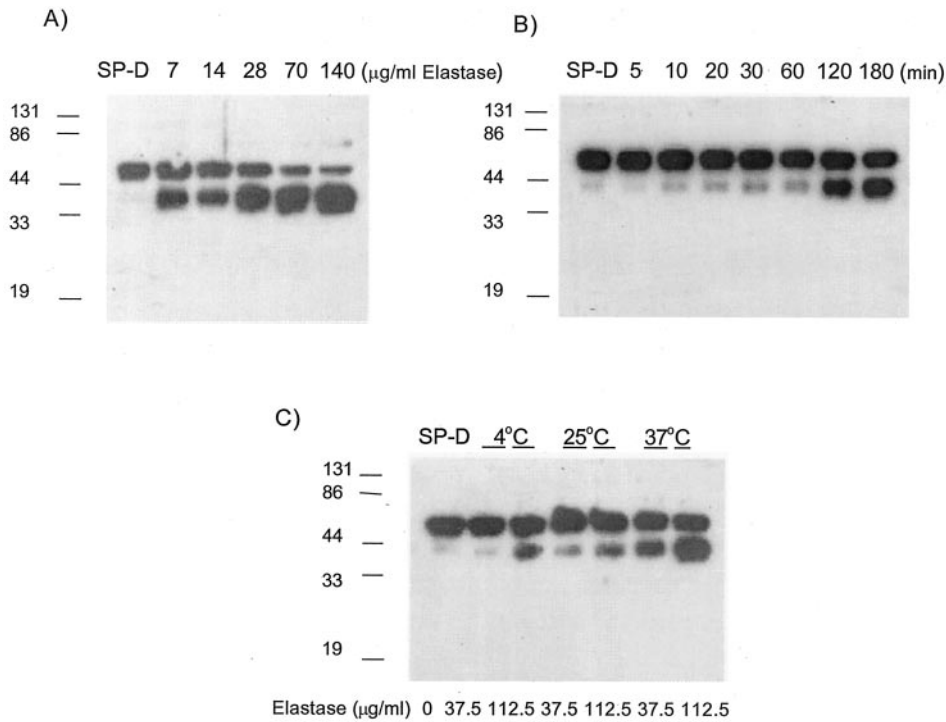


FIG. 1. *P. aeruginosa* elastase degrades SP-D in a dose-, time-, and temperature-dependent manner. Rat recombinant SP-D (14 µg/ml) in 50 mM TBS plus 2 mM EDTA was incubated with elastase, in 30 mM TBS, at concentrations ranging from 0 to 140 µg/ml for 3 h at 37 °C (A). SP-D (60 µg/ml) was incubated with 112.5 µg/ml elastase at 37 °C for variable time points (B). SP-D (60 µg/ml) was incubated with either 37.5 or 112.5 µg/ml elastase for 3 h at 4, 25, and 37 °C (C). In these assays, final EDTA concentrations were equal to or less than 0.5 mM. Degradation products were analyzed by SDS-PAGE and Western blot for SP-D and SP-D fragment.

ances. All statistics were performed using the Microsoft Excel software package. Analyses with a resultant $p < 0.05$ were determined significant.

RESULTS

P. aeruginosa Elastase Degrades SP-D Dose-, Time-, and Temperature-dependently—Our laboratory has previously reported that *P. aeruginosa* elastase degrades SP-D (19). In order to characterize further the kinetics of degradation, we examined the effects of dosage, time, and temperature on elastase activity. SP-D (14 µg/ml) was incubated with increasing concentrations of elastase for 3 h at 37 °C (Fig. 1A). At the lowest dose tested, 7 µg/ml, elastase produced fragments, whereas greater degradation was observed with concentrations up to 140 µg/ml elastase. SP-D, 60 µg/ml, was then incubated with 112.5 µg/ml of elastase at 37 °C for various time points (Fig. 1B). Immunoreactive fragment was detected as early as 10 min after the addition of elastase. Finally, elastase at either 37.5 or 112.5 µg/ml was added to SP-D (60 µg/ml) for 3 h at 4, 25, and 37 °C (Fig. 1C). SP-D was poorly degraded by elastase at both 4 and 25 °C. These data show that *P. aeruginosa* elastase degrades SP-D dose-, time-, and temperature-dependently.

P. aeruginosa Elastase Degradation of SP-A and SP-D Is Inhibited by Phosphoramidon—In order to confirm that the degradative activity observed in our elastase preparations was indeed due to elastase, we incubated SP-D (20 µg/ml) or SP-A (40 µg/ml) with elastase (75 µg/ml) in the presence or absence of increasing concentrations (0.05–5 mM) of phosphoramidon, an elastase inhibitor, for 18 h at 37 °C (Fig. 2). Phosphoramidon inhibited SP-D and SP-A degradation dose-dependently. In addition, a colorimetric assay (chromozym PL) specific for the *P. aeruginosa* serine protease, protease IV was performed to test the purity of our elastase preparations. The *P. aeruginosa* elastase utilized in these studies was negative for serine protease activity (data not shown). Furthermore, a commercial preparation of elastase resulted in production of an identical

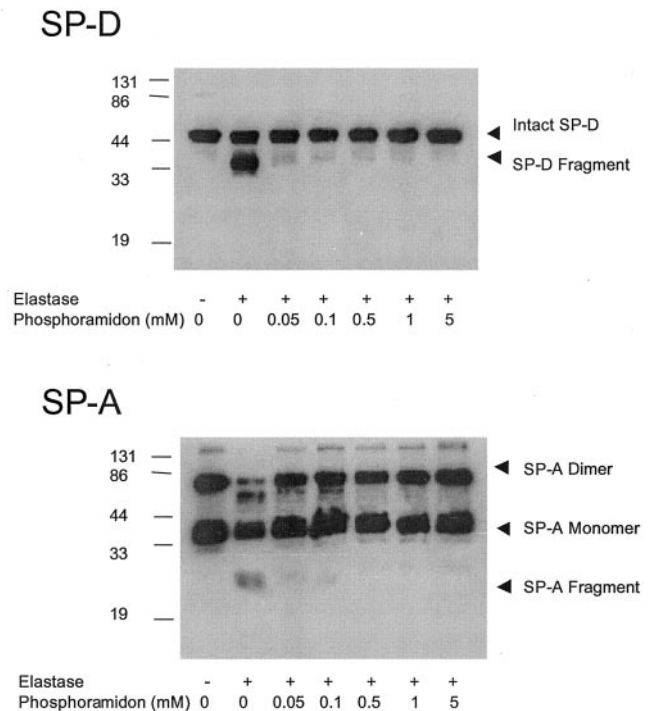


FIG. 2. Phosphoramidon, an elastase inhibitor, inhibits degradation of SP-A and SP-D by *P. aeruginosa* elastase. SP-D (20 µg/ml) or SP-A (40 µg/ml) was incubated with 75 µg/ml elastase for 18 h at 37 °C in the presence or absence of phosphoramidon. Degradation products were analyzed by SDS-PAGE and Western blot for SP-A, SP-D, and SP-A and SP-D fragments.

SP-D fragment. Degradation by commercial elastase was also inhibited by phosphoramidon at the same concentrations described above (data not shown).

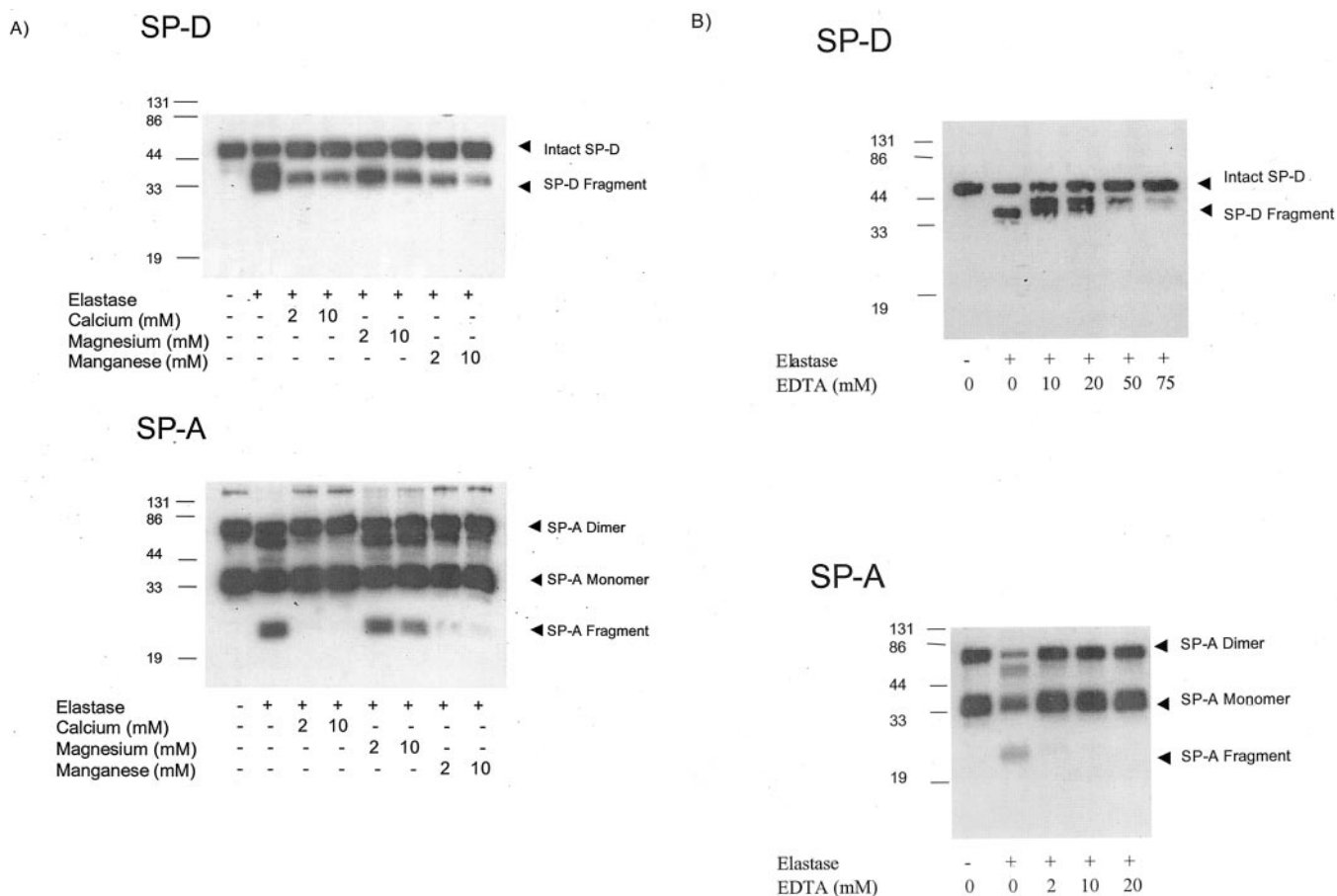


FIG. 3. Divalent metal cations and EDTA inhibit degradation of SP-D and SP-A by *P. aeruginosa* elastase. A, SP-D or SP-A (20 $\mu\text{g/ml}$) was incubated with 40 $\mu\text{g/ml}$ elastase in the presence or absence of 2 or 10 mM calcium, magnesium, or manganese for 3 h at 37 °C. B, SP-D (10 $\mu\text{g/ml}$) was incubated with 30 $\mu\text{g/ml}$ elastase, or SP-A (40 $\mu\text{g/ml}$) was incubated with 75 $\mu\text{g/ml}$ elastase in the presence or absence of increasing EDTA concentrations for 18 h at 37 °C. Degradation products were analyzed by SDS-PAGE and Western blot for SP-D, SP-A, SP-D fragment, and SP-A fragment.

Divalent Metal Cations and EDTA Inhibit Degradation of SP-D and SP-A by P. aeruginosa Elastase—SP-A has been shown to bind to divalent cations via its C-terminal lectin domain (26). Binding of SP-A and SP-D to oligosaccharides has been shown to be calcium-dependent (27, 28). SP-D binding to oligosaccharides requires either calcium, magnesium, or manganese, whereas SP-A lectin binding requires either calcium or manganese. We examined the effects of calcium, magnesium, and manganese on degradation of SP-D and SP-A by elastase. SP-D or SP-A at 20 $\mu\text{g/ml}$ were incubated with 40 $\mu\text{g/ml}$ elastase in the presence or absence of either 2 or 10 mM divalent metal cations (Fig. 3A). Degradation of SP-D by elastase was inhibited by manganese > calcium > magnesium. Contrastingly, SP-A degradation was inhibited by calcium and manganese but only slightly by magnesium. We then tested the ability of the metal chelator, EDTA, to inhibit elastase degradation of SP-A and SP-D. SP-D (10 $\mu\text{g/ml}$) was incubated with 30 $\mu\text{g/ml}$ elastase, or SP-A (40 $\mu\text{g/ml}$) was incubated with 75 $\mu\text{g/ml}$ elastase in the presence or absence of increasing EDTA concentrations for 18 h at 37 °C (Fig. 3B). Degradation of SP-A and SP-D was inhibited by the metal chelator, EDTA. However, much higher concentrations of EDTA were required to inhibit SP-D degradation as compared with SP-A degradation.

Purification of SP-D Fragment by Gel Filtration Chromatography—In order to examine the functional characteristics of the SP-D 35-kDa degradation fragment, we purified the fragment by gel filtration chromatography. SP-D (230 $\mu\text{g/ml}$) was incubated with 150 $\mu\text{g/ml}$ *P. aeruginosa* elastase for greater

than 24 h at 37 °C. The degradation solution was then applied to a Superose-6 fast protein liquid chromatography column in sequential 200- μl aliquots followed by successive elutions. SP-D fragment eluted from the column at an approximate mass of 400 kDa, suggesting a multimer. As shown by the Western blot under reducing conditions in Fig. 4, the degradation assay resulted in nearly 100% conversion of intact SP-D to the 35-kDa fragment monomer which was collected, pooled, and concentrated for use in functional protein assays.

Sequence Analysis of the SP-D Fragments—Previous work in our laboratory has suggested that elastase cleaves SP-D in the C-terminal lectin domain (19). To map the cleavage site, the SP-D degradation products were identified by Edman degradation. Sequencing identified five novel N termini generated by elastase cleavage of SP-D (Fig. 5). Cleavage of SP-D occurred at two alternate sites at the beginning of the lectin domain, Val-259 and Ala-267. In addition, two alternate cleavage sites were identified in the middle region of the lectin domain, Phe-323 and Trp-336. A fifth cleavage site was identified in the collagen domain of SP-D, Ile-112. The relevance of this cleavage was not determined.

The SP-D Fragment Does Not Aggregate S. typhimurium or E. coli—Aggregation of bacteria has been shown to be an important immune function of SP-D that is mediated by the lectin domain of SP-D. In order to test the hypothesis that SP-D fragment would fail to aggregate bacteria, we incubated a clinical isolate (S470) and a laboratory strain (PA01) of *P. aeruginosa*, *S. typhimurium*, and *E. coli* with intact SP-D

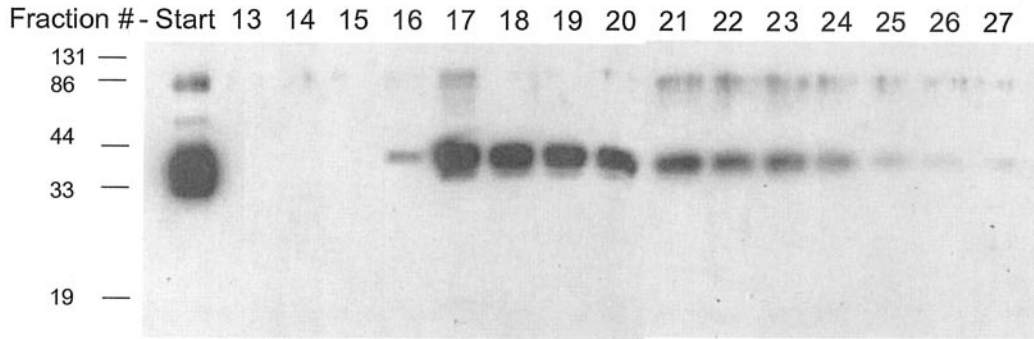


FIG. 4. Purification of SP-D fragment by gel filtration chromatography. SP-D was degraded with 150 µg/ml *P. aeruginosa* elastase for 48 h. The resultant solution was then applied to a Superose 6 gel filtration column. Column fractions were then analyzed by SDS-PAGE and Western blot for SP-D and SP-D fragment. Start, the original degradation solution before the column. SP-D fragment was localized to fractions 17–23.

Peptide Number	Sequence	Amino Acid for SP-D
1	WAPGEPNNGGAEN_VEIFT	336-355
2	FTYPT_E	323-329
3	ANSEAGFEDAKEMQRQAGGQ	267-286
4	VGDKIFR_A	259-267
5	ISGP_KAGPSG_G	112-126

1 MLHFLSMLVL LVQPLGLDGA EMKTLRSQSI TNTCTLVLCSP TENGSLPGRD
 51 GRDGRGPRG EKGDPLGPG MGLSGLPGR GPVGPKEGNG SAGEPGPKGE
 101 RGLVGPSPGISG PAGKEG PSKGQGNIGP QGKPGPKGEA GPKGEVGPAG
 151 MQGSAGAKGP AGPKGERGAP GEQGAPGNAG AAGPAGPAG QGAPGSRGPP
 201 GLKGDRGAPG DRGIKGESGL PDSAALRQOM EALNGKLQRL EAAFSRYKKA
 251 ALFPDQSGVG DKIFRAANSE EPFEDAKEMC ROAGGQLASP RSATENAAVQ
 301 QLVTAHAKAA FLSMTDVGTE GKFTYPTGEE LVYSNWAPE PNNNGGAENC
 351 VEIFTNGQWN DKACGEQRLV ICEF

FIG. 5. Sequence analysis of the SP-D cleavage fragments. Rat recombinant SP-D was degraded with *P. aeruginosa* elastase and analyzed by Edman degradation. Five novel N termini were identified. The boldface amino acids indicate the position of cleavage sites. The C-terminal lectin domain of rat SP-D is underlined.

and SP-D fragment. Consistent with prior observations from our laboratory (7), SP-D (2 µg/ml) did not aggregate *P. aeruginosa* (data not shown). SP-D at 1 µg/ml was effective at aggregating both *S. typhimurium* and *E. coli*, whereas the SP-D degradation fragment had no effect on either bacteria (Fig. 6). These data show that SP-D fragment no longer retains the lectin-dependent ability to aggregate bacteria.

SP-D Fragment Fails to Bind to *P. aeruginosa* or *S. typhimurium*—The inability to aggregate bacteria suggests that the SP-D fragment may exhibit impaired binding to bacteria. To examine bacterial binding, we incubated SP-D or SP-D fragment at 1 or 2 µg/ml with bacteria. Binding was determined by detection of SP-D by fluorescently labeled antibody. Intact SP-D bound dose-dependently to both *S. typhimurium* and *P. aeruginosa* (Fig. 7). The SP-D fragment at 1 or 2 µg/ml failed to bind to either bacteria. Due to the fact that SP-D fragment preparations contain EDTA and are more dilute than intact SP-D stocks, we confirmed that the lack of binding was not due to EDTA inhibition by adding SP-D fragment to bacteria in 10 mM CaCl₂ buffer. SP-D fragment still showed impaired binding to bacteria in high calcium buffer. These results confirm that the SP-D degradation fragment does not have a functional lectin domain.

SP-D Fragment Does Not Stimulate Phagocytosis of *E. coli* by Alveolar Macrophages—As a consequence of deficient aggregation and binding activity by SP-D fragment, we proposed that

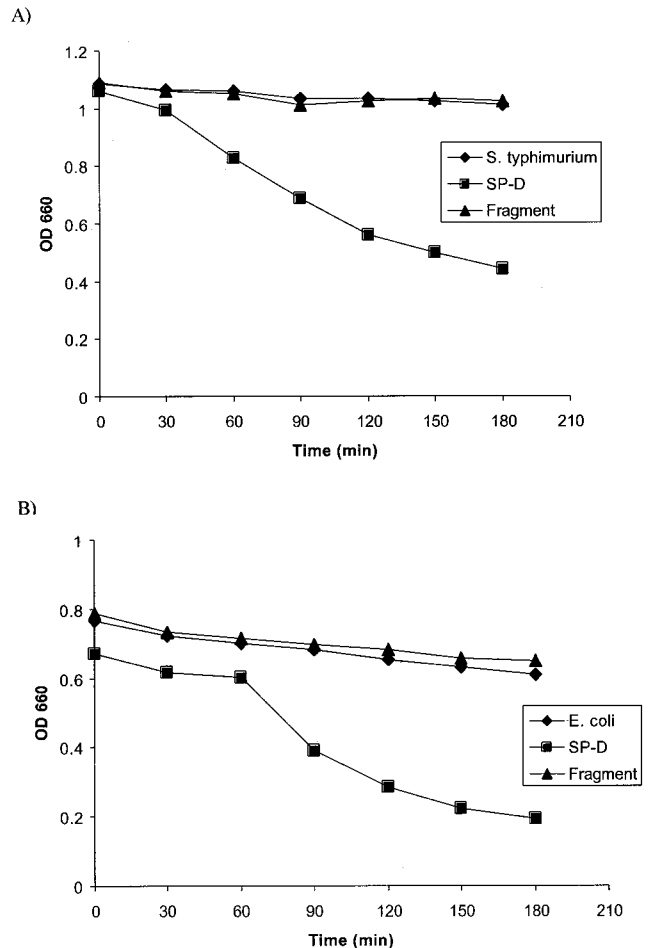


FIG. 6. SP-D fragment does not aggregate *S. typhimurium* or *E. coli*. Intact SP-D or SP-D fragment (1 µg/ml) was incubated with *S. typhimurium* (A) or *E. coli* (B) in 1 ml of TBS plus 2 mM CaCl₂. Aggregation of bacteria was assessed by reading the A_{660 nm} every 30 min for 180 min. A decrease in optical density indicates the precipitation of bacterial aggregates over time. The results presented represent one of several experiments with each bacteria.

SP-D fragment does not enhance phagocytosis of bacteria by alveolar macrophages as does intact SP-D. Alveolar macrophages were incubated with FITC-labeled *E. coli* for 1 h at 37 °C. Uptake of bacteria was then measured by FACS. Intact SP-D dose-dependently increased uptake of *E. coli* as measured by mean fluorescence and percentage of positive cells (Fig. 8). SP-D fragment had no effect on uptake of bacteria by alveolar macrophages at either dose tested. These data combined with

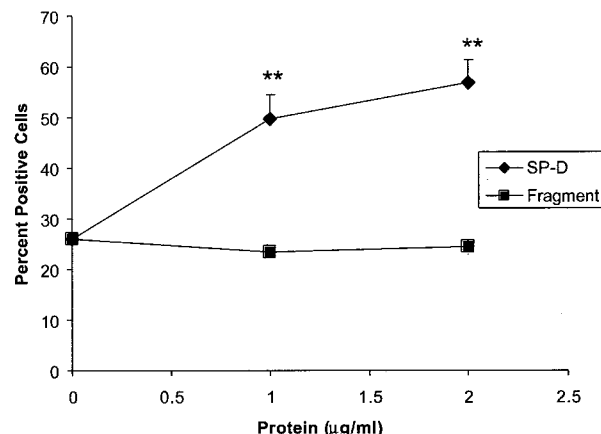
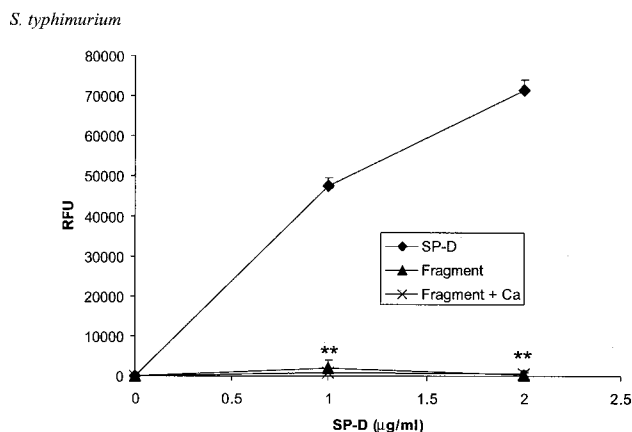
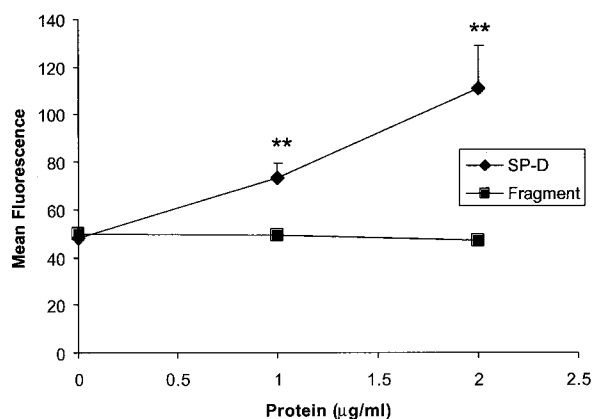
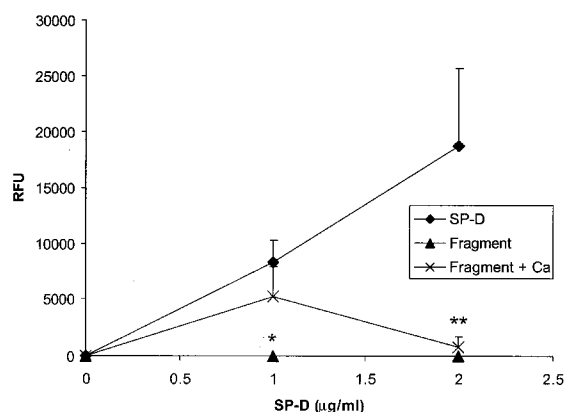
*P. aeruginosa*

FIG. 7. SP-D fragment fails to bind to *P. aeruginosa* or *S. typhimurium*. Intact SP-D or SP-D fragment was incubated at the indicated concentrations with *P. aeruginosa* ($n = 3$) or *S. typhimurium* ($n = 3$) in 250 μ l of TBS plus 2 mM (fragment) or 10 mM CaCl_2 (fragment plus calcium) for 1 h at 4 $^\circ\text{C}$. SP-D binding was then detected by the addition of 25 μ l of FITC-labeled anti-SP-D antibody for 30 min at 4 $^\circ\text{C}$. *, the decrease in SP-D fragment binding was statistically significant compared with intact SP-D; $p < 0.05$. **, the decrease in SP-D fragment and fragment plus calcium binding was statistically significant compared with intact SP-D ($p < 0.05$, except for fragment plus calcium with *P. aeruginosa*, where $p = 0.06$).

FIG. 8. SP-D fragment does not affect phagocytosis of *E. coli* by alveolar macrophages. FITC-labeled *E. coli* (5×10^7 cfu) were added to rat alveolar macrophages (5×10^5 cells) for 1 h at 37 $^\circ\text{C}$. SP-D or SP-D fragment was added at a concentration of 1 or 2 $\mu\text{g/ml}$ ($n = 3$ for all). Bacterial uptake was measured by FACS analysis and expressed as the percentage of positive macrophages for FITC and the mean fluorescence of the positive macrophages. Intact SP-D dose-dependently increased uptake at both doses tested. **, the effect of intact SP-D was significantly different from either no protein or SP-D fragment ($p < 0.05$).

earlier results show that degradation of SP-D by *P. aeruginosa* elastase abrogates SP-D immune function.

SP-D Fragment Increases Uptake of *P. aeruginosa* Deficient in Elastase Production by Alveolar Macrophages—In order to examine the potential contribution of elastase in abrogating SP-D-mediated uptake of *P. aeruginosa*, we tested the effects of intact SP-D on alveolar macrophage uptake of wild-type (PA01) and elastase deficient (PA01-B1) *P. aeruginosa*. Alveolar macrophages were incubated with FITC-labeled *P. aeruginosa* for 1 h at 37 $^\circ\text{C}$ in the presence or absence of SP-D. Intact SP-D had no effect on the uptake of wild-type PA01; however, SP-D dose-dependently enhanced the uptake of elastase-deficient *P. aeruginosa* (Fig. 9). These data suggest that the absence of elastase production by isolates of *P. aeruginosa* may alter the ability of SP-D to enhance phagocytosis of bacteria by macrophages.

Degradation of Rat BAL Results in the Production of a 35-kDa SP-D Fragment—SP-D normally exists *in vivo* in the alveolar hypophase in the presence of surfactant lipids. For our observed SP-D degradation to be relevant *in vivo*, *P. aeruginosa* elastase must be able to degrade normal rat SP-D in BAL. We incubated concentrated normal rat BAL with increasing con-

centrations of elastase overnight at 37 $^\circ\text{C}$. Aliquots of the reaction mixture were analyzed by Western blot to detect SP-D and SP-D fragment (Fig. 10). *P. aeruginosa* elastase dose-dependently degraded intact SP-D, resulting in production of a 35-kDa fragment. This observation indicates that the 35-kDa SP-D fragment is probably produced *in vivo*.

P. aeruginosa* Infection Is Associated with Degradation and Decreased Concentration of SP-D *in Vivo—Analysis of BAL from CF patients who have undergone bilateral lung transplantation demonstrates degradation fragments of human SP-D (arrow) as well as decreased concentrations of intact SP-D (arrow) (Fig. 11). Due to the fact that our anti-human SP-D antibody cross-reacts with human SP-A monomers, we measured immunoreactive SP-D by SDS-PAGE under nonreducing conditions followed by Western blot. Under these conditions, SP-D and SP-D fragment migrate as a trimer of different size than intact SP-A. SP-D that was degraded with elastase overnight at 37 $^\circ\text{C}$ was analyzed as a positive control (lane 2). Intact human alveolar proteinosis SP-A was analyzed as a negative control for antibody cross-reactivity (lane 1). In addition, the CF samples were probed with anti-human SP-A antibody under the same gel conditions to exclude the possibility that SP-A

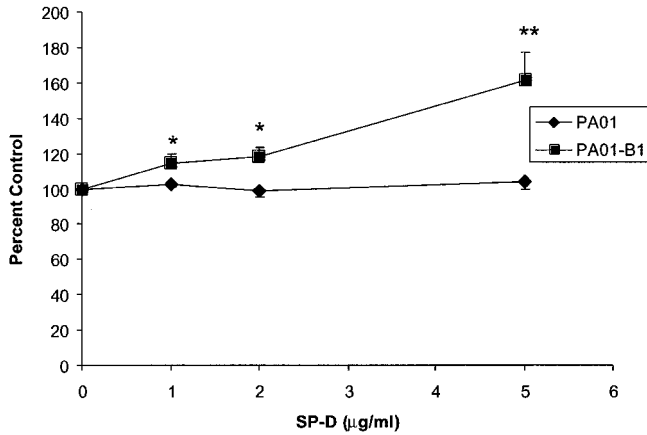


FIG. 9. Intact SP-D enhances uptake of elastase-deficient *P. aeruginosa*. FITC-labeled *P. aeruginosa* and 5×10^7 cfu of PA01 or elastase-deficient PA01-B1 were added to rat alveolar macrophages (5×10^5 cells) for 1 h at 37 °C. SP-D was added at a concentration of 1, 2, or 5 µg/ml ($n = 4$ for all). Bacterial uptake was measured by FACS analysis, which measures the amount of fluorescence associated with the macrophages. The data are expressed as the percentage change in mean fluorescence of the positive macrophages when treated with SP-D compared with the absence of SP-D. Intact SP-D dose-dependently increased uptake of PA01-B1 at the highest dose tested. *, the effect of SP-D on PA01-B1 was significantly different from that on PA01 ($p < 0.05$). **, the effect of intact SP-D was significantly different from either no protein or PA01 ($p < 0.05$).

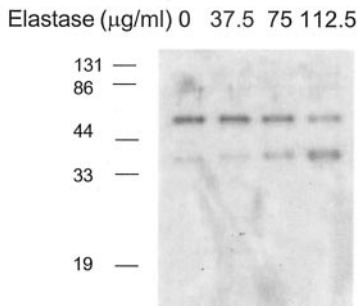


FIG. 10. Degradation of rat BAL results in the production of a 35-kDa SP-D fragment. Concentrated rat BAL (>1 mg/ml protein) was incubated with increasing concentrations of *P. aeruginosa* elastase for 24 h at 37 °C. Degradation products were analyzed by SDS-PAGE and Western blot for SP-D and SP-D fragment.

fragments were cross-reacting with the anti-SP-D antibody (data not shown). These CF samples were obtained from a prospective, pilot study in which the investigators were blinded to the clinical outcomes of the patients undergoing lavage until after results of the Western blots were obtained. Five of six patients had apparent degradation fragments of SP-D; two patients, (patients 3 and 5) had *P. aeruginosa* infections, and both had SP-D degradation fragments.

DISCUSSION

The data presented in this study demonstrate that degradation of SP-D by *P. aeruginosa* elastase results in the loss of protein function. Elastase degrades SP-D dose-, time-, and temperature-dependently to produce a 35-kDa fragment that can be detected by immunoblotting. Sequence analysis of the SP-D cleavage fragments indicates that the C-terminal lectin domain of SP-D is cleaved by *P. aeruginosa* elastase. Cleavage of the SP-D lectin domain resulted in loss of lectin-dependent protein functions including bacterial aggregation, bacterial binding, and regulation of bacterial uptake by alveolar macrophages. Thus, the 35-kDa fragment of SP-D lacks the normal innate immune properties of intact SP-D.

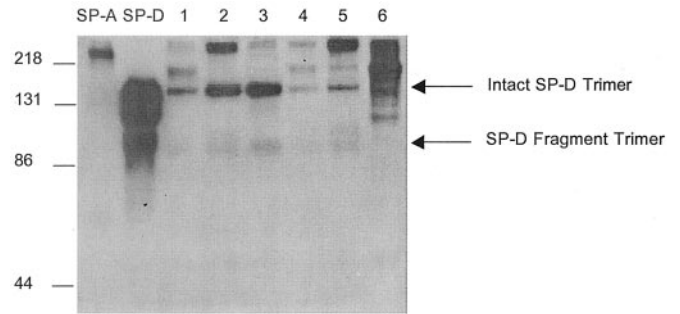


FIG. 11. BAL from CF patients who had undergone bilateral lung transplant contain SP-D degradation fragments. BAL from CF patients who had undergone bilateral lung transplantation were obtained, and immunoreactive SP-D was detected by gel electrophoresis under nonreducing conditions followed by Western blot analysis of equal aliquots of cell-free BAL from each patient. SP-A, human alveolar proteinosis SP-A; SP-D, rat recombinant SP-D incubated with elastase for 24 h; 1–6, CF patients. Patients 3 and 5 were positive for *P. aeruginosa* infection.

P. aeruginosa elastase is secreted *in vivo* as demonstrated by the presence of antibody titers to the protein in both serum and bronchial secretions (20). In addition, Doring and co-workers reported that 31 of 34 clinical isolates obtained from CF patients produced elastase at concentrations ranging from 0.02 to 490 µg/ml *in vitro*. Additionally, elastase mRNA has been isolated from bacteria obtained from the sputa of CF patients (30). More recent studies have shown that CF isolates can produce elastase at concentrations greater than 1 mg/ml *in vitro* (31). Although direct sampling of the lung interstitial space is not possible, several studies have measured elastase activity in sputum and BAL. Azghani *et al.* (32) measured elastase activity in the sputum of CF patients ranging from 3 to 110 µg/mg sputum. Two additional studies reported BAL elastase levels of 136 ± 98 ng/ml and 4.2 units/ml, which is equivalent to an ~16 µg/ml concentration of a commercial elastase preparation (33, 34). Whereas the concentration of elastase produced in the lung is not definitively known, these studies suggest that quantities of elastase in the µg/ml range utilized in our *in vitro* studies may be present.

The concentrations and time course of *P. aeruginosa* elastase activity observed in this study are within the ranges previously reported concerning the proteolytic activity of elastase on immunologic substrates. For example, Heck *et al.* (35) reported that elastase degradation of 100 µg/ml IgA occurs at concentrations of elastase ranging from 5 to 500 µg/ml at an incubation time of 20 h. Maximal degradation of IgA was seen at 500 µg/ml elastase. Fick *et al.* (36) reported that degradation of 125 µg of IgG was dose-dependent over elastase concentrations ranging from 0.5 to 4350 µg/ml for 16 h at 37 °C. Schultz and Miller (37) reported degradation of serum complement components at a concentration of 125 µg/ml *P. aeruginosa* elastase. Therefore, we conclude that the elastase concentrations utilized in this study (7–140 µg/ml) are within the levels normally reported for this enzyme.

We observed that calcium inhibits the elastase-mediated degradation of SP-A and SP-D, as does magnesium and manganese. The inhibition of degradation by the three metal ions tested is probably due to effects on the lectin domains of SP-A and SP-D rather than a direct effect on the enzyme, since the proteolytic activity of *P. aeruginosa* elastase is poorly inhibited by calcium, magnesium, and manganese (1 mM) *in vitro* (29). It has been shown that binding of calcium to SP-A causes a conformational change of the octadecamer (38). Furthermore, calcium induces a conformational change and aggregation of SP-A *in vitro*, which may alter availability of the protein for

degradation (26). Degradation of SP-D by neutrophil elastase is also inhibited by physiologic calcium levels (39). However, SP-D degradation fragments are still produced in the presence of 2 mM calcium albeit at lower levels in their study and in our own. It seems likely that degradation of SP-D can occur *in vivo*, where the calcium concentration is normally 1.2 mM in the interstitial fluid (40). Although EDTA inhibited elastase-mediated degradation of both SP-A and SP-D in our study, much higher concentrations of EDTA were required to inhibit SP-D degradation compared with SP-A. These data are consistent with the observation that *P. aeruginosa* elastase is a metalloprotease inhibited by EDTA (29). It is unclear why more EDTA was required to inhibit SP-D degradation compared with SP-A degradation. If the effects of EDTA were on elastase alone we would expect that the same EDTA concentration would have similar effects on degradation. It is possible that EDTA has direct effects on the substrate, in this case SP-D, altering its availability for degradation.

Degradation of SP-D by elastase results in the production of a 35-kDa fragment under reducing conditions. Under nonreducing conditions, the SP-D cleavage product migrates as a trimer, suggesting that the fragment has an intact N-terminal region, which contains cysteine residues at positions 34 and 39 that are required for multimerization (19). Furthermore, degradation of intact SP-D with bacterial collagenase results in the production of a collagenase-resistant fragment containing the C-terminal lectin domain. Degradation of SP-D first with elastase followed by collagenase results in the absence of an intact collagenase-resistant fragment. These data suggest that *P. aeruginosa* elastase cleaves SP-D in the C-terminal domain. Sequencing of the SP-D degradation fragments confirmed that *P. aeruginosa* elastase cleaves the lectin domain of intact SP-D. The four C-terminal cleavage sites identified are consistent with the formation of a 35-kDa N-terminal fragment, as we have shown. The peptide product of the cleavage site at Ile-112 within the collagen N-terminal domain has not been observed by Western blot or Coomassie stain. We are unaware of any functional consequences of this cleavage. It was not possible to determine the relative abundance of these cleavage products due to the multiple peptides detected; however, the only product observed by Western blot or Coomassie stain is the 35-kDa fragment described herein. Human neutrophil elastase, a serine protease, also cleaves SP-D in the C-terminal lectin domain to produce three primary fragments of 38, 5, and 7 kDa (39), and these fragments did not aggregate bacteria, a function mediated by the carbohydrate recognition domain (CRD). The conclusion that SP-D is cleaved in the CRD by *P. aeruginosa* elastase is consistent with our functional observations that the purified 35-kDa fragment failed to aggregate either *E. coli* or *S. typhimurium*, both of which were aggregated effectively by intact SP-D. Our clinical isolate of *P. aeruginosa* was not aggregated by intact SP-D consistent with previous reports from our laboratory (7).

Intact SP-D has been shown to bind to Gram-negative bacteria via an interaction with the core oligosaccharides of lipopolysaccharide (41). This binding is dependent on the CRD of SP-D. Our results are consistent with these data in that cleavage of the CRD by *P. aeruginosa* elastase results in a loss of binding activity, since the SP-D fragment did not bind to or enhance uptake of the Gram-negative bacteria tested, whereas intact SP-D was active in these assays. Furthermore, the observation that SP-D enhances uptake of an elastase-deficient *P. aeruginosa*, but not wild-type *P. aeruginosa*, suggests that degradation of SP-D by elastase may be an important mechanism by which *P. aeruginosa* escapes macrophage clearance.

The N-terminal SP-D cleavage fragment has a functionally

intact N terminus as demonstrated by the ability of the 35-kDa SP-D fragment to form trimers under nonreducing SDS-PAGE (19). In addition, the elution profile from the Superose column shows that SP-D fragment can form higher order multimers (data not shown). Several studies have attempted to determine the role of the N-terminal domain in SP-D function. SP-D has been shown to bind to membrane lipids such as phosphatidylinositol and glucosylceramide on host cells (42, 43). The N-terminal collagen domain of SP-D has been shown to be required for dodecamer formation and consequently is partially required for binding to phosphatidylinositol and glucosylceramide (44). However, it has more recently been determined that the minimum structural sequences for both phosphatidylinositol and glucosylceramide binding are in the CRD of SP-D (45). These data suggest that the N-terminal collagen domain of SP-D is insufficient for binding to bacterial cell membranes.

The composition of surfactant in CF patients has been analyzed by a number of investigators. For example, Postle *et al.* (15) showed that both SP-A and SP-D protein levels are decreased in the BAL of CF patients. In these studies, surfactant phospholipid levels were unchanged. Additional studies have demonstrated decreased SP-A levels in CF BAL as well as a reduction in the surface tension-reducing properties of CF surfactant (16, 17). The decrease in lipid associated SP-A in these patients may contribute to the loss of surface activity. Levels of the hydrophobic protein SP-B, which functions in the surface active properties of surfactant, were unchanged in CF BAL (17). A separate study conducted in infants with CF showed elevated SP-A levels, whereas SP-B levels were unchanged (46). A definitive explanation for these contrasting results is not available; however, the median age of the subjects in the latter study (31 months) was much younger than subjects in the other studies, a factor that may be contributory. We have previously shown that SP-A can be degraded by purified *P. aeruginosa* elastase in the presence of BAL lipids, and SP-A fragments are present in CF BAL samples (19). In the current study, we show that SP-D is also degraded in human BAL to produce a 35-kDa fragment under reducing conditions that is similar in size to that produced *in vitro* by elastase degradation of purified recombinant rat SP-D. In addition, SP-D fragments were detectable in CF BAL samples. It is likely that at least a portion of the observed decreases in SP-A and SP-D levels in CF BAL are due to *P. aeruginosa* elastase activity.

Whereas changes in SP-A and SP-D levels may have a role in surfactant function, it is likely that their roles as immune molecules are more critical to the inflammation seen in CF. In the present study, we confirm that degradation of SP-D abrogates protein function. Two key roles for SP-A and SP-D in response to bacterial challenge are to attenuate production of inflammatory cytokines and promote clearance of bacteria and apoptotic neutrophils by alveolar macrophages. Loss of these functions due to protein degradation by elastase, as we have shown for SP-D, would be predicted to lead to decreased bacterial clearance and greater inflammation. Recent studies in a mouse model of chronic *P. aeruginosa* infection (47) support this hypothesis. In this study, mice were infected with either PA01 *P. aeruginosa*, which express elastase, or PA01-E64, which has greatly reduced elastase activity, for 90 days. Whereas the numbers of viable bacteria per lung were the same in both groups, the mice infected with elastase producing PA01 had a 3–4-fold greater number of lymphocytes than the mice infected with elastase-deficient *P. aeruginosa*. This study demonstrates the potential contribution of elastase as a virulence factor in *P. aeruginosa* infections, although the impact on SP-A and SP-D was not determined. Future studies in animal models utilizing an elastase null *P. aeruginosa* strain will be needed to

assess the effects of elastase production on SP-A and SP-D levels *in vivo* and the contribution to changes in inflammatory infiltrate in the lungs.

In summary, our data demonstrate a mechanism by which *P. aeruginosa* can specifically degrade and inactivate an important lung pattern recognition molecule, SP-D. This potential mechanism of virulence may contribute to the pathogenic ability of *P. aeruginosa* in the lung. Degradation of SP-D by elastase may represent a novel strategy employed by *P. aeruginosa* to evade normal innate immune defenses and may help explain why CF patients preferentially establish infections with *P. aeruginosa* and why mortality is often due to this bacteria despite the presence of other bacteria in the lung.

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