Pseudomonas aeruginosa protease IV degrades surfactant proteins and inhibits surfactant host defense and biophysical functions

Jaret L. Malloy, Richard J. O’Callaghan, and Jo Rae Wright

1Department of Cell Biology, Duke University Medical Center, Durham, North Carolina; 2Department of Physiology and Pharmacology and Medicine, Lawson Health Research Institute, University of Western Ontario, London, Ontario, Canada; and 3Department of Microbiology, Immunology, and Parasitology, Louisiana State University Medical Center, New Orleans, Louisiana

Submitted 26 August 2004; accepted in final form 25 October 2004

PULMONARY SURFACTANT IS SYNTHESIZED and secreted by alveolar type II cells and is composed of 90% lipids (~80% phospholipids) and 10% proteins, the latter consisting primarily of four surfactant-associated proteins designated SP-A, SP-B, SP-C, and SP-D (9). Pulmonary surfactant has two distinct functions within the lung; reduction of surface tension at the air-liquid interface and participation in innate host defense. Both functions are dependent on surfactant-associated proteins. Pseudomonas aeruginosa is primarily responsible for respiratory dysfunction and death in cystic fibrosis patients and is also a leading pathogen in nosocomial pneumonia. P. aeruginosa secretes a number of proteases that contribute to its virulence. We hypothesized that P. aeruginosa protease IV degrades surfactant proteins and results in a reduction in pulmonary surfactant host defense and biophysical functions. Protease IV was isolated from cultured supernatant of P. aeruginosa by gel chromatography. Incubation of cell-free bronchoalveolar lavage fluid with protease IV resulted in degradation of surfactant proteins (SP)-A, -D, and -B. SPs were degraded in a time- and dose-dependent fashion by protease IV, and degradation was inhibited by the trypsin-like converting enzyme (TLCK). Degradation by protease IV inhibited SP-A- and SP-D-mediated bacterial aggregation and uptake by macrophages. Surfactant treated with protease IV was unable to reduce surface tension as effectively as untreated surfactant, and this effect was inhibited by TLCK. We speculate that protease IV may be an important contributing factor to the development and propagation of acute lung injury associated with P. aeruginosa via loss of surfactant function within the lung.

bacterial aggregation; bacterial uptake; macrophages; surface tension; surfactant aggregates

Address for reprint requests and other correspondence: J. R. Wright, Box 3709, Dept. of Cell Biology, Duke Univ. Medical Center, Durham, NC 27710 (E-mail: j.wright@cellbio.duke.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
be conclusively identified at that time. Since this initial observation, we have determined that this additional secreted protein is the recently described *P. aeruginosa* protease IV, a secreted serine protease that is regulated in a manner distinct from the other *P. aeruginosa* proteases (5, 39). Protease IV has been shown to be a significant virulence factor in keratitis caused by *P. aeruginosa* (6, 28). The significance of protease IV in lung virulence of *P. aeruginosa* has not been investigated. We hypothesize that protease IV degrades surfactant proteins, decreasing both the host defense and biophysical properties of pulmonary surfactant, therefore suggesting that protease IV may be a significant virulence factor in the development of acute lung injury associated with *P. aeruginosa*.

**MATERIALS AND METHODS**

**Materials and animals.** All chemicals were purchased from Sigma (St. Louis, MO) except where noted. All experiments utilizing bronchoalveolar lavage fluid (BALF) and alveolar macrophages were conducted with pathogen-free male Sprague-Dawley rats (Taconic, Germantown, NY) ranging from 250 to 350 g. All animal procedures were approved by the Duke University Medical Center Institutional Animal Care and Use Committee.

**Purification of *P. aeruginosa* protease IV.** Protease IV was initially purified from concentrated culture supernatants of *P. aeruginosa* strain PA103-29 using ion exchange and molecular sieve chromatography as previously described (5). Additional protease IV was isolated from culture supernatants of PAO1-B1 (kindly provided by Dr. B. Iglewski; Univ. of Rochester, Rochester, NY) by another chromatography method. Briefly, *P. aeruginosa* PAO1-B1 was grown in 2-liter cultures of nutrient broth (Difco, Detroit, MI), shaken (250 rpm) for 24 h at 37°C, and subsequently centrifuged at 7,000 g for 20 min to pellet the cells. Resulting supernatant was filtered over a 0.45-μm Durapore membrane filter (Millipore, Marlborough, MA) and concentrated by ultrafiltration with tangential flow using a Pellicon XL device with a 10-kDa cut-off membrane (Millipore) to a volume of 200 ml. The supernatant was applied to a DEAE Sepharose CL-6B column (50 × 1.6 cm) in 30 mM Tris-HCl, pH 8.3, and proteins were eluted with a gradient formed by the addition of 10 mM ammonium acetate buffer at pH 6.8. Purity of the gradient formed by the addition of 10 mM ammonium acetate buffer at pH 9 to 10 mM ammonium acetate buffer at pH 6.8, and proteins were eluted with a continuous linear gradient of NaCl (0 – 0.5 M) in the same buffer. Protease IV-positive fractions were pooled and applied to a CM Sepharose Fast Flow column (Amersham Biosciences) in 10 mM ammonium, pH 6.8, and proteins were eluted with a reaction buffer. The protease was inactivated by electrophoresis of equal volumes of reaction mixture (5 μl) on 15% SDS-PAGE under reducing conditions and analyzed by Western blot using well-characterized anti-human SP-A antibody (kindly provided by ALTANA Pharma, Konstanz, Germany) and anti-rat SP-D antibody (26). Inhibition studies were performed in an identical manner as the degradation assays except protease IV was incubated with 1 μM Nα-p-tosyl-L-lysine-chloromethyl ketone (TLCK) for 30 min at room temperature before the addition of the SPs.

**Degradation of SPs in BALF.** Normal rat BALF was obtained by a single whole lung lavage using 150 mM NaCl with 0.2 mM EDTA. The resultant BALF was spun at 150 g for 10 min to remove alveolar cells. Cell-free BALF (0.15 mg phospholipid/ml) was incubated with purified protease IV (5 μg/ml) for 24 h at 37°C. Control samples replaced protease IV with reaction buffer. The protease was inactivated by electrophoresis of equal volumes of reaction mixture (5 μl) on 15% SDS-PAGE under reducing conditions and analyzed by Western blot using well-characterized anti-human SP-A antibody (kindly provided by ALTANA Pharma) and anti-rat SP-D antibody. For analysis of SP-B, equal volumes of reaction mixture (30 μl) were resolved by 15% SDS-PAGE under nonreducing conditions and analyzed by Western blot using an anti-sheep SP-B antibody.

**Degradation of *S. typhimurium* in BALF.** Normal rat BALF was obtained by a single whole lung lavage using 150 mM NaCl with 0.2 mM EDTA. The resultant BALF was spun at 150 g for 10 min to remove alveolar cells. Cell-free BALF (0.15 mg phospholipid/ml) was incubated with purified protease IV (5 μg/ml) for 24 h at 37°C. Control samples replaced protease IV with an equal volume of 30 mM Tris, pH 7.75. For analysis of SP-A and SP-D, equal volumes of reaction mixture (30 μl) were resolved by 15% SDS-PAGE under reducing conditions and analyzed by Western blot using well-characterized anti-human SP-A antibody (kindly provided by ALTANA Pharma) and anti-rat SP-D antibody. For analysis of SP-B, equal volumes of reaction mixture (30 μl) were resolved by 15% SDS-PAGE under nonreducing conditions and analyzed by Western blot using an anti-sheep SP-B antibody.

**Degradation of *S. typhimurium* in BALF.** Normal rat BALF was obtained by a single whole lung lavage using 150 mM NaCl with 0.2 mM EDTA. The resultant BALF was spun at 150 g for 10 min to remove alveolar cells. Cell-free BALF (0.15 mg phospholipid/ml) was incubated with purified protease IV (5 μg/ml) for 24 h at 37°C. Control samples replaced protease IV with an equal volume of 30 mM Tris, pH 7.75. For analysis of SP-A and SP-D, equal volumes of reaction mixture (30 μl) were resolved by 15% SDS-PAGE under reducing conditions and analyzed by Western blot using well-characterized anti-human SP-A antibody (kindly provided by ALTANA Pharma) and anti-rat SP-D antibody. For analysis of SP-B, equal volumes of reaction mixture (30 μl) were resolved by 15% SDS-PAGE under nonreducing conditions and analyzed by Western blot using an anti-sheep SP-B antibody.

**Degradation of *S. typhimurium* in BALF.** Normal rat BALF was obtained by a single whole lung lavage using 150 mM NaCl with 0.2 mM EDTA. The resultant BALF was spun at 150 g for 10 min to remove alveolar cells. Cell-free BALF (0.15 mg phospholipid/ml) was incubated with purified protease IV (5 μg/ml) for 24 h at 37°C. Control samples replaced protease IV with an equal volume of 30 mM Tris, pH 7.75. For analysis of SP-A and SP-D, equal volumes of reaction mixture (30 μl) were resolved by 15% SDS-PAGE under reducing conditions and analyzed by Western blot using well-characterized anti-human SP-A antibody (kindly provided by ALTANA Pharma) and anti-rat SP-D antibody. For analysis of SP-B, equal volumes of reaction mixture (30 μl) were resolved by 15% SDS-PAGE under nonreducing conditions and analyzed by Western blot using an anti-sheep SP-B antibody.

**Degradation of *S. typhimurium* in BALF.** Normal rat BALF was obtained by a single whole lung lavage using 150 mM NaCl with 0.2 mM EDTA. The resultant BALF was spun at 150 g for 10 min to remove alveolar cells. Cell-free BALF (0.15 mg phospholipid/ml) was incubated with purified protease IV (5 μg/ml) for 24 h at 37°C. Control samples replaced protease IV with an equal volume of 30 mM Tris, pH 7.75. For analysis of SP-A and SP-D, equal volumes of reaction mixture (30 μl) were resolved by 15% SDS-PAGE under reducing conditions and analyzed by Western blot using well-characterized anti-human SP-A antibody (kindly provided by ALTANA Pharma) and anti-rat SP-D antibody. For analysis of SP-B, equal volumes of reaction mixture (30 μl) were resolved by 15% SDS-PAGE under nonreducing conditions and analyzed by Western blot using an anti-sheep SP-B antibody.

**Degradation of *S. typhimurium* in BALF.** Normal rat BALF was obtained by a single whole lung lavage using 150 mM NaCl with 0.2 mM EDTA. The resultant BALF was spun at 150 g for 10 min to remove alveolar cells. Cell-free BALF (0.15 mg phospholipid/ml) was incubated with purified protease IV (5 μg/ml) for 24 h at 37°C. Control samples replaced protease IV with an equal volume of 30 mM Tris, pH 7.75. For analysis of SP-A and SP-D, equal volumes of reaction mixture (30 μl) were resolved by 15% SDS-PAGE under reducing conditions and analyzed by Western blot using well-characterized anti-human SP-A antibody (kindly provided by ALTANA Pharma) and anti-rat SP-D antibody. For analysis of SP-B, equal volumes of reaction mixture (30 μl) were resolved by 15% SDS-PAGE under nonreducing conditions and analyzed by Western blot using an anti-sheep SP-B antibody.
were performed which included bacteria plus an equivalent amount of protease IV that would have been associated with the SP degradation assays.

**Bacterial association with alveolar macrophages.** Escherichia coli strains K12 (rough) and HB101 were grown overnight in 5 ml of LB broth, collected at 2,000 g for 10 min, and resuspended in 1 ml of Na2CO3, pH 9.0. Bacteria were labeled with FITC 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 removed by washing the bacteria three times with PBS. Bacteria concentration was determined by measurement of absorbance at 660 nm utilizing known extinction coefficients.

SPs were incubated in the presence or absence of protease IV for 24 h at 37°C before the bacterial association assay was performed. SP-A at a concentration of 50 μg/ml was incubated with 5 μg/ml of protease IV. SP-D at a concentration of 12.5 μg/ml was incubated with 5 μg/ml of protease IV.

Alveolar macrophages from normal rats were isolated by whole lung lavage, and resultant lavage fluid was centrifuged at 150 g for 10 min. The cell pellet was resuspended in PBS plus 2 mM CaCl2 plus 0.1% BSA, and cell numbers were determined by counting on a hemocytometer. Purity of isolated cells was determined by cytoospin and hemacolor staining.

Eppendorf tubes were initially incubated with PBS plus 1% BSA (1 ml) at 4°C for 1 h before addition of macrophages and bacteria to block nonspecific binding sites. Freshly isolated alveolar macrophages (5 × 10⁷) were then added and incubated with FITC-labeled bacteria (5 × 10⁸ cfu) in 250 μl of PBS plus 1 mM CaCl₂ plus 0.1% BSA for 1 h at 37°C. For SP-A experiments, a volume of the degradation assay resulting in a final concentration of 5 μg/ml was added to the macrophages and E. coli K12. For SP-D experiments, a volume of the degradation assay resulting in a final concentration of 1 μg/ml was added to the macrophages and E. coli HB101. Assay was terminated by addition of 1 ml of ice-cold PBS plus 0.1% BSA. The alveolar macrophages were washed twice with ice-cold PBS buffer (150 g, 10 min, 4°C), fixed in 300 μl of 1% formaldehyde in PBS, and analyzed for fluorescence by fluorescence-activated cell sorting (FACS). An additional control was performed that included alveolar macrophages plus bacteria plus an equivalent amount of protease IV that would be associated with the SP degradation assay.

**Surface activity analysis.** In vitro surface tension measurements of LA were performed using a pulsating bubble surfactometer (Electronetics, Amherst, NY) as described by Enhorning (8). LA were pelleted after a 15-min, 40,000 g centrifugation of cell-free BALF recovered from healthy rats. LA were prepared to obtain a final concentration of 2.5 mg of phospholipids per milliliter in 0.15 M NaCl and 1.5 mM CaCl₂ and frozen for later use. Three experimental conditions were analyzed: LA were incubated for 72 h at 37°C without protease IV, LA were incubated with protease IV (10 μg/ml), and LA were incubated with protease IV in the presence of 1 mM TLCK. After the incubation period, the samples were immediately frozen for later analysis. During this time period, the LA were kept in suspension with gentle agitation. After the incubation period, the samples were immediately frozen for later analysis, at which time the samples were incubated for 90 min at 37°C before surface activity was measured. Briefly, a bubble was created in the suspension containing the LA fraction. Surfactant adsorption kinetics was measured during the initial 10 s after bubble formation. Subsequently, the bubble was pulsed for a period of 5 min between a minimum radius of 0.44 mm and a maximum radius of 0.55 mm at a rate of 20 pulsations/min and a temperature of 37°C. Pressure was monitored across the air-liquid interface by a pressure transducer, and surface tension was calculated at the minimum and maximum bubble radii. All samples were analyzed at the same time, and the investigator was blinded to the experimental group.

**Statistics.** Data are expressed as means ± SD, and values between groups were compared using an unpaired Student’s t-test. A probability level of P < 0.05 was considered statistically significant.

**RESULTS**

**Identification of P. aeruginosa protease IV.** Previously, we determined that the surfactant collected from cultures of P. aeruginosa degrades SP-A (25). After separation of the secreted proteins in the cultured supernatant on an anion exchange column, two separate pooled fractions were determined to contain degradative activity toward SP-A. One of the fractions contained P. aeruginosa elastase; we were unable at that time to identify definitively the protein responsible for the degradation in the second fraction (25). Subsequently, Edman degradation analysis showed that the sequence of the peptide fragments (Fig. 1) corresponds to the recently described P. aeruginosa virulence factor, protease IV (5, 39). Protease IV has been determined to be a serine protease that demonstrates activity for the COOH side of lysine-containing peptides (5).

**SPs are degraded in the presence of protease IV.** To characterize the degradation of SPs by purified protease IV, we initially examined the time dependence of degradation. Figure 2A shows SP-A is degraded in a time-dependent manner by protease IV. Reduced human alveolar proteinosis SP-A migrates as both the dimer and monomer. Within 1 h of incubation, there is a decrease in the amount of both the SP-A dimer and monomer and the appearance of an intermediate band of ~65 kDa. After a 12-h incubation period, approximately one-half of the immunoreactive SP-A is degraded, and within 24 h, no immunoreactive protein can be detected. Figure 2B demonstrates that SP-D degradation is also time dependent. Incubation of recombinant rat SP-D with protease IV for 1 h results in the loss of any intact SP-D protein (~49 kDa) and produces a SP-D fragment of ~33 kDa. With increasing incubation time, this SP-D fragment is progressively degraded until no immunoreactive SP-D fragment is observed after a 24-h incubation period.

The effect of protease IV dose on the degradation of these proteins was investigated. Figure 3A demonstrates that incubation of SP-A with increasing concentrations of protease IV for 24 h results in increased degradation of the protein. At the lowest dose of protease IV (2.5 μg/ml), we observe degrada-
of both the monomer and dimer of SP-A and the appearance of the ~65-kDa intermediate band. The highest dose of protease IV tested (10 μg/ml) resulted in almost complete degradation of both SP-A forms. To implicate a specific effect of protease IV, the ability of TLCK, an irreversible inhibitor of the serine protease trypsin and many trypsin-like serine proteases, to inhibit degradation was analyzed. When protease IV was preincubated with TLCK, there was complete inhibition of the degradation of SP-A. Figure 3B demonstrates that degradation of SP-D by protease IV is also dose dependent, similar to SP-A degradation. The lowest dose of protease IV tested (2.5 μg/ml) results in the complete loss of the intact proteins and the appearance of the ~33-kDa SP-D fragment. Increasing the concentration of protease IV resulted in the complete degradation of this SP-D fragment. As demonstrated with SP-A, preincubation with TLCK resulted in the complete inhibition of protease IV activity against SP-D.

SPs are degraded by protease IV in the presence of surfactant lipids. Pulmonary surfactant is primarily composed of phospholipids (~80%), whereas the proteins comprise 10% by weight. SP-A and SP-D are hydrophilic proteins; SP-A avidly binds dipalmitoylphosphatidylcholine, the most abundant surfactant phospholipid (17), and SP-D binds phosphatidylinositol, a minor component of surfactant (29). The other SPs, SP-B and SP-C, are hydrophobic proteins that are tightly associated with surfactant lipids. To determine whether protease IV can degrade SPs in whole pulmonary surfactant, BALF was obtained from normal healthy rats and incubated with or without protease IV. Immunoreactive SP-A, SP-D, and SP-B were detected in aliquots of the reaction mixture as shown in Fig. 4, A–C, respectively. In Fig. 4A, rat SP-A migrates as a monomer (~38 kDa), and after incubation of BALF with protease IV, there is a considerable loss of immunoreactive SP-A. Figure 4B demonstrated that degradation of SP-D in the presence of surfactant lipids also results in the appearance of an ~33-kDa SP-D fragment similar to the isolated SP-D experiments. Non-reduced SP-B is displayed in Fig. 4C and demonstrates that this hydrophobic SP is also susceptible to degradation by protease IV.

Degradation of SPs by protease IV inhibits bacterial aggregation. Aggregation of bacteria by SP-A and SP-D has been shown to be a function of these innate immune molecules. To test a functional consequence of degradation of SPs by protease IV, isolated SP-A and SP-D were incubated with and without protease IV for 24 h at 37°C and subsequently tested for its
ability to aggregate *S. typhimurium*. Bacterial aggregation was measured by the decrease in absorbance (OD 660 nm) as the aggregated bacteria precipitate out of solution.

Figure 5A displays the effect of SP-A (10 μg/ml) on bacterial aggregation over a 60-min time course for three independent experiments. *S. typhimurium* alone did not self-aggregate as shown by stable absorbance readings over the 60-min experimental time period. Incubation of *S. typhimurium* with intact SP-A (not incubated with protease IV) resulted in aggregation of the bacteria as demonstrated by a significant decrease in absorbance at the 30- and 60-min time points compared with the *S. typhimurium* control (*P < 0.01*). In contrast, incubation of *S. typhimurium* with degraded SP-A (incubated with protease IV for 24 h before) inhibited bacterial aggregation as demonstrated by a significantly higher absorbance at the 30- and 60-min time points compared with the intact SP-A (*P < 0.01*). There was also no significant difference in absorbance compared with the control *S. typhimurium* group at any time point. Incubation of *S. typhimurium* with protease IV alone did not affect bacterial aggregation (data not shown). Figure 5B displays an individual bacterial aggregation experiment with the corresponding degree of SP-A degradation shown by Western blot.

Figure 6A displays the effect of SP-D (2.5 μg/ml) on the mean absorbance (OD ± SD) of a bacterial suspension over a 60-min time course for three independent experiments. *S. typhimurium* alone did not result in self-aggregation as shown by a nonsignificant decrease in absorbance over the 60-min experimental time period. Incubation of *S. typhimurium* with intact SP-D (not incubated with protease IV) resulted in aggregation of the bacteria as demonstrated by a significant decrease in absorbance at the 30- and 60-min time points compared with the *S. typhimurium* control (*P < 0.05*). In contrast, incubation of *S. typhimurium* with degraded SP-D (incubated with protease IV for 24 h before) reduced bacterial aggregation as demonstrated by a significantly higher absorbance at the 30- and 60-min time points compared with the intact SP-D (*P < 0.05*). There was also no significant difference in absorbance compared with the control *S. typhimurium* group at any time point. Incubation of *S. typhimurium* with protease IV alone did not affect absorbance of the bacterial suspension (data not shown). Figure 6B displays an individual bacterial aggregation experiment with the corresponding degree of SP-D degradation shown by Western blot. Interestingly, even in the presence of the SP-D degradation fragment formed after incubation with protease IV, bacterial aggregation was still abolished. Therefore, initial degradation of SP-D to the fragment would abrogate this immunological function.

---

**Fig. 4.** PIV degrades SP-A, SP-D, and SP-B in bronchoalveolar lavage fluid (BALF). Rat BALF was incubated in the absence or presence of PIV (1 μg) for 24 h at 37°C. A: SP-A was measured in equal volumes of reaction mixture, resolved by 15% SDS-PAGE under reducing conditions, and analyzed by Western blot. B: SP-D was measured in equal volumes of reaction mixture, resolved by 15% SDS-PAGE under reducing conditions, and analyzed by Western blot. C: SP-B was measured in equal volumes of reaction mixture, resolved by 15% SDS-PAGE under nonreducing conditions, and analyzed by Western blot.

**Fig. 5.** Degradation of SP-A by PIV inhibits bacterial aggregation. SP-A (50 μg/ml) was incubated in the presence or absence of PIV (5 μg/ml) at 37°C for 24 h. Reaction mixture was incubated with *Salmonella typhimurium* [5 × 10⁷ colony-forming units (cfu)], and aggregation was measured as a decrease in absorbance at 660 nm over 60 min. A: the mean and SD of 3 independent experiments for the 3 experimental groups: *S. typhimurium* control (*S. typh*), *S. typhimurium* with intact SP-A (*S. typh + SP-A*), and *S. typhimurium* with degraded SP-A (*S. typh + D SP-A*). B: individual experiment with the corresponding SP-A Western blot displaying the degree of degradation of SP-A in the presence of PIV. *P < 0.05* vs. *S. typh* and *S. typh + D SP-A*.
Degradation of SPs by protease IV inhibits their ability to enhance bacterial association with alveolar macrophages. SP-A and SP-D can act as opsonins within the lung to promote bacterial clearance by alveolar macrophages. To investigate the functional consequence of protease IV on this host defense function of SP-A and SP-D, the uptake of FITC-labeled bacteria with alveolar macrophages in the presence of intact or protease IV-degraded SPs proteins was investigated by FACS.

Figure 7A displays the effect of SP-A (5 μg/ml) on the association of FITC-labeled E. coli with alveolar macrophages as measured by mean fluorescence (± SD) and displayed as a percentage of the alveolar macrophages plus E. coli control group. Addition of intact SP-A (not incubated with protease IV) to the alveolar macrophage-bacteria suspension resulted in a significant increase in the association of E. coli with this cell type (P < 0.01). In contrast, degraded SP-A (incubated with protease IV for 24 h before assay) did not enhance bacterial association as did intact SP-A (P < 0.01). Of note, incubation of alveolar macrophages and E. coli with protease IV alone did not have an effect on the mean fluorescence level associated with alveolar macrophages after the experimental period (data not shown). Figure 8A displays an individual bacterial association experiment with the corresponding degree of SP-A degradation shown by Western blot.

Figure 8B displays an individual bacterial association experiment with the corresponding degree of SP-A degradation shown by Western blot.

Degradation of SPs by protease IV inhibits their ability to enhance bacterial association with alveolar macrophages. SP-A and SP-D can act as opsonins within the lung to promote bacterial clearance by alveolar macrophages. To investigate the functional consequence of protease IV on this host defense function of SP-A and SP-D, the uptake of FITC-labeled bacteria with alveolar macrophages in the presence of intact or protease IV-degraded SPs proteins was investigated by FACS.

Figure 7A displays the effect of SP-A (5 μg/ml) on the association of FITC-labeled E. coli with alveolar macrophages as measured by mean fluorescence (± SD) and displayed as a percentage of the alveolar macrophages plus E. coli control group. Addition of intact SP-A (not incubated with protease IV) to the alveolar macrophage-bacteria suspension resulted in a significant increase in the association of E. coli with macrophages (P < 0.01). In contrast, addition of degraded SP-A (incubated with protease IV for 24 h before assay) had no effect on the bacterial association with alveolar macrophages as shown by the same level of fluorescence as the control group and significantly less fluorescence associated with macrophages than when intact SP-A was added (P < 0.01). Additionally, incubation of alveolar macrophages and E. coli with protease IV alone did not affect the mean fluorescence level associated with alveolar macrophages after the experimental period. Figure 7B displays an individual bacterial association experiment with the corresponding degree of SP-A degradation shown by Western blot.

Figure 8A illustrates the effect of SP-D (1 μg/ml) on the association of FITC-labeled E. coli with alveolar macrophages as measured by mean fluorescence (± SD) and displayed as a percentage of the alveolar macrophages plus E. coli control group. Addition of intact SP-D (not incubated with protease IV) to the alveolar macrophage-bacteria suspension resulted in a significant increase in the association of E. coli with this cell type (P < 0.01). In contrast, degraded SP-D (incubated with protease IV for 24 h before assay) did not enhance bacterial association as did intact SP-D (P < 0.01). Of note, incubation of alveolar macrophages and E. coli with protease IV alone did not have an effect on the mean fluorescence level associated with alveolar macrophages after the experimental period (data not shown). Figure 8B displays an individual bacterial associa-
Experimental period between the control group and the group there was no difference in surface tensions over the entire biophysical function was specifically due to protease IV as group (protease IV with both the control LA group and the inhibited protease IV were significantly greater in the protease IV group compared with protease IV, and rat LA incubated with protease IV and plays the surface tensions of normal rat LA, rat LA incubated with protease IV and demonstrates to degrade a number of important biological proteins, including fibrinogen, plasminogen, immunoglobulin G, and complement components C3 and C1q; its activity is demonstrated to degrade a number of important biological proteins; however, the second protein could not be identified. As presented in this paper, the other degradative protein has now been identified by Edman degradation as P. aeruginosa protease IV (lysyl endopeptidase; EC 3.4.21.50; Fig. 1). In this paper, it is demonstrated in vitro that protease IV degrades SP-A and SP-D in a time- and concentration-dependent manner (Figs. 2 and 3) and, moreover, degrades SP-A, SP-D, and SP-B in BALF (Fig. 4). The degradation of SP-A and SP-D resulted in the inhibition of their ability to aggregate bacteria and enhance bacterial uptake by alveolar macrophages (Figs. 5–8). Moreover, incubation of surfactant with protease IV impacted the biophysical function of surfactant such that protease IV inhibited the surface tension-lowering function of LA (Fig. 9).

This is the first paper to demonstrate the effect of a P. aeruginosa protease, namely protease IV, on both the innate host defense functions and the biophysical function of pulmonary surfactant.

**DISCUSSION**

The original observation that P. aeruginosa degraded SP-A and SP-D was made by Mariencheck et al. (25) when investigating binding interactions between SP-A and P. aeruginosa. In these initial experiments, it was shown that two separate pooled fractions obtained by separation of P. aeruginosa culture supernatants on an anion exchange column degraded SP-A and SP-D. At that time, P. aeruginosa elastase, a zinc metalloprotease, was positively identified as one of the degradative proteins; however, the second protein could not be identified.

As presented in this paper, the other degradative protein has now been identified by Edman degradation as P. aeruginosa protease IV (lysyl endopeptidase; EC 3.4.21.50; Fig. 1). In this paper, it is demonstrated in vitro that protease IV degrades SP-A and SP-D in a time- and concentration-dependent manner (Figs. 2 and 3) and, moreover, degrades SP-A, SP-D, and SP-B in BALF (Fig. 4). The degradation of SP-A and SP-D resulted in the inhibition of their ability to aggregate bacteria and enhance bacterial uptake by alveolar macrophages (Figs. 5–8). Moreover, incubation of surfactant with protease IV impacted the biophysical function of surfactant such that protease IV inhibited the surface tension-lowering function of LA (Fig. 9).

This is the first paper to demonstrate the effect of a P. aeruginosa protease, namely protease IV, on both the innate host defense functions and the biophysical function of pulmonary surfactant.

**P. aeruginosa** secretes a number of virulence factors that aid in its survival and are crucial in the pathogenesis of infection. Four secreted proteases are a component of these factors and include the aforementioned elastase (LasB protease), a LasA protease, alkaline protease, and the recently described protease IV (5, 18). Protease IV is a serine protease that cleaves on the COOH side of lysine-containing peptides as determined by substrate susceptibility test. This 26-kDa protease has been demonstrated to degrade a number of important biological proteins, including fibrinogen, plasminogen, immunoglobulin G, and complement components C3 and C1q; its activity is inhibited by TLCK and the reducing agents dithiothreitol and 2-mercaptoethanol (5). Interestingly, protease IV has been shown to be the iron-regulated protein PrpL, which suggests that its expression is differentially regulated compared with the other P. aeruginosa extracellular enzymes (39). To date, protease IV has been implicated as an important virulence factor that contributes to the pathogenesis of Pseudomonas keratitis (6, 7, 28). For example, purified protease IV induced corneal
epithelial damage within 3 h after injection into the corneal stoma, and *Pseudomonas* strains that produce protease IV are highly damaging to rabbit and mouse corneas, whereas strains that are deficient in protease IV have reduced corneal virulence. To date, the effect of protease IV on other organ systems has not been investigated.

The current study demonstrates that protease IV degraded the pulmonary innate host defense molecules SP-A and SP-D in a time- and dose-dependent manner. Incubation of SP-A with protease IV resulted in qualitatively decreased concentrations of SP-A dimer and monomer with the appearance of an intermediate SP-A species as early as 1 h after incubation with protease IV. Previously, neutrophil elastase and *P. aeruginosa* elastase were shown to degrade SP-A in a similar manner (22, 25, 30); however, *P. aeruginosa* elastase resulted in the appearance of a 20-kDa SP-A fragment, whereas degradation of SP-A by protease IV resulted in only very small peptide fragments as confirmed by silver staining (data not shown). From the complete degradation of SP-A, it would be assumed that its immunological functions would be abrogated. To confirm this speculation, we examined two different SP-A functions after degradation by protease IV by utilizing two different types of bacteria. Degradation of SP-A after incubation with protease IV impaired its ability to aggregate *S. typhimurium* and also abolished SP-A-mediated increased uptake of *E. coli* by alveolar macrophages. In vivo, this would translate into decreased clearance of bacteria from the lung. Of note, SP-A-null mice, when inoculated with *P. aeruginosa*, have impaired clearance of the bacteria, further enforcing the importance of SP-A in the protection from this pathogen (20). Therefore, this opportunistic organism may secrete protease IV to aid in its survival and colonization by degrading a component of the innate host defense within the lung.

Recently, there has been a series of reports describing the degradation of SP-D by different biologically produced proteases. *P. aeruginosa* elastase, a zinc metalloprotease, degraded SP-D by cleaving in the COOH-terminal carbohydrate recognition domain, resulting in an ~35-kDa fragment (1). In addition, three active neutrophil serine proteases: neutrophil elastase, proteinase-3, and cathepsin G also degraded SP-D with protease IV degraded SP-D to a slightly larger fragment; however, more specifically, *P. aeruginosa* protease IV degraded SP-D to a relatively stable 35-kDa fragment (15). In the current study, it was demonstrated that *P. aeruginosa* protease IV also degraded SP-D to an ~35-kDa fragment within 1 h of incubation time. However, in the presence of protease IV, this fragment was relatively unstable and degraded over time, whereas the degradation by the previous proteases resulted in a relatively stable fragment. Interestingly, all of these biologically relevant proteases degraded SP-D to a similar-sized fragment; however, more specifically, *P. aeruginosa* protease IV degraded SP-D to a slightly larger fragment than *P. aeruginosa* elastase as observed by Western blot analysis (unpublished observation). These data suggest that there may be a region of SP-D that is highly accessible to different proteases resulting in comparable sized fragments but with distinct cleavage sites.

As with SP-A, we investigated the functional significance of SP-D degradation by protease IV. As seen with SP-A, degraded SP-D failed to aggregate *S. typhimurium* and no longer enhanced *E. coli* uptake by alveolar macrophages. Interestingly, when examining the degree of SP-D degradation that corresponded with individual aggregation and bacterial uptake assays, we discovered individual experiments in which the 35-kDa fragment was still present after 24 h of incubation with protease IV. In these experiments, as shown in Figs. 6B and 8B, the SP-D fragment formed by protease IV had lost its host defense functions, which is in agreement with observations from the previous studies (1, 15). Of particular importance, this SP-D fragment was observed within the first hour of incubation with protease IV, and together with the previous observations, this suggests that protease IV can rapidly inhibit the host...
defense function of SP-D, which could be an important initiating step in colonization of the lung by *P. aeruginosa*.

Investigations of pulmonary surfactant recovered from cystic fibrosis patients and patients with severe pneumonia have documented decreased lavage levels of SP-A and SP-D, unchanged phospholipids levels but diminished surface tension-reducing properties (11, 13, 27, 31). In the current study, *P. aeruginosa* protease IV decreased the adsorption kinetics and increased minimum surface tension of isolated LA. This effect was attributed to protease IV since specific inhibition of its proteolytic activity by TLCK abrogated any decrease in biophysical function of the LA. SP-A was degraded in the LA incubated with protease IV, and this degradation was absent when TLCK inhibited protease IV activity. Unfortunately, it was not possible to convincingly demonstrate that SP-B was degraded in these samples and therefore we cannot definitively conclude whether impaired biophysical properties are due to degradation of SP-A alone or SP-A and SP-B. Importantly, a study by Liao et al. (22) reported that degradation of SP-A, SP-B, and SP-C resulted in higher adsorption rates and higher minimum surface tension values compared with controls, whereas degradation of SP-A alone resulted in only altered adsorption rates. These previous findings in combination with the detectable degradation of SP-B in our BALF experiments (Fig. 4C) suggest that there may have been a nondetectable degradation of SP-B in our LA samples. Nevertheless, we have documented that *P. aeruginosa* protease IV specifically alters the biophysical function of pulmonary surfactant by impairing adsorption and increasing minimum surface tension. Importantly, protease IV had no effect on the concentration of phospholipids, and the restoration of LA function when protease IV was inhibited with TLCK indicates the observed decrease in function was not the consequence of nonspecific protein inhibition (2). In this context, proteolytic degradation of SPs, such as that reported in the current study, may explain the loss of surfactant function even when overall phospholipid levels are unchanged in a situation of acute lung injury.

In conclusion, protease IV can be included with elastase as *P. aeruginosa* proteases that degrade SP-A and SP-D. Importantly, though, production of protease IV can be regulated in a host defense function of these SPs, which may contribute to bacterial virulence. In the current study, protease IV degrades SP-A and SP-D, resulting in reduction in host defense functions of these SPs, which may contribute to colonization of the lung. Additionally, protease IV alters the biophysical function of surfactant by inhibiting the surface tension-reducing property of this material, thus potentially contributing to decreased lung function. Together, these in vitro observations suggest that *P. aeruginosa* protease IV may play a significant role in both colonization of the lung and the progression of the associated acute lung injury. Future in vivo studies are important to determine the contribution of protease IV to the pathophysiology of the lung injury associated with *P. aeruginosa*.

ACKNOWLEDGMENTS

The authors thank Drs. John Alcorn and Bill Mariencheck for critical input and Kathy Evans for isolating SP-A and SP-D. Authors also thank Dr. J. Michael Cook and the staff of the Duke Cancer Center flow facility.

GRANTS

Funding was provided by National Institutes of Health Grants HL-51134 (to J. R. Wright) and EY-12961 (to R. J. O’Callaghan). J. L. Malloy is a Parker B. Francis fellow and was previously funded by the Canadian Lung Association and the Canadian Institutes of Health Research.

REFERENCES


