Elafin is a neutrophil serine protease inhibitor expressed in lung and displaying anti-inflammatory and anti-bacterial properties. Previous studies demonstrated that some innate host defense molecules of the cystic fibrosis (CF) and chronic obstructive pulmonary disease airways are impaired due to increased proteolytic degradation observed during lung inflammation. In light of these findings, we thus focused on the status of elafin in CF lung. We showed in the present study that elafin is cleaved in sputum from individuals with CF. Pseudomonas aeruginosa-positive CF sputum, which was found to contain lower elafin levels and higher neutrophil elastase (NE) activity compared with P. aeruginosa-negative samples, was particularly effective in cleaving recombinant elafin. NE plays a pivotal role in the process as only NE inhibitors are able to inhibit elafin degradation. Further in vitro studies demonstrated that incubation of recombinant elafin with excess of NE leads to the rapid cleavage of the inhibitor. Two cleavage sites were identified at the N-terminal extremity of elafin (Val-5—Lys-6 and Val-9—Ser-10). Interestingly, purified fragments of the inhibitor (Lys-6—Gln-57 and Ser-10—Gln-57) were shown to still be active for inhibiting NE. However, NE in excess was shown to strongly diminish the ability of elafin to bind lipopolysaccharide (LPS) and its capacity to be immobilized by transglutamination. In conclusion, this study provides evidence that elafin is cleaved by its cognate enzyme NE present at excessive concentration in CF sputum and that P. aeruginosa infection promotes this effect. Such cleavage may have repercussions on the innate immune function of elafin.

Elafin is a cationic 6-kDa non-glycosylated serine protease inhibitor belonging to the cheloniains, a distinct family of the canonical inhibitors also including secretory leukoprotease inhibitor (SLPI). It is also known as SKALP (skin-derived anti-leukoprotease) or ESI (elastase-specific inhibitor). The molecule displays a compact structure maintained by four conserved disulfide bridges characteristic of WAP (whey acidic protein) family and shares 40% sequence identity with SLPI. In addition to its ability to inhibit porcine pancreatic elastase, elafin is a potent inhibitor of two neutrophil serine proteases, neutrophil elastase (NE) and proteinase 3 (1, 2), and is therefore thought to protect tissue from degradation by these enzymes. Elafin is released by proteolytic cleavage from a larger molecule called trappin-2 or pre-elafin, which possesses at the N terminus of the whey acidic protein domain a cementoin domain containing several motifs having the consensus sequence GQDPVK that can act as transglutaminase substrate, allowing the cross-linking of the inhibitor to extracellular matrix proteins (3–5). It has been shown that trypaste, a mast cell-derived protease, may be involved in the proteolytic processing of trappin-2 into elafin (6). In vivo, elafin is mainly detected under inflammatory conditions, suggesting that the inhibitor is inducible. Several studies have demonstrated that elafin expression can be up-regulated in response to pro-inflammatory stimuli such as lipopolysaccharide (7), NE (8), and pro-inflammatory cytokines like IL-1β or TNF-α (9–12). Elafin/trappin-2 is mainly expressed by epithelial surfaces such as skin (13–15) or lung epithelium (10, 16) where the inhibitor acts as an antiprotease to protect tissue against proteolytic damages caused during inflammatory events. Inflammatory cells such as alveolar macrophages (17) and neutrophils (18) have also been shown to express the inhibitor. In addition to an antiprotease property, it has recently been demonstrated that trappin-2 and elafin possess anti-inflammatory and anti-bacterial activities. The two molecules display anti-bacterial activities against Pseudomonas aeruginosa (Gram-negative) and Staphylococcus aureus (Gram-positive) (11, 19), which appear to be independent of their anti-elastase activity or charge properties. In mice, trappin-2 has been shown to dose-dependently reduce LPS-induced neutrophil influx into alveoli in vivo.
addition to inhibiting LPS-induced production of matrix metalloproteinase-9 and the potent neutrophil attractants Cxc11 and Cxcl2 (chemokine ligands 1 and 2), suggesting an immunomodulatory role in innate immunity (20). By reducing NF-κB activation, trappin-2 has been demonstrated to attenuate IL-8 secretion by endothelial cells in response to various pro-inflammatory stimuli (TNF-α, LPS, oxidized low density lipoprotein) as well as LPS-induced TNF-α secretion by macrophages (21). Recently, our group demonstrated that elafin inhibits the LPS-induced production of MCP-1 in monocytes by inhibiting AP-1 and NF-κB activation (22).

During lung inflammation some components of the innate immune response have been shown to be sensitive to exacerbated host proteolytic activity emanating from dysregulated elastolytic enzymes (23). We have demonstrated that elastolytic cysteine cathepsins present in the lung under inflammatory conditions are involved in the inactivation of several host defense molecules such as SLPI, defensins, and lactoferrin. Cysteine cathepsins were shown to cleave and inactivate SLPI and defensins (human β-defensin 1 and 3), respectively, in epithelial lining fluid from individuals with emphysema (24) and CF (25). Additionally, lactoferrin degradation observed in P. aeruginosa-infected CF sputum was found to be due to an excess of cathepsin activity (26). Furthermore, other elastolytic proteases are potentially involved in the cleavage and inactivation of host defense molecules. Pseudomonas elastase, also referred as pseudolysin, has been demonstrated to cleave SLPI (27), and high concentrations of pseudolysin, Pseudomonas alkaline protease, and NE were also able to inactivate lactoferrin after a prolonged exposure (28).

In the present study we demonstrate that levels of elafin are lower in P. aeruginosa-positive as opposed to P. aeruginosa-negative CF sputa and that recombinant elafin incubated in P. aeruginosa-positive CF sputa is rapidly cleaved. Our data provide evidence that NE is involved in the cleavage of elafin in CF sputum as only NE inhibitors are able to inhibit this process. Furthermore, NE activity is higher in P. aeruginosa-positive CF sputa compared with P. aeruginosa-negative sputa, confirming that the low levels of elafin observed in P. aeruginosa-positive CF sputa are due to elevated NE levels with subsequent increased cleavage of elafin. We also demonstrate that purified NE in excess can cleave elafin at two distinct sites in its N-terminal extremity and that both fragments of the inhibitor generated upon NE activity still retain inhibitory activity. Although NE preserves the antiprotease activity of elafin, we show on the other hand that the protease considerably affects the capacities of elafin to cross-link with fibronectin by transglutamination and to bind LPS.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant human elafin was obtained from Proteo Biotech AG (Kiel, Germany). Goat anti-human elafin antibody and biotinylated anti-human elafin antibody were purchased from R&D Systems (Abingdon, Oxon, UK). Aprotinin, benzamidine, Pefabloc, N-terminal-l-lysine chloromethyl ketone hydrochloride (TLCK), N-p-tosyl-l-phenylalanine chloromethyl ketone (TPCK), N-(methoxysuccinyl)-Ala-Ala-Pro-Val-chloromethyl ketone (MeOSuc-AAPV-CMK), leupeptin, pepstatin A, EDTA N-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide (MeOSuc-AAPV-pNA), and guinea pig liver transglutaminase were purchased from Sigma-Aldrich. α1-Antichymotrypsin, α1-antitrypsin, E-64, GM6001, phosphoramidon, soybean trypsin inhibitor, and plasma fibronectin were purchased from Merck. Human neutrophil elastase, cathepsin G, and porcine pancreatic elastase were from Elastin Products Co., Inc. (Owensville, MO). Human proteinase 3 was from Athens Research (Athens, GA). Horseradish peroxidase-conjugated streptavidin was obtained from Zymed Laboratories Inc. (San Francisco, CA). SuperSignal West Femto Maximum Sensitivity Substrate was purchased from Pierce. All other reagents were of analytical grade.

**CF Sputum Processing**—The protocol for sputum processing was modified from that described elsewhere (29). Sputum was weighed and then treated with 4.5 volumes of normal saline per weight. Each sample was briefly vortexed and then rocked for 15 min at room temperature. An additional 4.5 volumes of normal saline were added to each sample and rocked for 5 min more. The sample was then filtered through sterile 48-μm nylon gauze, and the soluble sputum phase was obtained by centrifugation of the filtrate for 10 min at 790 × g. The supernatant was stored at −80 °C until required.

**Determination of Elafin Levels in CF Sputum by Enzyme-linked Immunosorbent Assay (ELISA)**—Goat anti-human elafin antibody (R&D Systems, AF1747, 1:500 in Voller’s buffer, 100 μl per well) was added to Immunlon-2 plates and left overnight at 4 °C. The plate was washed 3 times with PBS, 0.05% Tween 20 (PBS-T) and blocked in PBS-T containing 0.1% bovine serum albumin for 1 h at room temperature. After washing 3 times in PBS-T, elafin standards (100 μl per well, concentration ranged from 62.5 to 20,000 pg/ml) and CF sputum samples (100 μl per well) were added to the plate for 2 h at room temperature. The plate was then washed, and biotinylated anti-human elafin antibody (R&D Systems, BAF1747, 1:250 in PBS-T, 100 μl per well) was added to the plate for 2 h at room temperature. After washing, the plate was incubated with horseradish peroxidase-conjugated streptavidin (Zymed Laboratories Inc., 1:2500 in PBS-T, 100 μl per well) for 20 min at room temperature and washed with PBS-T. Peroxidase activity was measured by the addition of ABTS (100 μl per well) and reading the absorbance at 405 nm.

**Western Blot Analysis of Recombinant Elafin Incubated with CF Sputum and NE**—Recombinant elafin (8.5 × 10⁻⁷ M) was incubated with 10 μl of CF sputum in 30 mM Tris-buffered saline to a final volume of 20 μl for 0, 10 min, 1 h, 6 h, or 24 h at 37 °C. In some cases, CF samples were preincubated for 1 h at 37 °C with the following protease inhibitor before adding elafin: 10 mM Pefabloc, 0.13 mM TLCK, 13 μM aprotinin, 0.6 mM leupeptin, 0.4 mM E-64, 0.4 mM pepstatin A, 13 mM EDTA, 0.5 mM GM6001 or 0.2 mM phosphoramidon, 0.3 mM TPCK, 3 mM benzamidine, 9 μM soybean trypsin inhibitor, 1 μM α1-antitrypsin (AA’T), 1 μM α1-antichymotrypsin, 4 μM SLPI, or 1 mM MeOSuc-AAPV-CMK. Additionally, recombinant elafin (8.5 × 10⁻⁷ M) was incubated with various concentrations of neutrophil elastase, proteinase 3, or cathepsin G (from 3.5 × 10⁻⁹ to 8.5 × 10⁻⁶ M) for 24 h in 0.1 μM Hepes, 0.5 mM NaCl, pH 7.5, in a 20-μl volume at 37 °C. All incubations were stopped by adding...
sample treatment buffer containing reducing or non-reducing agent and boiling samples for 5 min. Samples were separated by Tricine SDS-PAGE using a 17.5% polyacrylamide gel and blotted onto a 0.2-μm nitrocellulose membrane (Sigma-Aldrich). The membrane was blocked for 1 h at room temperature with 3% bovine serum albumin in PBS containing 0.1% Tween 20. Elafin was detected by using a biotinylated anti-elafin antibody (R&D Systems, 1:500, overnight at 4 °C) followed by peroxidase-conjugated streptavidin (Zymed Laboratories Inc., 1:2500, 20 min at room temperature). Peroxidase activity was detected with a chemiluminescent substrate (SuperSignal West Femto Maximum Sensitivity Substrate, Pierce).

**HPLC Mass Spectrometry**—Cleavages of elafin by neutrophil elastase were assessed by incubating recombinant elafin (1 μg; 4.1 × 10⁻⁶ M) with purified NE (10 μg; 8.3 × 10⁻⁶ M) for 1 h in 30 mM Tris-buffered saline, pH 7.5, in 40 μl final volume at 37 °C. Elastase activity was neutralized with 1 μl of phenylmethylsulfonyl fluoride, 100 mM, for 30 min at room temperature. Samples were lyophilized until analysis when they were redissolved in 10 μl of 6 M guanidine HCl, 100 mM Tris, pH 8.5, 1 mM EDTA. 1 μl of 10% trifluoroacetic acid was added to each sample to bring the pH to <3. Samples were then analyzed by reverse phase HPLC coupled to electrospray mass spectrometry as described (30).

**Protease Assay**—Recombinant elafin (1 μg; 4.1 × 10⁻⁶ M) was incubated with purified NE (10 μg; 8.3 × 10⁻⁶ M) for 2 h in 30 mM Tris-buffered saline, pH 7.5, in 40 μl final volume at 37 °C. Elafin fragments were purified by reverse phase HPLC, dried, and reconstituted with 50 μl of 0.1 M Heps, 0.5 mM NaCl, pH 7.5, containing 0.1% Brij-35. Various volumes of reconstituted fractions F1 and F2 (from 0 to 5 μl) were incubated for 30 min at 37 °C with 3.3 mM NE in 0.1 M Heps, 0.5 mM NaCl, pH 7.5, containing 0.1% Brij-35. Residual activity of NE was detected by adding the chromogenic substrate MeOSuc-AAPV-pNA at a final concentration of 1 mM and by measuring absorbance at 405 nm over the time at 37 °C in a 96-well microplate reader (Victor2 1420 Multilabel Counter, Wallac). Residual activity was expressed in percentage as 100% activity is determined by control NE.

**Measurement of Elastase Concentration in CF Sputum**—10 μl of *P. aeruginosa*-positive and -negative CF sputum were diluted in 0.1 M Heps, 0.5 mM NaCl, pH 7.5, containing 0.13 mM E-64, 0.11 mM pepstatin A, and 5.4 mM EDTA and treated with or without NE inhibitor (1 mM MeOSuc-AAPV-CMK or 1.6 μM SLPI) for 1 h at 37 °C in a 100-μl volume. 50 μl of the chromogenic substrate MeOSuc-AAPV-pNA, was mixed with each sample to a final concentration of 1.4 mM, and absorbance at 405 nm of samples was measured over time at 37 °C in a 96-well microplate reader (Victor2 1420 Multilabel Counter). The concentration of NE in samples was determined by comparing the elastase activity (given by the rate of hydrolysis of the substrate) with a standard curve of purified NE. All measurements were performed in duplicate.

**Analysis of Elafin Cross-linking to Fibronectin by Transglutaminase**—Recombinant elafin (100 ng, 3 μM) was incubated with increasing concentrations of neutrophil elastase (0–1000 ng, 0–6.1 μM) in 30 mM Tris-HCl, 150 mM NaCl, pH 7.5 for 1 h at 37 °C. Neutrophil elastase was inactivated by 5 μg of α1-antitrypsin for 30 min. The resulting mix was incubated in 200 mM Tris acetate, pH 6, containing 5 mM CaCl₂ and 0.1 mM dithiothreitol for 1 h at 37 °C with 5 μg of plasma fibronectin and 0.38 milliunits of guinea pig liver transglutaminase (one unit catalyzes the formation of 1.0 μmol of hydroxamate per minute from Nε-CBZ-glutaminylglycine and hydroxylamine at pH 6.0 at 37 °C). The reaction was stopped by adding sample treatment buffer without reducing agent and by boiling samples for 5 min at 100 °C. Samples were separated by 4–12% Bis-Tris SDS-PAGE and analyzed by Western blotting using a biotinylated anti-elafin antibody according to the method described above.

**LPS ELISA**—A microtiter plate was coated with 100 ng/well LPS (*Sigma, Escherichia coli* 055:B5) which had been diluted in serum-free RPMI media and incubated at 37 °C for 3 h. Unbound LPS was washed off the plate with distilled water. Excess water was gently tapped off, and the plate was left to air-dry overnight at room temperature. The next day the plate was blocked with 200 μl/well blocking buffer (PBS with 1% (w/v) bovine serum albumin) for 2 h at 37 °C. The plate was washed 3 times with PBS, 0.05% (v/v) Tween, and 100 μl/well of the appropriately diluted proteins (diluted in serum-free RPMI media) were added to the plate at 37 °C for 2 h. Control wells, to which serum-free RPMI media alone was added in place of proteins, were included on each ELISA plate. Again, the plate was washed three times before primary antibody (R&D Systems, AF1747) was added at the appropriate dilution (1:100) for 37 °C for 2 h. After washing 100 μl/well of diluted horseradish peroxidase-conjugated secondary antibody was added to the plate at 37 °C for 2 h, and the plate was washed 3 times. Substrate, 100 μl/well (ABTS Single Solution, Zymed Laboratories Inc.), was added, and the plate was incubated at room temperature for 20 min. The A₄₀₅ of the wells were measured on a microtiter plate reader, and results were analyzed using Prism, Version 3.0 (Graphpad Software, San Diego).

**RESULTS**

Levels of Elafin in Soluble CF Sputum—Levels of elafin in soluble CF sputum from 11 patients with positive *P. aeruginosa* sputum cultures and 8 patients with negative *P. aeruginosa* sputum cultures were determined by sandwich ELISA. As shown in Fig. 1,
levels of elafin were found to be significantly lower in *P. aeruginosa*-positive CF sputa compared with *P. aeruginosa*-negative CF sputa (502 ± 204 versus 2761 ± 876 pg/ml, p < 0.05).

Incubation of Exogenous Elafin with CF Sputum—Effects of negative (*Ps−*) and positive (*Ps+*) *P. aeruginosa* CF sputa on elafin were assessed to determine their ability to potentially cleave or degrade the protease inhibitor. Recombinant elafin was incubated with *Ps−* and *Ps+* CF sputa for various times at 37 °C and analyzed by Western blot under reducing or non-reducing conditions using a biotinylated anti-elafin antibody (Fig. 2). As shown in Fig. 2A, no elafin was detectable after an incubation for 24 h with *Ps+* CF sputum, whereas a faint band corresponding to intact elafin could still be detected with *Ps−* CF sputum in the same conditions of incubation. Although no fragments of elafin were detectable under reducing conditions (Fig. 2A), Western blot analysis performed under non-reducing conditions resulted in the detection of three distinct bands (Fig. 2B); in addition to the upper band displaying a similar size to intact elafin, two lower bands corresponding to proteolytic fragments of elafin were detected. A time-course experiment was performed by incubating recombinant elafin with *Ps−* or *Ps+* CF sputum for 0, 10 min, 1 h, 6 h, or 24 h at 37 °C. As shown in Fig. 2C, levels of recombinant elafin detected by Western blot under reducing conditions decreased in both *Ps−* and *Ps+* CF sputa over time. However, elafin was more rapidly cleaved in *Ps+* than in *Ps−* CF sputum. Most of the elafin was cleaved after incubation for 10 min in *Ps+* CF sputum, whereas intact elafin was still clearly detected after 6 h of incubation in *Ps−* CF sputa (Fig. 2C).

These findings show that the proteolytic activity directed against elafin was higher in *Ps+* than in *Ps−* CF sputum.

Identification of the Protease(s) Involved in the Cleavage of Elafin in CF Sputum—To identify the protease(s) involved in the cleavage of elafin in CF sputa, different protease inhibitors were preincubated with *Ps+* CF sputum samples before adding recombinant elafin. After 24 h of incubation at 37 °C, samples were analyzed by Western blot under reducing or non-reducing conditions using an anti-elafin antibody. First, the use of nonspecific protease inhibitors targeting each protease family (serine, cysteine, acidic proteases, and metalloproteinases) allowed identification of the family of the CF sputum protease(s) involved in the cleavage of elafin. As shown in Fig. 3A, Pefabloc, a nonspecific serine protease inhibitor, inhibits elafin cleavage in *Ps+* CF sputa, whereas neither leupeptin, E-64 (cysteine protease inhibitor), pepstatin A (acidic protease inhibitor), nor metalloproteinase inhibitors (EDTA, GM6001, phosphoramidon) had any effect. In addition to Pefabloc, several other serine protease inhibitors were used to identify more precisely the serine protease involved in the cleavage of elafin. As shown in Fig. 3B, no effect was observed with trypsin-like (TLCK, benzamidine, soybean
trypsin inhibitor) and chymotrypsin-like (TPCK) inhibitors, suggesting that neither trypsin-like nor chymotrypsin-like proteases are involved in elafin cleavage in Ps+ CF sputa. However, a slight inhibition of elafin cleavage by the non-specific serine protease inhibitor aprotinin was observed (Fig. 3B). Because no trypsin-like or chymotrypsin-like proteases mediated elafin cleavage in CF sputum, we hypothesized that elastase-like proteases, particularly neutrophil elastase and proteinase 3, could be involved in this process. Among inhibitors targeting neutrophil serine proteases (e.g. neutrophil elastase, proteinase 3, and cathepsin G) that have been tested in our study, only AAT, SLPI, and MeOSuc-AAPV-CMK inhibited elafin cleavage in Ps+ CF sputa (Fig. 3C). According to our previous results, α1-antichymotrypsin, a chymotrypsin-like inhibitor targeting cathepsin G, did not prevent elafin cleavage (Fig. 3C). Non-reducing Western blot analysis provided similar results as Pefabloc, and the neutrophil elastase inhibitor MeOSuc-AAPV-CMK completely prevented elafin cleavage (not shown). Taken together, these results suggest that NE is involved in the cleavage of NE in CF sputum.

Given that NE is responsible of the cleavage of elafin in CF sputum and that the inhibitor is more rapidly degraded in Ps+ than in Ps− CF sputa, NE activity was measured in both CF sputa by using the chromogenic substrate MeOSuc-AAPV-pNA. Elastase activity in CF sputa was calculated using a standard curve of purified NE to determine the concentration of free NE in samples. As shown in Fig. 4, the concentration of free NE was increased 3.9-fold in Ps+ compared with Ps− CF sputa (2.86 versus 0.73 μM). Additionally, free elastase activity in both samples was totally abrogated using the NE inhibitors MeOSuc-AAPV-CMK and SLPI (Fig. 4), thus confirming the specificity of the measurements.

**Effects of Neutrophil Serine Proteases on Elafin Integrity—**

The ability of human neutrophil serine proteases to cleave elafin was evaluated in vitro (Fig. 5). Recombinant elafin was incubated at 8.5 × 10^{-7} M with purified proteases and analyzed by Western blot with an anti-elafin antibody. Firstly, dose-response incubations were carried out for 24 h at 37 °C using concentrations of neutrophil serine proteases ranged from 3.5 × 10^{-9} to 8.5 × 10^{-6} M. As shown in Fig. 5A, human NE and Pr3 were found to cleave recombinant elafin only in conditions with excess of proteases; although a partial cleavage of the inhibitor was observed at equimolarity, excess of neutrophil elastase and proteinase 3 completely cleaved elafin (Fig. 5A, 1 and 2). In contrast to these proteases, human cathepsin G appeared less effective in cleaving elafin as 10-fold excess of the enzyme was necessary to partially cleave elafin (Fig. 5A, 3). Time-course incubations of elafin with NE at equimolarity (3.5 × 10^{-6} M) and in excess (3.5 × 10^{-5} M) were performed over 24 h at 37 °C to better characterize the proteolytic cleavage observed previously. As shown in Fig. 5B, NE in excess cleaved recombinant elafin quickly. Under these conditions, a complete cleavage of elafin was observed after only 30 min of incubation, whereas significant amounts of intact elafin were still detected after 24 h of incubation with equimolar concentrations of elastase. Cleavage products of elafin after a 24-h exposure to 8.5 × 10^{-7} M and 3.5 × 10^{-6} M NE could be detected under non-reducing conditions.
Cleavage of Elafin by Neutrophil Elastase in CF Sputum

A

B

FIGURE 6. Identification of NE cleavage sites in recombinant elafin. A, HPLC analysis of elafin incubated with an excess of human neutrophil elastase. Human neutrophil elastase (8.3 × 10⁻⁶ M) was incubated with elafin (4.1 × 10⁻⁶ M) for 1 h at 37°C. The samples were neutralized with 5 mM phenylmethylsulfonyl fluoride, dried, and reconstituted in 6 M guanidine, 100 mM Tris (pH 8.5). The samples were then separated by HPLC. Three peaks (1, 2, and 3) were obtained corresponding to various elafin products. Peaks were analyzed by mass spectrometry, and results revealed that peak 1 corresponded to the fragment Ala-1—Val-5, peak 2 to Lys-6—Gln-57, and peak 3 to Ser-10—Gln-57. This indicates the residue in P1 position within the protease binding loop. Arrows represents cleavages sites generated by 2-times excess of NE for 1 h. The arrowhead indicates the partial cleavage site detected after a 24-h NE exposure using a 2.5× excess of elafin. This arrowhead also represents the scissile bond in the elafin inhibitor loop.

Reducing conditions (Fig. 5B). Of note, patterns of cleavage of the inhibitor in these conditions were similar to that observed in Ps− and Ps+ CF sputa, respectively (Fig. 2B).

To further investigate the elastase-mediated cleavage of elafin, products from NE-elafin incubations using two distinct [enzyme]:[inhibitor] molar ratios (1:2.5 or 2:1) were analyzed by HPLC and mass spectrometry. HPLC separation (under non-reducing conditions) of elafin products generated within 1 h by a slight excess of NE ([enzyme]:[inhibitor] molar ratio = 2:1) resulted in the formation of three distinct peaks (Fig. 6A, peaks 1, 2, and 3). Identification of elafin fragments was carried out by analyzing peaks in mass spectrometry (data not shown). The measured mass of the peak 1 was 542.27 Da, identifying it as elafin residues 1—5 (calculated mass = 542.27 Da). Likewise, the measured masses for the peaks 2 and 3 were 5474.9 and 5093.4 Da, respectively, identifying them as elafin residues 6—57 (calculated mass = 5474.6 Da) and 10—57 (calculated mass = 5093.1 Da). Although native elafin used as a control exhibited an observed mass of 5999.1 Da (calculated mass = 5999.2 Da) with all cysteines in disulfide leakage, no full-length inhibitor as the intact form or possessing a mass with additional 18 Da was detected with an excess of NE. The extracted ion chromatogram was examined for the presence of peptides released from elafin by NE under these conditions (not shown). Only peptides corresponding to elafin residues 1—5 (observed mass = calculated mass = 542.27 Da) and 6—9 (observed mass = 399.25 Da, calculated mass = 399.24 Da) were found in the extracted ion chromatogram. Conversely, the peptide 1—9 was not detected using this method. These results indicate that a 2× excess of NE cleaved elafin at Val-5—Lys-6 and Val-9—Ser-10 peptide bonds. In contrast, such cleavages were not detected using conditions with a slight excess of elafin ([enzyme]:[inhibitor] molar ratio = 1:2.5), even after 24 h of incubation. Instead, a partial cleavage of the inhibitor was identified at the scissile peptide bond Ala-24—Met-25 (not shown). Taken together, these findings indicate that NE-mediated cleavages of elafin at Val-5—Lys-6 and Val-9—Ser-10 peptide bonds only occur with excess of NE. All the cleavage sites generated by NE within elafin are summarized in Fig. 6B.

Effects of Excess of NE on Elafin Properties—Inhibitory activity of elafin cleaved by NE in excess was investigated to determine whether the NE treatment abolished the antiprotease activity of the inhibitor. Elafin fragments 5—10 and 10—57 obtained by incubating elafin with a 2× excess of NE for 2 h were separated by HPLC in two fractions (Fig. 7A, fractions F1 and F2) and investigated for their anti-NE activity by protease assay. As shown in Fig. 7A2, fractions F1 and F2 corresponding to elafin fragments 5—10 and 10—57 were able to inhibit NE in a dose-dependant manner.

The ability of NE in excess to cleave elafin and to remove an N-terminal peptide containing the transglutaminase substrate motif AQEPVK prompted us to examine the effect of such treatment on the capacity of the inhibitor to covalently cross-link to fibronectin by transglutamination. Like elafin, fibronectin is a substrate of transglutaminases (31) and was recently demonstrated to bind recombinant elafin in vitro by transglutation (5). After treating elafin with increasing concentrations of NE, the inhibitor was incubated with fibronectin in the presence or absence of guinea pig liver transglutaminase and analyzed by Western blot. As demonstrated in Fig. 7B (lane 2), the treatment of elafin and fibronectin with transglutaminase led to the generation of an immunoreactive band of high molecular mass characteristic of elafin-fibronectin complexes. Elafin was preincubated with increasing amounts of NE to assess the ability of elafin and NE-cleaved elafin to bind fibronectin by transglutamination. As shown in Fig. 7B, treatment with NE in excess led to the cleavage of elafin (lanes 7—10), whereas sub-molar amounts of NE preserved elafin integrity (lanes 3—6). Under these latter conditions, elafin was shown to covalently bind to fibronectin by transglutamination in contrast to the conditions using excess of NE that abolished the cross-linking reaction between the inhibitor and the fibronectin. To confirm that this effect was only due to the cleavage of elafin and not to the cleavage of fibronectin as NE was previously shown to degrade fibronectin (32), all samples were treated by AAT to neutralize NE, and the nitrocellulose membrane was stripped and reprobed with an anti-fibronectin antibody. The Western blot revealed no cleavage of fibronectin (data not shown). Taken together, these results indicated that NE-mediated cleavage of elafin abolished the capacity of the inhibitor to cross-link to fibronectin by transglutation.

Elafin is an antibacterial component possessing the capacity to interact with the endotoxin LPS of Gram-negative bacteria and to modulate macrophage responses after LPS stimulation (33). We, therefore, investigated the effect of NE on the ability of the inhibitor to bind to LPS. Recombinant elafin was incubated alone or with an excess of NE and then examined for its capacity to bind to the endotoxin by ELISA using an LPS-coated plate and an anti-elafin antibody (Fig. 7C). Our results showed
Cleavage of Elafin by Neutrophil Elastase in CF Sputum

Although all incubations performed in our study were carried out at pH 7.5, we found that the incubation of elafin with CF sputum at pH 5.2 also led to the cleavage of elafin, although it did not improve hydrolisis rate of the inhibitor (data not shown). Our findings provide evidence that NE plays a pivotal role in the cleavage of elafin in CF sputum. NE inhibitors (AAT, SLPI, MeO-Suc-AAPV-CMK) were able to inhibit the cleavage of elafin, and purified NE was shown to rapidly cleave elafin in vitro in conditions of excess enzyme. Two cleavage sites were identified in elafin at Val-5—Lys-6 and Val-9—Ser-10 peptide bonds using a 2× excess of NE. These cleavage sites are in accordance with the specificity of NE, as this enzyme preferentially cleaves at the C terminus of small aliphatic residues including valine and alanine (35, 36). The ability of excessive NE to cleave elafin suggests that NE preferentially interacts with the protease-binding loop rather than cleaving the N-terminal extremity of elafin. Structural studies of elafin (37, 38) indicated that the N-terminal extremity of the inhibitor is at the opposite side of the protease binding loop (inhibitory loop). Therefore, free NE can likely cleave the inhibitor complexed with its target enzyme.

The rate of elafin cleavage in CF samples correlates with NE activity. Samples containing greater amounts of NE activity degraded elafin more quickly. NE activity was shown to be highest in P. aeruginosa-positive CF sputum where elafin was degraded rapidly compared with P. aeruginosa-negative samples where NE activity was lower. A previous study demonstrated similar variation in NE activity (26). This observation can be explained at least in part by bacterial infection. P. aeruginosa is an opportunistic Gram-negative bacterium that frequently infects the lungs of CF patients. This human pathogen is known to induce expression of neutrophil chemoattractants including the CXC chemokine IL-8, and a prolonged IL-8 expression is observed in CF epithelial cells compared with non-CF cells after P. aeruginosa exposure (39). Therefore, increased recruitment and activation of neutrophils at inflammatory sites in response to bacterial infection leads to increased secretion of NE, a potent mediator of lung inflammation involved in a number of pro-inflammatory processes. In addition, we have previously demonstrated that CF neutrophils are more sensitive than control neutrophils to TNF-α and IL-8 contained in CF sputum resulting in secretion of higher levels of NE (40).

that native elafin (not treated with NE) was able to interact to LPS in a dose-response manner. On the contrary, little or no elafin was detected when the inhibitor was treated with NE. These results, thus, indicated that NE in excess suppresses the ability of elafin to bind to LPS.

**DISCUSSION**

Elafin is an inducible and multifunctional peptide expressed by mucosal surfaces including lung epithelium. In addition to being a potent inhibitor of NE and proteinase 3, two neutrophil serine proteases, the molecule displays anti-bacterial and anti-inflammatory properties and, thus, participates in the innate defense of the lung during inflammatory events. In the present study we have demonstrated that elafin is rapidly cleaved in P. aeruginosa-positive CF sputum compared with P. aeruginosa-negative CF samples. Airways from CF individuals is known to be low (pH 5–6), as demonstrated in exhaled breath condensate from patients with exacerbated and stable CF (34).
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In this report we have detected elafin in CF sputum by ELISA, and our results further demonstrate that the levels of the inhibitor are decreased in *P. aeruginosa*-positive CF samples. We believe that this is due to increased cleavage rather than down-regulation of the inhibitor. IL-1, LPS, and NE are pro-inflammatory components playing a crucial role in the regulation of inflammation in cystic fibrosis (41), and several studies showed that these inflammatory mediators up-regulate the expression of elafin (7, 8, 10). Moreover in this study experiments related to elafin incubation with CF sputum demonstrated proteolytic cleavage of the molecule in CF samples, thus confirming our hypothesis. In addition, we found by ELISA that recombinant elafin treated with an excess of purified NE was less immunoreactive than native elafin (data not shown). Therefore, increased hydrolysis of elafin at its N terminus can explain at least in part the diminution of elafin detection observed in *P. aeruginosa*-positive CF sputum.

Functional investigations of elafin fragments Lys-6—Gln-57 and Ser-10—Gln-57 revealed that NE-mediated cleavages at Val-5—Lys-6 and Val-9—Ser-10 peptide bonds have no dramatic consequences on the inhibitory capacity of elafin. Some fragments of elafin cleaved at the N terminus were previously identified in *vivo* in biological fluids such as urine from patients with psoriasis (42) and purulent sputum (43). Interestingly, the fragment found in purulent sputum started after Val-9 and was found to display inhibitory activity (43). The preservation of antiprotease properties of elafin fragments Lys-6—Gln-57 and Ser-10—Gln-57 can be explained by the analysis of elafin structure. NMR analysis of elafin indicates that the N-terminal extremity of elafin (Ala-1—Pro-13) is a flexible region linked to a rigid and flat structure, also referred as “four-disulfide core” possessing the inhibitory activity (38). A previous structure-activity study of synthetic elafin using variants of the inhibitor demonstrated that the N-terminal part of elafin is not essential for expressing full inhibitory activity (44). Therefore, it is likely that cleavages occurring in the N-terminal part of elafin will not perturb the structure of the four-disulfide core, thus preserving the inhibitory properties of elafin. We also found a partial cleavage in the disulfide core (at Ala-24—Met-25) mediated by NE within 24 h of incubation using elafin in slight excess (data not shown). The x-ray crystallographic analysis of elafin complexed with porcine pancreatic elastase (37) indicated that this peptide bond is located in the inhibitory loop of elafin and corresponds to the scissile peptide bond, also called P1-P1’ bond, according to the nomenclature of Schechter and Berger (45). Such a cleavage occurring at the scissile bond can be observed for protease inhibitors that obey the standard mechanism described by Laskowski *et al.* (46). A complex between a canonical inhibitor and its target enzyme is reversible and can dissociate the enzyme from the inhibitor in an intact (virgin) or cleaved form. According to the standard mechanism, the modified inhibitor, which is thermodynamically identical to the virgin inhibitor, can react with the enzyme and inhibit it completely. This, thus, suggests that the partial cleavage observed at Ala-24—Met-25 of the elafin inhibitory loop is the result of the standard interaction of the inhibitor with its target enzyme and may not lead to a dramatic loss of the antiprotease activity.

Elafin and its precursor trappin-2 (pre-elafin) possess transglutaminase substrate motifs having the consensus sequence GQDPVK and allowing the inhibitors to be covalently cross-linked to various extracellular matrix (ECM) proteins by transglutamination. Elafin contains one transglutaminase substrate motif located in its N-terminal extremity, whereas trappin-2 possesses five motifs, four of which are in the N-terminal moiety (cementoتن domain), and one is located in the C-terminal part (elafin domain). Transglutaminases are a family of enzymes catalyzing the formation of an isopeptide bond between the side chains of a Gln and a Lys residue belonging to two different proteins. By this mechanism, both elafin and trappin-2 are able to be cross-linked with a variety of proteins, although trappin-2 was shown to be more rapidly immobilized, likely due to a higher amount of transglutaminase substrate motif (5). In *vivo*, trappin-2/elafin have been found covalently complexed in several epithelia and colocalized with type-1 transglutaminase, suggesting that the immobilization process results from transglutaminase activities. In the cornified cell envelope of the epidermis, both molecules are cross-linked to several proteins (including involucrin, keratin-1, loricrin, cystatin α, filaggrin) and are thought to function as cross-bridging molecules (47). In tracheal epithelium, trappin-2/elafin is also found in a complexed form (4). Previous *in vitro* studies showed that trappin-2 and/or elafin can covalently bind to a number of ECM proteins including elastin, fibronectin, laminin, fibrinogen, collagen, and β-crystallin by transglutamination (4, 5, 48). The transglutaminase-mediated cross-linking of trappin-2 and elafin to fibronectin and elastin was demonstrated to preserve their antiprotease activity and to protect the associated ECM molecule against proteolysis mediated by NE (5, 48). Hence, the immobilization of trappin-2/elafin ECM proteins in elastic tissues such as lung and skin may play a protective role by preserving the structural integrity of the tissue against damages caused by neutrophilic infiltrations during inflammation. In the present study, a cleavage of the elafin N terminus containing the transglutaminase substrate motif AQEPVK was demonstrated to likely occur *in vivo* by NE in the sputum of patients with CF. Functional investigation of this cleavage showed that it completely suppresses the ability of elafin to cross-link with fibronectin by transglutamination *in vitro*. Therefore, it is likely that the cleavage of elafin by excess of NE observed in CF sputum can regulate *in vivo* the amounts of immobilized and soluble forms of the inhibitor in the CF lung. Furthermore, the regulation of the attachment of elafin to ECM proteins may also affect the protection of these proteins against neutrophil-mediated proteolysis and the structural integrity of the lung tissue.

A number of cationic antimicrobial peptides and proteins are able to bind the endotoxin LPS and modulate the cell inflammatory response induced by the endotoxin LPS (49). Some molecules like BPI (bactericidal/permeability-increasing protein), granulysin/NK lysis, histatins, histone H2A, LL-37, and SLPI can neutralize the pro-inflammatory effects of LPS, whereas another molecule like azurocidin/HBP (heparin-binding protein) can enhance the capacity of LPS to induce the release of pro-inflammatory mediators by cells. A recent study demon-
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strated that trappin-2 (pre-elafin) and a fragment of elafin starting from Pro-13 were able to bind LPS and modulate the macrophage response to LPS (33). Although trappin-2 binds LPS much more strongly than the elafin fragment, both molecules can inhibit and enhance the LPS-induced activation of macrophages, respectively, in the presence and in absence of serum (33). The reason for these antagonistic effects remains unknown. More intriguingly, the elafin fragment, which is less potent than trappin-2 to bind LPS, is much more effective in phages, respectively, in the presence and absence of serum (33). We and others have shown the importance of elafin as an antiprotease, immunomodulatory, and anti-microbial. That an antiprotease such as elafin can be cleaved by its cognate protease underlines the delicate protease-antiprotease balance which exists in the CF lung. Although the cleavage mediated by NE preserves the antiprotease activity of elafin, it may affect the innate immunity properties of the molecule in CF by altering the capacity of elafin to be immobilized by transglutamination and to bind LPS. The degree to which elafin may be cleaved is determined by the inflammatory milieu and the underlying disease process as illustrated by the role of P. aeruginosa in perpetuating the NE burden in the CF lung.

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