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Decreased Levels of Secretory Leucoprotease Inhibitor in the Pseudomonas-Infected Cystic Fibrosis Lung Are Due to Neutrophil Elastase Degradation

Sinéad Weldon,‡ Paul McNally, † Noël G. McElvaney, † J. Stuart Elborn, † Danny F. McAuley, † Julien Wartelle,‡ Abderrazzaq Belaaouaj,‡ Rodney L. Levine,§ and Clifford C. Taggart2*

Secretory leucoprotease inhibitor (SLPI) is a neutrophil serine protease inhibitor constitutively expressed at many mucosal surfaces, including that of the lung. Originally identified as a serine protease inhibitor, it is now evident that SLPI also has antimicrobial and anti-inflammatory functions, and therefore plays an important role in host defense. Previous work has shown that some host defense proteins such as SLPI and elafin are susceptible to proteolytic degradation. Consequently, we investigated the status of SLPI in the cystic fibrosis (CF) lung. A major factor that contributes to the high mortality rate among CF patients is Pseudomonas aeruginosa infection. In this study, we report that P. aeruginosa-positive CF bronchoalveolar lavage fluid, which contains lower SLPI levels and higher neutrophil elastase (NE) activity compared with P. aeruginosa-negative samples, was particularly effective at cleaving recombinant human SLPI. Additionally, we found that only NE inhibitors were able to prevent SLPI cleavage, thereby implicating NE in this process. NE in excess was found to cleave recombinant SLPI at two novel sites in the NH2-terminal region and abrogate its ability to bind LPS and NF-κB consensus binding sites but not its ability to inhibit activity of the serine protease cathepsin G. In conclusion, this study provides evidence that SLPI is cleaved and inactivated by NE present in P. aeruginosa-positive CF lung secretions and that P. aeruginosa infection contributes to inactivation of the host defense screen in the CF lung. The Journal of Immunology, 2009, 183: 8148–8156.

C ystic fibrosis (CF) is an autosomal recessive disease caused by loss of expression/function mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) protein. Lung disease causes 95% of the morbidity and mortality in CF patients and is associated with the failure of pulmonary innate immune functions leading to a vicious cycle of continual infection, inflammation, and remodeling of lung tissue. A major factor that contributes to this mortality rate is infection with Pseudomonas aeruginosa. Once chronic infection is established, it is virtually impossible to eradicate and is associated with reduced survival. Another contributing factor is the presence of large numbers of neutrophils and ensuing high concentrations of neutrophil proteases, particularly neutrophil elastase (NE), in the airways of CF patients that overwhelm the host’s antiprotease screen. During the past three decades it has become clear that a number of proteins involved in defending the lung against proteases possess multiple, yet seemingly independent, functions that under normal circumstances serve to protect the lung from infection and inflammation as well as protease-induced degradation.

One such protein is human secretory leucoprotease inhibitor (SLPI), a cationic 11.7 kDa serine protease inhibitor constitutively expressed at mucosal surfaces, primarily the upper respiratory tract. SLPI consists of 107 amino acids organized in two whey acidic protein four disulfide core (WFDC) domains, each with four disulfide bridges (4, 5). SLPI is produced by a number of cell types, including neutrophils, macrophages, and epithelial cells, and expression can be altered by various stimuli, including LPS (6, 7), NE (8, 9), and both pro- and anti-inflammatory cytokines (10, 11). Overall, the evidence to date suggests that the function of SLPI is to protect local tissue from the detrimental consequences of inflammation not only as a result of its well-documented antiprotease activities but also via its antimicrobial and anti-inflammatory properties. SLPI can inhibit a variety of proteases released during inflammation such as elastase, cathepsin G (CatG), trypsin, chymotrypsin, chymase, and tryptase (12). Meanwhile, a number of studies have found that SLPI displays antimicrobial effects against pathogens such as Escherichia coli, P. aeruginosa, and Staphylococcus aureus, and it also inhibits the growth of fungi such as Aspergillus fumigatus and Candida albicans (13–15).

SLPI also possesses immunomodulatory activity both in vivo and in vitro. Administration of aerosolized SLPI to CF patients suppressed levels of both NE and IL-8 in the lung (16, 17). In response to LPS, SLPI-deficient mice showed increased mortality.
from endotoxin shock (18). Furthermore, in a mouse model of acute lung injury, prior administration of SLPI decreased lung injury and down-regulated NF-κB activation by preventing degradation of the NF-κB inhibitor protein IκBβ (19, 20). These findings have been confirmed in vitro and mechanisms of action continue to be elucidated. SLPI binds bacterial LPS extracellularly, thereby down-regulating the uptake of LPS and subsequent production of proinflammatory mediators (21–23). However, as a consequence of the internalization of SLPI into the cytoplasm and nucleus of cells such as monocytes, it appears that SLPI also has intracellular sites of action (24). In the cytoplasm, SLPI inhibited LPS- and lipoteichoic acid-induced NF-κB activation in human monocytes, by preventing degradation of key regulatory proteins such as IκBα, IκBβ, and IKK-1 receptor activated kinase (IRAK) (25, 26). Additionally, previous findings from our group have shown that SLPI also exerts effects on the NF-κB signaling cascade in the nucleus of monocytes, where SLPI can compete with p65 for binding to the p50NF-κB binding sites in the promoter regions of proinflammatory genes such as IL-8 and TNF-α (24).

Although SLPI can inhibit a variety of proteases released during inflammation, it appears that SLPI levels and activity may be compromised in numerous disease states where the body is overwhelmed by infection and concomitant proteases. Evidence of SLPI cleavage has been reported in individuals with frequent chronic obstructive pulmonary disease (COPD) exacerbations (27), emphysema (28), and CF (29). It has become clear that SLPI and other important host defense proteases are susceptible to proteolytic cleavage by a range of proteases of both endogenous and bacterial origin. We have previously shown that cysteine proteases called cathepsins cleave and inactivate SLPI and human β-defenseins in epithelial lining fluid from individuals with emphysema (28) and CF (30), respectively, while cathepsin-mediated degradation of lactoterrin was observed in P. aeruginosa-infected CF sputum and bronchoalveolar lavage fluid (BALF) (31). Additionally, exogenous elastolytic proteases of bacterial origin may play a role in the cleavage and inactivation of host defense molecules. For example, Pseudomonas elastase or pseudolysin has been shown to cleave SLPI (32), and high concentrations of pseudolysin, Pseudomonas alkaline protease, and NE were also able to inactivate lactoterrin (33).

Recent findings from our laboratory have demonstrated that recombinant human elafin (another well-characterized WFDC-containing protein found in the lung) is cleaved by NE in CF sputum and that P. aeruginosa infection promotes this degradation (34). In the present study, we expand this research to investigate the hypothesis that the high levels of NE present in the P. aeruginosa-infected CF lung correlate with low levels of SLPI and that NE is responsible for cleaving and inactivating the multifunctional, host defense protein SLPI. Our findings show that Pseudomonas infection increases NE levels, which subsequently cleave SLPI in the CF lung and may have repercussions on the innate immune functions of this protein.

### Materials and Methods

**Materials**

Recombinant human SLPI was obtained from Amgen. Biotinylated anti-human SLPI Ab was purchased from R&D Systems. N-(methoxyuccinyl)-Ala-Ala-Val-chloromethyl ketone (MeOSuc-AAPV-CMK or CMK) and EDTA were purchased from Sigma-Aldrich. α-1-Antichymotrypsin (ACT), N-(methoxuccinyl)-Ala-Ala-Pro-Val-paranitroanilide (MeOSuc-AAPV-pNA), N-(methoxuccinyl)-Ala-Ala-Pro-Met-paranitroanilide (MeOSuc-AAPM-pNA), CatG, and E64 were purchased from Merck Biosciences. Human SLPI was from Elastin Products. Human protease 3 (Pr3) was from Athens Research. HRP-conjugated streptavidin was obtained from BioLegend. SuperSignal West Femto maximum sensitivity substrate was purchased from Pierce. All other reagents were of analytical grade.

**CF BALF processing**

We studied a total of 26 patients diagnosed with CF (F508del), 15 of whom had P. aeruginosa infection (Table I). CF BALF samples were obtained from individuals undergoing flexible bronchoscopy for clinical reasons. Ethical approval for the use of these samples was obtained from the Institutional Review Board of the Adelaide and Meath Hospital incorporating the Children’s National Hospital. Bronchoscopy was performed via a laryngeal mask airway. The bronchoscope was directed to the lingual and right middle lobe. Bronchoalveolar lavage was performed by instilling 1 ml/kg of sterile normal saline per lobe. Return was typically in the region of 40%. Specimens from right and left lobes were pooled. BALF was centrifuged at 4000 rpm for 10 min and the supernatant was aliquoted and stored at −80°C. Bacteria were cultured from the BALF sample on the day of the procedure.

**Determination of SLPI levels in Pseudomonas-positive and -negative CF BALF**

SLPI levels were determined by ELISA. Rabbit anti-human SLPI (1/1000 in Voller’s buffer) was added to Maxisorp 96-well plates (Nunc) and left overnight at 4°C. The plate was washed three times with PBS/0.05% (v/v) Tween 20 (PBST) and blocked in PBST containing 1% BSA for 1 h at room temperature. After washing three times in PBST, SLPI standards (26 pM to 1.66 nM) and CF BALF samples (100 μl per well of 1/100 diluted sample) were added in 100-μl aliquots to the plate for 2 h at room temperature. The plate was then washed and biotinylated anti-human SLPI Ab (1/1000 in PBST, R&D Systems) was added to the plate for 2 h at room temperature. The plate was blocked for 1 h at room temperature. After washing, the plates were incubated with HRP-conjugated streptavidin (1/2500 in PBST) for 20 min at room temperature. After washing, the plates were incubated with HRP-conjugated streptavidin (1/2500 in PBST) for 20 min at room temperature and washed with PBST. After the third wash, peroxidase activity was measured by the addition of substrate (ABTS Single Solution; Zymed Laboratories). The absorbance at 405 nm of the wells was measured on a microtiter plate reader (Genios using Magellan software) and results were converted to SLPI concentrations (nM) using GraphPad Prism version 5.1 (GraphPad Software).

**Western blot analysis of SLPI in CF BALF**

Individual P. aeruginosa-positive and -negative CF BALF (20 μl) samples were separated by denaturing SDS-PAGE using 15% polyacrylamide gels and blotted onto nitrocellulose membrane (Sigma-Aldrich). The membrane was blocked for 1 h at room temperature with 3% BSA in PBS containing 0.1% (v/v) Tween 20 (PBST) and blocked in PBST containing 1% BSA for 1 h at room temperature. After washing, the plates were incubated with HRP-conjugated streptavidin (1/2500 in PBST) for 20 min at room temperature and washed with PBST. After the third wash, peroxidase activity was measured by the addition of substrate (ABTS Single Solution; Zymed Laboratories). The absorbance at 405 nm of the wells was measured on a microtiter plate reader (Genios using Magellan software) and results were converted to SLPI concentrations (nM) using GraphPad Prism version 5.1 (GraphPad Software).

**Table I. Clinical information for patients included in the study**

<table>
<thead>
<tr>
<th></th>
<th>Pseudomonas-Negative</th>
<th>Pseudomonas-Positive</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>15</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>7.75 (1.6)</td>
<td>14 (9.9)</td>
<td>0.0047</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>16.7 (0.6)</td>
<td>17 (0.9)</td>
<td>0.7529</td>
</tr>
<tr>
<td>FEV1 (% predicted)</td>
<td>71.8 (7.8)</td>
<td>92 (8.7)</td>
<td>0.1080</td>
</tr>
<tr>
<td>Neutrophils/ml BALF</td>
<td>2.37 (1.5)</td>
<td>9.01 (1.9)</td>
<td>0.0114</td>
</tr>
</tbody>
</table>

*Values represent means (SEM). BMI indicates body mass index; FEV1, forced expiratory volume in 1 s.*
Additionally, recombinant SLPI (0.42 μM) was incubated with various concentrations of NE, P3, or CatG (from 16 nM to 4.2 μM) for 24 h in TBS in a 20 μl volume at 37°C. All incubations were stopped by adding sample treatment buffer containing reducing agent and boiling for 5 min. Samples were separated by SDS-PAGE using a 15% polyacrylamide gel and blotted onto nitrocellulose membrane (Sigma-Aldrich). SLPI was detected as described above.

Measurement of elastase activity in CF BALF

P. aeruginosa-positive and -negative CF BALF (10 μl) were diluted in 0.1 M HEPES, 0.5 M NaCl (pH 7.5) containing 0.13 mM E64, 0.11 mM pepstatin A, and 5.4 mM EDTA and treated with or without NE inhibitor (1 mM MeOSuc-AAPV-CMK) for 1 h at 37°C in a 100 μl volume. The chromogenic substrate, MeOSuc-AAPV-pNA (50 μl), was mixed with each sample to a final concentration of 1.4 mM and absorbance at 405 nm was measured over the time at 37°C in a 96-well microplate reader (Genios using Magellan software). The activity 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.

HPLC mass spectrometry

Cleavage of SLPI by NE was assessed by incubating recombinant SLPI (2 μM) with NE (8 μM) for 1 h in 0.1 M HEPES/0.5 M NaCl (pH 7.5) in a 40-μl final volume at 37°C. Elastase activity was neutralized with 1 μl of PMSF (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. One microliter 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 (34).

LPS ELISA

A Maxisorp plate (Nunc) was coated with 100 ng/well P. aeruginosa LPS (P. aeruginosa serotype 10; Sigma-Aldrich) diluted in serum-free RPMI 1640 media (SFM) and incubated at 37°C for 3 h. Unbound LPS was washed off the plate with distilled water and the plate was left to air dry overnight at room temperature. The next day, the plate was blocked with 1% BSA in PBS for 2 h at room temperature. The plate was washed three times with PBS/0.05% (v/v) Tween. Samples (100 μl/well) were added to the plate (0.42 μM SLPI alone, 0.42 μM SLPI incubated with 1.68 μM NE) and diluted 1/2 in SFM across the plate followed by incubation for 2 h. Control wells received SFM alone. The plate was washed three times before adding biotinylated anti-SLPI IgG (1/1000) and incubating for 2 h.

After washing, streptavidin peroxidase (1/2500) was added to the plate for 20 min. After washing, ABTS single solution substrate (Zymed Laboratories) was added and the plate was incubated at room temperature for 20 min. The absorbance at 405 nm of wells was measured on a microtiter plate reader (Genios using Magellan software) and results were analyzed using GraphPad Prism version 5.1 (GraphPad Software).

EMSA

EMSA was performed as described previously (24). Proteins were incubated with double-stranded biotinylated NF-κB consensus oligonucleotide (5'-AGTGGAGGCCCCTTCCAGGC-3'; MWG Biotech) for 30 min at room temperature in binding buffer (4% (v/v) glycerol, 0.1 mM EDTA, 10 mM Tris- HCl (pH 7.5), 100 mM NaCl, 5 mM DTT, and 0.1 μg/ml nucleoclease) and 2 μg of poly(dI-dC)-poly(dI-dC) (Sigma-Aldrich). Reaction mixtures were electrophoresed on native 15% polyacrylamide gels. The gels were transferred to nitrocellulose in 1× TBE for 30 min at 380 mA, 100 V, and then cross-linked on a UV transilluminator for 10 min. Detection of SLPI-DNA complexes was performed using a chemiluminescent nucleic acid detection module (Pierce Chemical). In brief, after crosslinking, the blot was incubated in blocking buffer for 1 h at 37°C. Streptavidin-peroxidase was added for 15 min in blocking buffer at room temperature, and the blot was then washed six times in wash buffer. The blot was incubated for 5 min in equilibration buffer and developed with the chemiluminescent reagents provided with the kit. SLPI-DNA binding was analyzed using the Syngene GeneSnap and GeneTools software on the ChemiDoc system.

Evaluation of the antiprotease activity of NE-cleaved SLPI

To evaluate the antiprotease activity of NE-cleaved SLPI, we tested the ability of cleaved SLPI to inhibit one of its target proteases, CatG. NE (13.3 μM) was incubated with SLPI (3.33 μM) in HEPES/NaCl buffer for 6 h, after which NE activity was inactivated with the addition of 1 mM MeOSuc-AAPV-CMK. Samples approximating to 0.166 and 0.666 μM SLPI were withdrawn from the mixture and incubated with CatG (0.333 μM) in HEPES/NaCl buffer for 30 min. After this time, CatG substrate MeOSuc-AAPM-pNA (1 mM) was added to the samples and turnover of substrate was evaluated at 405 nm over a 15 min period. Delta absorbance values were converted to the amount of paranitroaniline produced as an indicator of CatG activity. Control reactions included NE-cleaved SLPI (666 nM) incubated with CatG substrate alone and CatG (0.333 μM) alone.

Results

Levels of SLPI in CF BALF

SLPI levels in BALF from 11 CF patients with Pseudomonas infection and 15 CF patients not infected with Pseudomonas were determined by ELISA. As shown in Fig. 1A, levels of SLPI were found to be significantly lower in P. aeruginosa-positive CF BALF (Ps+) compared with P. aeruginosa-negative BALF (Ps−) (3.99 ± 2.11 vs 15.62 ± 5.74 nM, p < 0.05). Analysis of SLPI in Ps+ and Ps− CF BALF by Western blot revealed distinctive banding patterns between the samples. As illustrated in Fig. 1B, in the absence of Pseudomonas infection, SLPI remains intact and a full-length protein of ~12 kDa is detected. However, in Ps− CF BALF samples, cleaved SLPI and lower levels of full-length SLPI were found, suggesting that lower SLPI levels found in Ps− BALF are a consequence of degradation.

Effects of CF BALF incubation on recombinant SLPI integrity

The effects of Ps− and Ps+ CF BALF on the integrity of recombinant SLPI were investigated to determine and compare their ability to cleave SLPI. Recombinant SLPI was incubated with Ps− and Ps+ CF BALF for various times at 37°C and analyzed by Western blot. As shown in Fig. 2, the levels of recombinant SLPI detected decreased in Ps− CF BALF but not in Ps+ CF BALF over time. This degradation was visible but not complete after 1 h, with the presence of an upper band displaying a similar size to intact SLPI and a lower band corresponding to proteolytic fragments of SLPI. However, after 6 and 24 h proteolysis is almost complete, with no intact SLPI detectable in the samples; only the presence of a lower band could be visualized. Recombinant SLPI was incubated separately in the absence of CF BALF under the same conditions and no degradation was detected. SLPI incubated in the presence of
These findings show that the proteolytic activity directed against SLPI was higher in Ps- CF BALF than in Ps+ CF BALF.

Identification of the protease family involved in the cleavage of SLPI in CF BALF

To identify the protease family involved in the cleavage of SLPI, Ps+ CF BALF samples were preincubated with different nonspecific protease inhibitors targeting the serine, cysteine, or metalloprotease families before adding recombinant SLPI in buffer at pH 7.5. After 24 h of incubation at 37°C, samples were analyzed by Western blot for SLPI. As shown in Fig. 3A, PMSF, a nonspecific serine protease inhibitor, inhibits SLPI cleavage in Ps+ CF BALF, whereas neither the cysteine protease inhibitor E64 nor the metalloproteinase inhibitor EDTA had any effect. When this incubation was repeated at a lower pH (5.5), SLPI degradation was still prevented by PMSF but not by EDTA or E64 (Fig. 3B). Overall, these findings show that nonspecific inhibition of serine proteases in Ps+ CF BALF prevented cleavage of SLPI.

Identification of the serine proteases involved in the cleavage of SLPI in CF BALF

Given the previous findings, we expanded the number of serine protease inhibitors used to further classify the putative serine protease that mediates the Ps+ CF BALF cleavage of SLPI. The inhibitors chosen target various groups of serine proteases (NE, Pr3, and CatG) with different efficacies. ACT inhibits CatG and chymase, elafin inhibits elastase and Pr3, and MeOSuc-AAPV-CMK specifically targets elastase. As shown in Fig. 4A, ACT did not prevent SLPI cleavage. In contrast, elafin and MeOSuc-AAPV-CMK completely inhibited SLPI cleavage in Ps+ CF BALF, indicating that NE is most likely to be the serine protease responsible for SLPI cleavage in Ps+ CF BALF.

The ability of purified human neutrophil serine proteases to cleave SLPI was evaluated in vitro to clarify which protease is mediating the cleavage observed. Recombinant SLPI was incubated with purified proteases and analyzed by Western blot. A dose-response experiment was conducted for 1 h at 37°C using an increasing concentration of protease. As shown in Fig. 4B, incubating recombinant SLPI with NE, Pr3, and CatG resulted in the appearance of SLPI cleavage products in a dose-dependent manner. However, when the cleavage patterns are compared with those illustrated in Fig. 2 following SLPI/Ps+ CF BALF incubations, only NE and CatG produce a similar pattern of cleavage products to that found following incubation of SLPI with Ps+ CF BALF. When SLPI was incubated with Pr3, proteolysis is almost complete, with no intact SLPI detectable in the samples at the higher doses used in contrast to the banding pattern found with NE and CatG. However, as shown in Fig. 4A, the CatG inhibitor ACT does not inhibit Ps+ CF BALF-mediated cleavage of SLPI (Fig. 4A), indicating that NE is the serine protease involved in the cleavage of SLPI by Ps+ CF BALF.

Measurement of NE activity in CF BALF

Given that NE is responsible for the pattern of cleavage of SLPI in Ps+ CF BALF, and that the inhibitor is more rapidly degraded in Ps- than in Ps+ CF BALF, NE activity was measured in both CF BALF samples using the chromogenic substrate MeOSuc-AAPV-pNA. Elastase activity in CF BALF was calculated using a standard curve of purified NE to determine the concentration of NE in samples. As shown in Fig. 5, the concentration of free NE is increased >9-fold in Ps+ compared with Ps- CF BALF (18.76 vs 1.92 μM, Fig. 5). Additionally, free elastase activity in both samples is totally abrogated using the NE inhibitor MeOSuc-AAPV-CMK (Fig. 5), thus confirming the specificity of the measurements. These results reinforce our finding that NE is responsible for SLPI cleavage in Ps+ CF BALF.

Analysis of NE-SLPI cleavage sites

To further investigate the elastase-mediated cleavage of SLPI, products from NE-SLPI incubations were analyzed by HPLC and mass spectrometry. HPLC separation of SLPI products generated...
within 2 h by an excess of NE resulted in the formation of three distinct peaks (Fig. 6A, peaks 1–3). Identification of SLPI fragments was conducted by analyzing peaks by mass spectrometry. The deconvoluted mass of peak 1 was 10,152 Da, identifying it as SLPI residues Glu17-Ala107 (calculated mass of 10,152 Da). Likewise, the measured mass for peak 2 was 10,223 Da, identifying it as SLPI residues Ala16-Ala107 (calculated mass of 10,223.14 Da). The measured mass of peak 3 was 11,725 Da, identifying it as full-length SLPI residues Ser1-Ala107 (calculated mass of 11,725.93 Da). These results indicate that 2-fold excess of NE cleaved SLPI at the Ser15-Ala16 and Ala16-Glu17 peptide bonds. In contrast, such cleavages were not detected using conditions with a slight excess of SLPI ([enzyme]/[inhibitor] molar ratio of 1:2.5), even after 24 h of incubation (data not shown). Taken together, these findings indicate that NE-mediated cleavage of SLPI occurs only with excess of NE. The cleavage sites generated by NE within SLPI are summarized in Fig. 6B.

**FIGURE 5.** Free neutrophil elastase activity in Pseudomonas-negative and -positive CF BALF. Ps+/H11001 and Ps+/H11002 CF BALF samples diluted in HEPES buffer were preincubated alone or with the elastase inhibitor MeOSuc-AAPV-CMK (CMK) and mixed with the chromogenic substrate MeoSuc-AAPV-pNA. The absorbance of samples was measured at 405 nm over time. The activity of NE in samples was assessed by comparing the NE activity in Pseudomonas-infected and noninfected BALF with that of a standard curve of purified NE. Each measurement was performed in duplicate.

**FIGURE 6.** HPLC analysis of SLPI incubated with NE. A, Human NE (8 μM) was incubated with SLPI (2 μM) in 0.1 M HEPES/0.5 M NaCl (pH 7.5) for 2 h at 37°C. The samples were neutralized with 5 mM PMSF, dried, and reconstituted in 6 M guanidine, 100 mM Tris (pH 8.5). The samples were then separated by HPLC and three peaks were obtained corresponding to various SLPI products obtained from the incubation. Peaks were analyzed by mass spectrometry. The deconvoluted mass of peak 1 was 10,152 Da, identifying it as SLPI residues Glu17-Ala107; the deconvoluted mass of peak 2 was 10,223 Da, identifying it as SLPI residues Ala16-Ala107; and peak 3 corresponded with full-length SLPI residues Ser1-Ala107 (11,725 Da). B, Schematic representation of neutrophil elastase cleavage sites in the amino acid sequence of SLPI (Ser1-Ala107). Both WFDC domains present in SLPI are underlined. The lines represent disulfide bridges linking paired cysteine residues (10–39, 18–43, 26–38, 32–47, 64–95, 71–97, 80–92, 86–101), and the asterisk identifies the scissile peptide bond between Leu72 and Met73. The arrows represents the cleavage sites (Ser15-Ala16 and Ala16-Glu17) generated by excess neutrophil elastase for 2 h.

**Effects of excess NE on SLPI functions**

In addition to its antiprotease capabilities, SLPI has antibacterial and anti-inflammatory effects and can interact with the endotoxin LPS of Gram-negative bacteria such as *E. coli* and modulate macrophage responses after LPS stimulation (21–23). We therefore investigated the effect of NE-induced cleavage on the ability of SLPI to bind LPS. Recombinant SLPI was incubated alone or with an excess of NE and examined for its capacity to bind to *P. aeruginosa* LPS by ELISA. As illustrated in Fig. 7A, intact SLPI was able to interact with *P. aeruginosa* LPS in a dose-dependent manner. In contrast, little or no SLPI-LPS binding was detected when SLPI pretreated with NE or untreated NE were analyzed. Thus, these results suggest that NE suppresses the ability of SLPI to bind to *P. aeruginosa* LPS. Similar findings were obtained when *E. coli* LPS was used (data not shown). SLPI can inhibit LPS-induced proinflammatory responses in monocytes and macrophages, and previous work from our group has found that SLPI exerts inhibitory...
effects on nuclear events of the LPS-induced NF-κB signaling cascade by competing with NF-κB p65 for binding to the NF-κB binding sites in the promoter region of genes such as IL-8 and TNF-α (24). Therefore, we investigated whether NE-cleaved SLPI was able to bind NF-κB consensus oligonucleotides. The results show that while intact SLPI binds biotinylated NF-κB oligonucleotide, SLPI incubated with an excess of NE or NE alone does not bind to the oligonucleotides (Fig. 7B). Overall, these findings suggest that NE abrogates a number of mechanisms by which SLPI exerts its anti-inflammatory functions.

**Evaluation of the antiprotease activity of NE-cleaved SLPI**

To determine the effect of NE cleavage on the antiprotease activity of SLPI, we evaluated the ability of NE-cleaved SLPI to inhibit CatG using the CatG-specific substrate MeOSuc-AAPV-CMK. As before, SLPI was incubated with excess NE and cleavage of SLPI was confirmed by Western blot (data not shown). Samples were withdrawn from the mixture and incubated with a fixed amount of CatG (0.333 μM): a subinhibitory sample (equivalent of 0.166 μM cleaved SLPI) and a 2-fold excess sample (equivalent of 0.666 μM cleaved SLPI). As illustrated in Fig. 8, the subinhibitory sample of NE-cleaved SLPI reduced CatG activity, and the 2-fold excess sample of cleaved SLPI almost completely abolished CatG activity, similar to that observed with 2-fold excess of native full-length SLPI. Therefore, NE-cleaved SLPI retains its antiprotease activity against its target serine protease CatG.

**Discussion**

CF is characterized by chronic lung disease and recurrent bacterial infection. Infection with *P. aeruginosa* is associated with increased morbidity and mortality in patients with CF. Typically respiratory secretions are rich in multifunctional innate immunity proteins and peptides such as lactoferrin, LL-37, β-defensins, elafin, and SLPI. However, during lung inflammation it appears that a number of these host defense components are sensitive to exacerbated proteolytic activity emanating from dysregulated elastolytic enzymes as found in chronic lung diseases such as CF (27, 28, 30, 31, 33–35). It is important that the cause of this proteolytic activity is elucidated to facilitate the development of more efficient and robust therapies and to enable our understanding of how CF progresses. In the upper respiratory tract SLPI is thought to be primarily involved in protecting against human NE-induced damage, as it accounts for ~90% of the total molar concentration of elastase inhibitors in human bronchial secretions (36) and may in fact be up-regulated during times of inflammation. Nevertheless, there is a growing wealth of evidence purporting that despite its putative proteolytically stable structure due to the number of disulfide bridges present, SLPI is in fact prone to cleavage by pro tease of endogenous and bacterial origin.

In this study, we demonstrate the potent ability of *P. aeruginosa*-infected CF bronchial secretions to cleave exogenous recombinant human SLPI in an NE-dependent manner. In contrast, CF BALF from patients not infected with *P. aeruginosa* was not able to cleave SLPI. In vivo, SLPI concentrations have been found to be higher in upper respiratory airways than in lower airways, with reported levels of 0.1–0.2 μg/ml (8.33–16.66 nM) in BALF (29) and between 0.1 and 24 μg/ml (8.33 nM to 2 μM) in saliva (12, 37, 38). We have shown that SLPI levels in noninfected CF airways are generally lower than in infected CF airways, and we have also shown that SLPI is more stable in higher pH environments, which are typical of CF airways.

Thus, we predict that when chronic infection has taken hold, NE-cleaved SLPI would be up-regulated during times of inflammation. Nevertheless, there is a growing wealth of evidence purporting that despite its putative proteolytically stable structure due to the number of disulfide bridges present, SLPI is in fact prone to cleavage by proteases of endogenous and bacterial origin.
fragments of ~10 kDa in size in BALF from Ps⁺ patients. Although most Ps⁺ BALF samples displayed no evidence of SLPI cleavage, we detected the presence of cleavage products in two samples, suggesting that other proteases are present in these BALF samples that are capable of cleaving SLPI. The identity of this protease activity is unknown; however, we speculate that cathepsins may play a role given our previous demonstration of the ability of cathepsins to cleave SLPI (28). Overall, the findings suggest that the decrease in BALF SLPI levels as evidenced by ELISA is a consequence of degradation by factors present in Ps⁺ BALF. We further investigated this phenomenon by comparing the effects of CF BALF from P. aeruginosa-infected and non-infected patients on the integrity of recombinant human SLPI. Previous work by Vogelmeier et al. (29) demonstrated the presence of cleaved SLPI in BALF from patients with CF, but no details of the status of Pseudomonas infection were provided in the study. Our findings expand this observation, as we show that only Pseudomonas-infected BALF is capable of degrading SLPI. As Pseudomonas elastase (pseudolysin) has been reported to cleave SLPI (32), we examined the involvement of this bacterial protease by pretreating P. aeruginosa-infected BALF with EDTA, which inhibits Pseudomonas elastase activity. Pretreatment with EDTA was unable to inhibit P. aeruginosa-positive BALF-mediated cleavage of SLPI. However, we found that P. aeruginosa-positive BALF cleavage of SLPI was inhibited by the addition of human NE inhibitors (PMSF, elafin, and MeOSuc-AAPV-CMK), thus implicating human NE in the process. When recombinant human SLPI was incubated with an excess of purified NE (molar ratio of [enzyme]/[inhibitor] of 2:1), we observed a similar pattern of cleavage products to that found with P. aeruginosa-positive BALF.

Although CatG incubation with SLPI also gave a similar pattern of cleavage products to that obtained with NE and P. aeruginosa-positive BALF, ACT (which inhibits CatG and chymase) was unable to prevent this cleavage. Consequently, we concluded that neither CatG nor chymase mediated the cleavage we observed. Belkowski et al. (39) have reported the ability of chymase to cleave exogenous recombinant human SLPI at residues Leu72-Met73, the reactive site bond present in the second WFDC domain of the protein. Identification of Ser15-Ala16 as a cleavage site is in agreement with previous work by Masuda et al. (41), who reported the ability of excess NE to cleave SLPI at least two sites, Ser15-Ala16 and Thr57-Arg58. These cleavage sites are in contrast to those we previously found for cathepsin-mediated cleavage of SLPI, which occurred at Thr67-Tyr68 (28). We have previously demonstrated that cathepsin L in COPD BALF can cleave and inactivate SLPI; however, there is little or no NE activity in COPD BALF (28). In contrast, NE and cysteiny1 cathepsin activity are both present in CF BALF (30). Nevertheless, we postulate that SLPI has a much greater affinity for NE than cathepsin L, so in the Pseudomonas-infected lung, SLPI most likely binds preferentially to NE, thus masking the Thr67-Tyr68 bond from cleavage by cathepsins. However, under these circumstances, excess NE can cleave SLPI at the NH₂ terminus, as we have shown in this report.

The implications of NE-induced cleavage of SLPI in vivo became evident from a number of functional studies in which we tested the ability of NE-cleaved SLPI to exert well-known anti-inflammatory functions such as binding to LPS and NF-κB consensus binding sites. Ding et al. demonstrated that recombinant human SLPI binds to E. coli LPS and prevents the formation of LPS-CD14 complexes and slows the transfer of LPS from the complexes to macrophages (23). Following incubation with NE, the ability of SLPI to bind P. aeruginosa and E. coli LPS was dramatically reduced, which may contribute to enhanced cellular responsiveness to LPS. Additionally, we demonstrated that NE-cleaved SLPI is no longer capable of binding NF-κB consensus binding sites and is therefore unable to prevent NF-κB p65 binding and initiating proinflammatory cytokine expression as shown previously (24). Given that NE was found to cleave SLPI at two sites in the NH₂-terminal, these findings suggest that this region of the SLPI protein plays a vital role in at least some of the anti-inflammatory functions of SLPI. Previous work has highlighted the importance of the NH₂-terminal of SLPI in mediating its antimicrobial activities (14, 43). Consequently, it is reasonable to hypothesize that in addition to inactivating important anti-inflammatory effects of SLPI, NE is also capable of dampening the antibacterial ability of SLPI. It is interesting to note, however, that although NE can inactivate a number of SLPI’s anti-inflammatory mechanisms of action, it fails to significantly alter the ability of SLPI to inhibit one of its target serine proteases, CatG. The preservation of antiprotease properties of SLPI fragments may be a result of its structure. The antiprotease active site of SLPI is located in a loop (residues 67–74) in the COOH-terminal WFDC domain. It has been reported that the recombinant COOH-terminal domain of SLPI retains its elastase inhibitory activity (41, 44). As NE cleaves SLPI at the NH₂ terminus, we would expect the resulting fragment to retain its antiprotease activity as confirmed by our findings above.

The susceptibility of SLPI to proteolytic degradation is a major hindrance in treating diseases characterized by a protease burden such as CF. Given the vicious cycle of infection, inflammation, and proteolytic degradation that exists in CF, and the multifunctionality of SLPI, this protein is an obvious choice as a therapeutic for such a disease, but findings to date reveal only limited success. Intravenous administration of recombinant SLPI to animals resulted in rapid renal clearance hindering their use as i.v. agents (45). As an alternative, aerosolized recombinant versions have been evaluated and were found to be effective both in terms of delivery and in mediating therapeutic effects (16). McElvaney
et al. (16) demonstrated suppression of pulmonary NE and IL-8 levels following administration of aerosolized SLPI to CF patients. However, the use of SLPI in a clinical setting has been limited by enzymatic cleavage and failure to deposit efficiently in poorly ventilated, highly inflamed areas of the lung (46) and, as such, clinical studies have yet to progress to the next stage. Recent work by Gibbons et al. (47) may present an effective alternative to remedy some of these obstacles. By encapsulating recombinant human SLPI in biocompatible liposomes, the stability of SLPI was improved in response to cysteine protease exposure; however, whether encapsulation protects from NE-induced degradation remains to be determined.

Overall, these novel findings broaden our understanding of the destruction caused by P. aeruginosa infection and how it contributes to chronic damage seen in the CF lung. Despite aggressive antibiotic treatments, pulmonary infection with P. aeruginosa remains a leading cause of morbidity and mortality in CF patients. Pseudomonas aeruginosa infection is associated with higher elastase concentrations, and proteases, particularly NE, contribute to the pathology of CF by impairing mucociliary clearance, interfering with innate immune functions, and perpetuating neutrophilic inflammation (1, 48, 49). Our results confirm and expand previous work demonstrating that excessive NE activity in the Pseudomonas-aeruginosa-infected lung can cause the destruction of elafin, another serine anti-protease produced locally in the lung (34). In vivo, SLPI appears to be constitutively expressed and may provide a baseline host defense shield that can be up-regulated at times when inflammation or infection are anticipated (50). However, given its susceptibility to proteolytic degradation by not only cysteinyl proteases like cathepsins, but also the serine protease NE, the levels and biological activities may be compromised in vivo.

Disclosures

The authors have no financial conflicts of interest.

References


