LL-37 Complexation with Glycosaminoglycans in Cystic Fibrosis Lungs Inhibits Antimicrobial Activity, Which Can Be Restored by Hypertonic Saline

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LL-37 Complexation with Glycosaminoglycans in Cystic Fibrosis Lungs Inhibits Antimicrobial Activity, Which Can Be Restored by Hypertonic Saline

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There is an abundance of antimicrobial peptides in cystic fibrosis (CF) lungs. Despite this, individuals with CF are susceptible to microbial colonization and infection. In this study, we investigated the antimicrobial response within the CF lung, focusing on the human cathelicidin LL-37. We demonstrate the presence of the LL-37 precursor, human cathelicidin precursor protein designated 18-kDa cationic antimicrobial protein, in the CF lung along with evidence that it is processed to active LL-37 by proteinase-3. We demonstrate that despite supranormal levels of LL-37, the lung fluid from CF patients exhibits no demonstrable antimicrobial activity. Furthermore Pseudomonas killing by physiological concentrations of exogenous LL-37 is inhibited by CF bronchoalveolar lavage (BAL) fluid due to proteolytic degradation of LL-37 by neutrophil elastase and cathepsin D. The endogenous LL-37 in CF BAL fluid is protected from this proteolysis by interactions with glycosaminoglycans, but while this protects LL-37 from proteolysis it results in inactivation of LL-37 antimicrobial activity. By digesting glycosaminoglycans in CF BAL fluid, endogenous LL-37 is liberated and the antimicrobial properties of CF BAL fluid restored. High sodium concentrations also liberate LL-37 in CF BAL fluid in vitro. This is also seen in vivo in CF sputum where LL-37 is complexed to glycosaminoglycans but is liberated following nebulized hypertonic saline resulting in increased antimicrobial effect. These data suggest glycosaminoglycan–LL-37 complexes to be potential therapeutic targets. Factors that disrupt glycosaminoglycan–LL-37 aggregates promote the antimicrobial effects of LL-37 with the caveat that concomitant administration of antiproteases may be needed to protect the now liberated LL-37 from proteolytic cleavage. The Journal of Immunology, 2009, 183: 543–551.

Antimicrobial proteins and polypeptides of innate pulmonary host defense are a diverse assembly of molecules, including lactoferrin, secretory leucoprotease inhibitor (SLPI), lysozyme, and defensins, all of which are capable of killing microorganisms (1). In mammals, one of the main antimicrobial peptide families is the cathelicidin family (2). Peptides belonging to the cathelicidin family are either constitutively produced or inducible upon stimulation of keratinocytes (3) and bone marrow progenitor cells (4). The effect and importance of cathelicidins in host defense has been demonstrated in vivo by studies of microbial pathogens in cathelicidin knock-out mice (5), Shigella-infected dysenteric rabbits (6), and Morbus Kostmann’s syndrome in humans (7), which is associated with antimicrobial peptide deficiencies or decreased antimicrobial peptide activity. The human cathelicidin precursor protein designated 18-kDa cationic antimicrobial protein (hCAP18) has been identified in peripheral blood neutrophils (8), lung epithelium (9), and alveolar macrophages (10). The hCAP18 is composed of a conserved N-terminal prodomain called cathelin and a C-terminal antimicrobial peptide domain. The C-terminal domain is released from the parent molecule by proteolytic processing by proteinase 3 to the cationic α-helical peptide LL-37 in neutrophils (8, 11) and to ALL-38 by prostate-derived protease gastrin in the vagina (12). Furthermore, LL-37 acts as a multifunctional immunomodulator, which has been shown to be chemotactic to human neutrophils, monocytes, T cells, and mast cells (13–15) and stimulate phagocytosis, reactive oxygen species production (16) and the synthesis of leucotriene B4 by neutrophils (17), degranulation of mast cells (18), and induce chemokine response in monocytes, airway epithelia, and keratinocytes (19–21). The mature LL-37 has further been shown to be degraded to shorter peptic fragments, including KR-20, KS-30, and RK-31, by proteases in human sweat (22). Studies suggest proteolytically cleaved LL-37 peptide fragments to have altered antimicrobial activity and reduced immunomodulatory host response compared with the 37 amino acid long intact LL-37 (22).

The human cathelicidin can kill pathogens, which are important in the context of cystic fibrosis (CF) (23), a disease caused by mutations in the gene encoding the CF transmembrane conductance regulator. These mutations lead to bacterial colonization and chronic airway infection, which is a major cause of mortality and morbidity in CF individuals and suggest a poor antimicrobial host response.
response (24). Antimicrobial effector molecules, such as defensins and SLPI, have previously been shown to be inactivated in CF lungs because of their susceptibility to proteolytic degradation (25, 26). Proteinases of significant CF pathogens including aureolysin of *Staphylococcus aureus* (27) and elastase of *Pseudomonas aeruginosa* have been shown to degrade LL-37 (28). Previously, it has been shown that LL-37 is present in high concentrations in CF lungs (29), which are in fact associated with pulmonary inflammation and disease severity (30). In addition, it has been demonstrated that the antimicrobial activity of LL-37 is inhibited in vitro by LPS (31, 32) and mucins (33), in CF sputum by F-actin bundles and DNA, and in wound fluid by glycosaminoglycans (GAGs) (34), which also interact with proteases (35–37), chemokines (35), cytokines (35), and antimicrobial peptides (38, 39). Studies also show that CF sputum becomes antimicrobial after in vitro treatment with gelsolin and DNase (31, 32). In this study, we evaluate the presence, activation, and antimicrobial activities of LL-37 in the CF lung and attempt to determine whether its inability to prevent bacterial colonization in CF is due to limited supply, impaired activation, degradation, or complexation and evaluate means by which this relative inactivity can be reversed. In this study, we build on previous data and demonstrate for the first time a significant antimicrobial effect of CF spuata by exposure to GAG lyases in vitro or nebulized hypertonic saline in vivo without the addition of exogenous antimicrobials. This innovative study illustrates the ability of nebulized hypertonic saline to solubilize and activate the antimicrobial peptide LL-37 in the airways of CF patients. We also demonstrate, for the first time, that the addition of protease inhibitors that are specific against LL-37-degrading proteases in CF lungs, here shown to be neutrophil elastase and cathepsin D, can enhance this antimicrobial effect that can partially be neutralized by specific LL-37 Abs.

**Materials and Methods**

**Bronchoalveolar lavage (BAL) fluid sample collection**

CF patients were deemed noncolonized when no *P. aeruginosa* growth was detectable in sputum or cough swab during the previous 12 mo. Noncolonized (*n* = 11, average age of 7.78 ± 2.11 years and FEV1 of 61.00% ± 8.637 (mean ± SE)) and colonized (*n* = 18, average age of 14.47 ± 1.41 years and FEV1 of 64.65% ± 6.84 (mean ± SE)) CF BAL samples were obtained from individuals undergoing flexible bronchoscopy for clinical reasons. Non-CF healthy controls were obtained from children (*n* = 11, average age of 8.21 ± 1.22 years) without lung disease undergoing elective nonpulmonary surgical procedure. Full informed parental consent was obtained for all procedures and ethical approval for the use of these samples was obtained from the institutional review board of the Adelaide and Meath Hospital incorporating the National Children’s Hospital. Bronchoscopy was performed via a laryngeal mask airway and the bronchoscope was directed to the lingula and right middle lobe. BAL 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. BAL fluid was centrifuged at 2000 g for 10 min and the supernatant aliquoted and stored at −80°C. The pellet was resuspended in physiological saline and cell counts were performed using trypsin blue exclusion. Differential cell counts were performed microscopically after staining with May-Grunwald and Giemsa with at least 200 cells counted per slide.

**Sputum sample collection**

Sputum samples were collected before and after administration of 4 ml 7% saline via a Pari LC Plus turbo nebulizer. Samples from individuals with proven CF were evaluated (six male; six female, average age of 24.73 ± 6.24 years and FEV1 of 56.75% ± 32.17 (mean ± SE)). Immediately after collection of the first sputum sample, each patient received nebulized hypertonic saline for 15 min and the second sample then collected within 60 s. To every gram of expectorated sputum, 1 ml of physiological saline was added. Samples were rotated gently for 3 min, centrifuged at 836 g for 10 min at 4°C, and supernatants kept frozen at −70°C.

**FIGURE 1.** Quantification of hCAP18/LL-37 in BAL fluid from CF and healthy individuals. A, ELISA analyses of hCAP18/LL-37 in BAL fluid from *Pseudomonas* -colonized CF individuals (*n* = 18), noncolonized CF individuals (*n* = 10), and controls (*n* = 11). B, Correlation between hCAP18/LL-37 and neutrophil numbers in BAL fluid from individuals with CF was deemed significant when α = 0.05 (*n* = 23, Pearson r = 0.7293, *p* < 0.0001, R² = 0.5319). C, hCAP18/LL-37 levels after adjustment for neutrophil numbers in *Pseudomonas*-colonized CF individuals (*n* = 17) compared with noncolonized CF (*n* = 9). D, Antimicrobial effect of 0–5.6 µM LL-37 (average of two experiments) against *P. aeruginosa* after 1 h incubation at 37°C. §, *p* > 0.999 killing. SEM is indicated by the bars and two-tailed *p* value calculated by unpaired *t* test.
FIGURE 2. LL-37 is activated by proteinase 3 in CF BAL fluid. Western blot analyses of the proteolytic processing of hCAP18 in A, BAL fluid samples from control subjects (n = 8) and B, from CF (n = 5) individuals from 0 to 48 h. C. The proteolytic processing of hCAP18 in CF BAL fluid after 48 h incubation with addition of specific protease inhibitors including A1AT, SLPI, and ACT, the metallocprotease inhibitor EDTA, amidase inhibitor pepstatin A, and the cysteine protease inhibitor E-64. D, The inhibitory effects of 20 mM CMK and 1 g/L recombinant elafin on proteolytic processing of hCAP18 in CF BAL fluid from 6 to 48 h. E, Western blot analysis of hCAP18 and LL-37 after incubation for 0–80 min in control BAL to which was added 10⁻⁶ M proteinase 3 or 10⁻⁶ M neutrophil elastase. Each blot is representative of one of three experiments.

Gel electrophoresis and Western blot analyses

Samples were subjected to SDS-PAGE under denaturing conditions in 4–12% NuPAGE gels (Invitrogen) and to native gel electrophoresis in NativePAGE Novex Bis-Tris gel system (Invitrogen). Proteins were separated at 200 V for 35 min and at 150 V for 110 min for SDS-PAGE and native gel electrophoresis respectively, following the manufacturer’s instructions. After gel electrophoresis, proteins were blotted onto 0.2 µm nitrocellulose or polyvinylidene difluoride membrane (Sigma-Aldrich) for 60 min at 30 V, membranes were blocked for 1 h in 5% dry milk in PBS with 0.25% Tween 20 and then incubated with 0.3 µg/ml polyclonal rabbit LL-37 specific Ab (Santa Cruz Biotechnology). The secondary Abs were HRP-linked anti-rabbit and anti-mouse IgG (Cell Signaling Technology), respectively. Immunoreactive protein bands were visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce) after exposure to Kodak X-OMat LS Film (Sigma-Aldrich).

Quantification of LL-37 in CF BAL fluid

hCAP18/LL-37 ELISA kit was purchased from Hycult Biotechnology. Samples were diluted to the appropriate concentrations and ELISA performed following the manufacturer’s instructions.

Processing of hCAP18 in BAL fluid

CF BAL fluid (8.5 µl) incubated with an equal volume of 0.1 M HEPES buffer (pH 7.5) containing 0.5 M NaCl was incubated at 37°C for 0, 6, 24, and 48 h. In some cases, CF BAL fluid samples were preincubated with 1 µl of 1 g/L recombinant human elafin (Protease Biotech), 0.2 M Pefabloc (Sigma-Aldrich), 20 mM N-(methoxysuccinyl)-Ala-Ala-Pro-Val-chloromethyl ketone (CMK) (Sigma-Aldrich), 1 mM pepstatin A (Sigma-Aldrich), 0.1 g/ml EDTA (Sigma-Aldrich), 1 g/L α-1-antichymotrypsin (ACT) (Merck Biosciences), 1 g/L α-1-antitrypsin (A1AT) (Merck Biosciences), 5 g/L SLPI (R&D Systems), or 5 g/L E-64 (Merck Biosciences). The mixtures were incubated at 37°C, and samples were collected at specific time points and subjected to SDS-PAGE.

Proteolytic processing of hCAP18 in BAL fluid from healthy individuals was analyzed by incubation with 10⁻⁷ or 10⁻⁶ M of human neutrophil elastase (Athens Research and Technology) or human proteinase 3 (Athens Research and Technology) in Dulbecco’s PBS (DPBS) (Invitrogen). Reactions were incubated at 37°C and samples were collected at specific time points for Western blot analyses.

Proteolytic degradation of LL-37

LL-37 (5 µg) was incubated with 4 µl CF BAL fluid to a final volume of 10 µl for 24 and 48 h at 37°C. Experiments were repeated by inclusion of protease inhibitors as described above, added 1 h before the addition of LL-37.

For further analyses, 10⁻⁶ M neutrophil elastase (Athens Research and Technology), proteinase 3 (Athens Research and Technology), cathepsin D (Athens Research and Technology), or cathepsin G (Elastin Products Company) was added to 10⁻⁶ M LL-37 in 20 mM NaHPO₄ (pH 6.1). Reactions were incubated at 37°C, samples collected at specific time points, and subjected to Western blot analyses. For some reactions, LL-37 was preincubated for 1 h with heparan sulfate, chondroitin sulfate, and hyaluronic acid, at a ratio of 1:10 (w/w) for each GAG.

Acidic and serine protease activity assays

Cathepsin D assay kit (Sigma-Aldrich) was used to measure cathepsin D activity using the manufacturer’s instructions. In brief, 50 µl BAL fluid was incubated with 20 µl assay buffer, 10 µl H₂O or pepstatin A, and 20 µl substrate solution and the rate of reactions recorded at 460 nm after excitation at 340 nm in a 96-well microplate reader (Victor2 1420 Multi-label Counter, Wallac).

Neutrophil elastase activity in BAL fluid samples from CF and controls was estimated using the substrate N-(methoxysuccinyl)-Ala-Ala-Pro-Val-p-nitroanilide (Sigma-Aldrich). In brief, two aliquots of BAL fluid were mixed with an equal volume of 0.1 M HEPES buffer including 0.5 M NaCl (pH 7.5) and transferred to a 96-well cell culture plate (Nunclon). Saline mixed with buffer alone was used as a control. Fifty microliters of 2 mM N-(methoxysuccinyl)-Ala-Ala-Pro-Val-p-nitroanilide was added to each well, incubated for 5 min, and measured for liberation of p-nitroaniline spectrophotometrically at 405 nm. Neutrophil elastase activity was quantified by comparison with a neutrophil elastase standard (Elastin Products Company) of known activity.

Bacterial culture and antimicrobial assays

P. aeruginosa strain (PAO1) and S. aureus strain (8325–4) were used to analyze the antimicrobial activity of LL-37 and airway surface fluid. For each experiment, microbial colonies were seeded from frozen stocks and grown overnight at 37°C on Trypticase Soy Agar (BD Biosciences) plates. A single colony was suspended in BBL Trypticase Soy Broth (BD Biosciences) and incubated at 37°C and 200 rpm until mid-logarithmic phase was reached.
The antimicrobial effect of LL-37 in the presence of heparan sulfate (Sigma-Aldrich), chondroitin sulfate (Sigma-Aldrich), hyaluronic acid (Sigma-Aldrich), or CF BAL fluid was tested by colony forming unit (CFU) count. In brief, Pseudomonas and Staphylococcus cells were washed once in DPBS (Invitrogen) and 10^7–10^8 CFU/ml incubated with LL-37 for 1 h at 37°C with shaking (200 rpm). Samples were removed, 10-fold serial dilutions prepared and 10 or 100 μl of each dilution streaked on trypticase soy agar. DPBS were used as a control and colonies were counted after overnight incubation at 37°C.

Antimicrobial effect of sputum collected before and after nebulization of hypertonic saline was tested in the presence of 16 mM pefabloc and 0.4 g/l peptatin A against 10^5 CFU/ml bacteria for 2 h. In some experiments sputum was treated with GAG lyases, 0.5 unit of the hyaluronidase (Sigma-Aldrich), chondroitinase ABC, and heparinase II (Sigma-Aldrich) was pre-incubated with 50 μl sputum supernatant in the presence or absence of 2 mM pefabloc and 30 μM peptatin A for 6 h at 37°C. Microbes were then added to the mixture at a final concentration of 10^5 CFU/ml and the samples incubated for an additional 2 h at 37°C. The CFU count was performed as described above. In an additional experiment, antimicrobial effect of LL-37 sputum collected post nebulization was neutralized by incubation with 144 μg/ml LL-37 specific Ab for 1 h in the presence of 16 mM Pefabloc, 0.4 g/l peptatin A, and 0.2 g/L E-64. Heat-inactivated LL-37 Ab was used as controls and CFU count conducted as described above.

Inhibition zone assay was conducted as previously described (40). In brief, 1% agarose in Luria-Bertani broth containing increasing NaCl concentrations (0–600 mM) was mixed with Pseudomonas cells (10^5 CFU/ml), and 3 μl of LL-37 (1 g/L) dissolved in water was added to each well. Zones of bacterial growth inhibition were recorded using a jeweler’s eyepiece (The Binding Site) after an overnight incubation at 37°C.

Release of LL-37 from GAGs in BAL fluid

For Western blot analyses, 20 μl of CF BAL fluid was incubated at 37°C with 1 unit of the GAG lyases hyaluronidase, chondroitinase ABC, or heparinase II (Sigma-Aldrich) for 24 h in the presence or absence of 10 mM pefabloc and 50 μM peptatin A. Alternatively, CF BAL fluid was treated for 1 h at 37°C with an equal volume of increasing NaCl concentrations.

Statistical analyses

The data were analyzed with the GraphPad Prism version 4.03 for Windows (GraphPad Software) and results expressed as means ± SEM. Normally distributed data were analyzed by unpaired t test or Mann-Whitney U test. p values <0.05 were deemed significantly different.

Results

Quantification of hCAP18/LL-37 and neutrophil numbers in CF BAL fluid

hCAP18/LL-37 levels were raised in BAL fluid samples from CF patients compared with healthy controls (Fig. 1A). Furthermore, hCAP18/LL-37 levels correlated with the number of neutrophils in corresponding samples (Pearson’s r = 0.7293, r^2 = 0.5319, p < 0.0001) (Fig. 1B) and with the stage of Pseudomonas colonization (Fig. 1A). Interestingly, when cathelicidin levels were adjusted for neutrophil number (Fig. 1C), a more pronounced difference was observed between colonized and noncolonized CF samples (p = 0.0105). Taking into account that CF BAL fluid is ~100-fold dilated epithelial lining fluid (41), the average concentration of LL-37 in epithelial lining fluid from colonized CF individuals is potentially 6.47 μM. An in vitro antimicrobial killing assay illustrated that 5.6 μM LL-37 possessed significant anti-Pseudomonas properties reducing the CFU count by 99.99% (Fig. 1D). Ensuing experiments were designed to investigate why despite such high levels of antipseudomonal LL-37 the lungs of individuals with CF remodeled with Pseudomonas.

LL-37 is activated by proteinase 3 in CF BAL fluid

One possible reason for the relative inefficiency of LL-37 in the CF lung could be lack of activation. The proteolytic processing of the cathelicidin precursor protein hCAP18 was analyzed in BAL fluid samples from CF and healthy individuals by Western blot analyses (Fig. 2). There was no processing of hCAP18 in control BAL fluid

FIGURE 3. Antimicrobial activity of exogenous LL-37 is inhibited by cathepsin D and neutrophil elastase in CF BAL fluid. A. Anti-Pseudomonal effect of 32 μM LL-37 after 24 h incubation in BAL fluid from healthy controls (n = 4) and CF individuals (n = 6). B. Western-blot analyses of synthetic LL-37 after 24 h incubation in CF and control BAL fluid. C. Inhibition of proteolytic degradation of LL-37 after 24 h incubation by specific protease inhibitors pefabloc and pepstatin A but not by E-64 and EDTA. D. Incubation of synthetic LL-37 with the serine proteases proteinase 3, neutrophil elastase and cathepsin G and the acidic protease cathepsin D from 0 to 24 h. §, No viable bacteria remained in 100 μl of 10-fold diluted sample. SEM is indicated by the bars and two-tailed p value calculated by unpaired t test. Each experiment was performed at least three times.
FIGURE 4. LL-37 is resistant to proteolytic degradation when bound to glycosaminoglycans. A, Native gel electrophoresis and Western blot analyses of CF BAL fluid incubated with increasing concentrations of NaCl. B, Antimicrobial effect of 3 μg LL-37 in the presence of increasing NaCl concentrations against *P. aeruginosa* using the inhibition zone assay. C, CF BAL fluid exposed to GAG lyases with and without protease inhibitors (pefabloc and pepstatin A) and subjected to native gel electrophoresis. D, LL-37 pre-exposed to GAGs, and incubated in the presence of cathepsin D for 24 h analyzed by SDS-PAGE. E, Antipseudomonal effect of 7.4 μM LL-37 analyzed with and without preincubation with GAGs. Two-tailed p value was calculated by t test. Each experiment was performed at least three times.

(n = 8) (Fig. 2A). In contrast, cleavage of hCAP18 to LL-37 was observed in all CF BAL fluid samples analyzed (n = 5; Fig. 2B). Processing of hCAP18 to LL-37 in CF BAL fluid was inhibited by the serine protease inhibitor pefabloc and not by the metalloprotease inhibitor EDTA, the acidic protease inhibitor pepstatin A or the cysteine protease inhibitor E-64 (Fig. 2C). Processing was inhibited to a lesser extent by A1AT and SLPI but not by ACT (Fig. 2C).

Given the inhibitory effects of pefabloc on hCAP18 processing, implicating a serine protease, we next evaluated the inhibitory actions of recombinant elafin and CMK, which inhibit both neutrophil elastase and proteinase 3 (42). These inhibitors were effective for up to 48 h incubation at 37°C (Fig. 2D), implicating both neutrophil elastase and proteinase 3 in hCAP18 processing. Following this, proteinase 3 and neutrophil elastase were titrated to active concentrations in BAL fluid from a healthy individual and proteolytic processing of hCAP18 observed by Western blot analyses (Fig. 2E). Processing of LL-37 was observed in samples incubated with proteinase 3 but not with neutrophil elastase (Fig. 2E). Extracellular cleavage of hCAP18 by proteinase 3 has previously been documented (8) and verified within this study by the fact that proteinase 3 was the only protease capable of processing the cathelicidin precursor hCAP18 in healthy BAL fluid (Fig. 2E). Thus, within the CF lung setting, hCAP18 is a specific substrate for proteinase 3 and processing to the biologically active molecule LL-37 takes place, accordingly these results do not constitute a rationale for a lack of LL-37 antimicrobial activity.

Exogenous LL-37 is inactivated by cathepsin D and neutrophil elastase in CF BAL fluid

We next assessed the role of proteolytic cleavage in the degradation/inactivation of LL-37 in the CF lung. *Pseudomonas* killing by exogenously added LL-37 was assessed in BAL fluid from CF patients (n = 6) and healthy control individuals (n = 4). Reactions containing 32 μM LL-37 were incubated for 24 h at 37°C and then assayed for antipseudomonal activity (Fig. 3A). LL-37 incubated in healthy BAL fluid reduced the number of colonies by more than 99.99%. In contrast, the antimicrobial activity of CF BAL fluid was not improved upon addition of LL-37 (p = 0.1678). Reactions were subsequently subjected to SDS-PAGE, which demonstrated that the exogenous LL-37 was degraded in BAL fluid samples from CF patients (Fig. 3B).

To investigate proteases responsible for this degradation of LL-37, CF BAL fluid was incubated for 1 h with the protease inhibitors pefabloc, pepstatin A, E-64, or EDTA before addition of LL-37 (Fig. 3C). The proteolytic degradation of LL-37 in CF BAL fluid was inhibited by the serine and acidic protease inhibitors pefabloc and pepstatin A, respectively (Fig. 3C) but not by the metalloprotease inhibitors EDTA or the cysteine protease inhibitor E-64. LL-37 was subsequently incubated with the serine proteases proteinase 3, neutrophil elastase, or cathepsin G or the acidic protease cathepsin D (Fig. 3D). A hierarchy in protease efficiency in LL-37 degradation was observed whereby cathepsin D was the most efficient, followed by neutrophil elastase, while proteinase 3 and cathepsin G proved to be the least efficient at degrading the peptide. CF BAL fluid was analyzed for the presence of cathepsin D by Western blot analyses. Three immunoreactive bands corresponding to the procathespins D, the cathepsin D H chain, and the cathepsin D L chain were detected in CF BAL fluid samples (data not shown). Cathepsin D and neutrophil elastase activities were analyzed in CF BAL fluid with a mean of 0.42 ± 0.09 U/ml and 16.95 ± 4.79 nM detected, respectively. These data suggest that the aspartic endopeptidase cathepsin D is the major component responsible for proteolytic degradation of LL-37 in the CF lung.
followed by neutrophil elastase. However in most of the CF samples evaluated (Fig. 2), LL-37, while inactive, was not proteolytically degraded in vivo suggesting that proteolytic degradation is not the main reason for the inability of LL-37 to act against P. aeruginosa in vivo.

**LL-37 is inactivated and the peptide impervious to proteolytic degradation when bound to GAGs in CF BAL fluid**

Due to the cationic and amphiphatic characteristics of LL-37, we evaluated whether this peptide is bound to anionic molecules found in CF BAL fluid through electrostatic interactions. To break these electrostatic bonds CF BAL fluid was incubated with increasing concentrations of NaCl (0 mM, 150 mM, 300 mM, 450 mM, 600 mM, 750 mM and 900 mM) and samples were subjected to native gel electrophoresis and western blot analysis (Fig. 4A). The results indicate a correlation between increased NaCl concentration and the intensity of an immunoreactive band corresponding to the free molecular form of LL-37. Furthermore, the immunoreactivity against a high m.w. smear decreased when samples were treated with 900 mM NaCl solution (Fig. 4A), most likely representing a reduction in the amount of the complexed form of the peptide. To study whether high NaCl concentrations interfere with the antimicrobial effect of LL-37 we analyzed the anti-*Pseudomonas* effect in a NaCl-rich microenvironment (Fig. 4B). In the absence of NaCl, antimicrobial activity of LL-37 was abolished, most likely due to the random coil structure of the peptide in water (43). The activity gradually increased with increased NaCl concentrations, which supports the antimicrobial amphipathic α-helical conformation of LL-37 (43), and reached maximum activity at 300–450 mM NaCl concentration. At NaCl concentrations as high as 600 mM, LL-37 activities were reduced but the peptide still active.

Previous studies have shown that long linear negatively charged chains of sulfated and nonsulfated disaccharides designated GAGs are increased in CF (44–46) and lung epithelial shedding of GAG proteoglycans can be stimulated by pathogenic virulence factors (47–49). In the present study, we hypothesized that LL-37 interacts with GAGs within the CF lung milieu. After digestion of GAGs with chondroitinase ABC, heparitinase II, and hyaluronidase, samples were subjected to native gel electrophoresis and Western blot analyses (Fig. 4C). An immunoreactive band corresponding to LL-37 was visualized in material belonging to samples that were treated with GAG lyases. Interestingly, samples treated with GAG lyases in the absence of pefabloc and pepstatin A showed no immunoreactivity against LL-37 (Fig. 4C), supporting our previous observation that exogenous uncomplexed LL-37 is susceptible to proteolytic degradation by cathepsin D and neutrophil elastase in the CF lung (Fig. 3). To analyze this further, we pre-expressed synthetic LL-37 to a mixture of GAGs including heparan sulfate, chondroitin sulfate, and hyaluronic acid. The results show that exposure to GAGs inhibited proteolytic degradation by cathepsin D (Fig. 4D). We further hypothesized that the electrostatic interactions between LL-37 and GAGs inhibited the antimicrobial activity of the peptide. Pre-exposure of 7.4 μM LL-37 to GAGs used at a 1:10 (w:w) ratio inhibited bacterial killing (p = 0.0022) (Fig. 4E). Taken together, these data suggest complex formation between GAGs and LL-37 in the CF lung, leading to LL-37 inactivation and microbial persistence.

**Digestion of GAGs accompanies antimicrobial activation in CF sputum**

The importance of the interaction of LL-37 with GAGs within the CF lung was addressed by assessing the ability of sputum to kill bacteria following digestion of GAGs by GAG lyases. CF sputum antimicrobial effect was increased when exposed to GAG lysases (hyaluronidase, heparitinase II, and chondroitinase ABC), with less survival of *S. aureus* (n = 10) (Fig. 5A) and *P. aeruginosa* (n = 9) (Fig. 5B), with bacterial counts reduced on average by 37.9% (p = 0.1333) and >99.9% (>1000-fold), respectively. The additional dividend of treating sputum with GAG lysates in the presence of the protease inhibitors pepstatin A and pefabloc targeted against cathepsin D and neutrophil elastase, which are implicated in LL-37 degradation (Fig. 3), was the enhanced killing effect against *S. aureus*, reducing the CFU count by further 24.5%, resulting in a significant reduction of 62.5% (p = 0.0172, Fig. 5A).

**Nebulized hypertonic saline treatment liberates LL-37 and coincides with enhanced bacterial killing capacity**

Given the demonstrated effect of increasing sodium concentrations on LL-37–GAG complexes (Fig. 4), we next evaluated the effects of nebulized hypertonic saline treatment ex vivo (Fig. 6). Sputum samples were collected from CF individuals pre- and post-treatment with aerosolized hypertonic saline (7% NaCl). Sputum supernatants were subjected to native gel electrophoresis followed by Western blot analysis, and results revealed higher concentrations of uncomplexed LL-37 post treatment (Fig. 6A). Furthermore, antimicrobial analysis of the sputum extracts against *S. aureus* (n = 4) (Fig. 6B) and *P. aeruginosa* (n = 5) (Fig. 6C) demonstrated...
Antimicrobial effect of sputum is significantly ($p = 0.0192$) and $78.3\%$ ($p = 0.0361$), respectively. These findings illustrate that targeting of electrostatic interactions by inhaled hypertonic saline frees cationic antimicrobial peptides such as LL-37 thereby restoring the disabled bactericidal capacity. To demonstrate that LL-37 contributes to the antimicrobial activity of sputum post treatment with hypertonic saline, we analyzed the samples in the presence of heat inactivated LL-37 Ab, an effect not seen in the presence of heat inactivated (hi) LL-37 Ab.

Antimicrobial effect of sputum collected after treatment with nebulized hypertonic saline was partially neutralized when samples were preincubated with LL-37 specific Ab and two tailed Mann-Whitney $U$ test used to calculate $p$ values. Control experiments used heat inactivated (hi) LL-37 Ab.

**Discussion**

Since the initial description of hCAP18/LL-37 up-regulation in BAL of individuals with pulmonary infection (30, 50), only a limited understanding exists as to why LL-37, which illustrates low minimum inhibitory concentration values (0.2 $\mu$M) against $P$. aeruginosa (23) and is present in large quantities in the CF BAL, fails to target microbial infections in individuals with CF. The results of this study go some way toward elucidating this paradox. We demonstrate that anionic GAGs present within the airways bind LL-37 electrostatically, effectively inhibiting antimicrobial activity. Incubation with GAG lyases has a pronounced solubilizing effect, releasing LL-37 and simultaneously improving antimicrobial activity. Similarly, high sodium concentrations either in vitro or in vivo, post nebulization of hypertonic saline to CF individuals, release LL-37 from anionic matrices and improves antimicrobial efficiency, an effect inhibited by inclusion of specific Ab against LL-37. However, this antimicrobial effect is temporary as the CF lung contains proteases such as cathepsin D and neutrophil elastase, which can inactivate the now liberated LL-37. This suggests that a strategy whereby nebulized hypertonic saline augmented with antiproteases may provide optimization of the innate antimicrobial activity of LL-37 in the setting of CF.

**FIGURE 6.** Antimicrobial effect can be induced in sputum of CF patients by inhalation of hypertonic saline. A, Free LL-37 abundance was analyzed by native gel immunoblots in sputum from individuals with CF before and after inhalation of hypertonic saline. The blot presented is representative of four separate experiments. B and C, Sputa isolated from CF subjects pre and post hypertonic saline treatment was incubated with $B$. $S$. aureus ($n = 4$) or C, $P$. aeruginosa ($n = 5$) and CFU determined after 2 h. Two-tailed $p$ values calculated by unpaired $t$ test. D, Antimicrobial effect of sputum collected after treatment with nebulized hypertonic saline was partially neutralized when samples were preincubated with LL-37 specific Ab and two tailed Mann-Whitney $U$ test used to calculate $p$ values. Control experiments used heat inactivated (hi) LL-37 Ab.

The potential antimicrobial effects of LL-37 are manifold and can be expected to be of major importance in the CF lung. LL-37 exhibits antimicrobial activity against a wide range of microbes including viruses (51), fungus (52), and both Gram-positive and Gram-negative bacteria, such as $S$. aureus and $P$. aeruginosa (23, 51), important pathogens that colonize the lungs of individuals with CF. LL-37 is activated efficiently in the CF lung most notably by proteinase 3 and is present in large quantities in this antimicrobial active form. In normal circumstances, this should mean increased antimicrobial killing of a wide range of microorganisms. In contrast and as demonstrated in this study, LL-37 is inactivated in two different fashions in CF, either by adsorption onto GAGs, which although protective against proteolysis renders the LL-37 inactive, or by proteolysis of the unconjugated LL-37 by cathepsin D and neutrophil elastase. This processing-degradation cycle by PR-3 and cathepsin D may be unique to the lung as other studies have shown different proteases involved in these processes in other organ systems (12, 53).

With regards to GAGs, these glycomolecules are a major component of the extracellular matrix, distributed in the subepithelial tissue and bronchial walls (54). Increased concentrations of GAGs have been found in BAL fluid from children with CF (55) and secretion of heparan sulfate, chondroitin sulfate, and hyaluronic acid is markedly increased in bronchial cells and CF tissues (44–46, 56–58). In addition, various pathogenic bacteria including $P$. aeruginosa cause release of GAGs from epithelial surfaces (47, 48) and it has been shown that chondroitinase ABC has an important effect on the viscosity of CF sputa, suggesting a major role for GAGs in viscoelasticity of CF purulent sputa (56). GAGs can impact upon the immune response on a number of levels and have been shown to interact with proteases (35–37), chemokines (35), cytokines (35), and antimicrobial peptides (38, 39). In the present study, another therapy illustrating the potential to disrupt molecular interactions imposed by GAGs is investigated, namely nebulized hypertonic saline. It has previously been documented that hypertonic saline treatment of individuals with CF is associated with an improvement in lung function, respiratory symptoms, and...
with marked benefits with respect to exacerbations (59, 60). In addition to osmotically restoring the liquid layer lining the airways (61), our studies indicate that hypertonic saline may improve lung function by disrupting interactions between glycosaminoglycan polysaccharides and antimicrobial peptides. By subjecting CF BAL fluid and sputum samples to GAG lyses or to increasing concentrations of NaCl or by treating CF individuals with nebulized hypertonic saline we can effect release of LL-37 from glycosaminoglycans and subsequent activation. In addition, hypertonic saline could potentially cause release of LL-37 from other anionic molecules such as DNA and mucins. These data argue a parallel between Na\(^+\) concentration and protein displacement and mirrors a functional role for monovalent K\(^+\) in affecting the release of antimicrobial proteins from anionic matrix within the confines of the neutrophil phagocytic vacuole (62).

The microenvironment, including ionic strength, has considerable influence on the activity of various antimicrobial peptides (63) and increased NaCl concentration is known to enhance the antimicrobial activity of LL-37 (23, 43) but reduce the effect of defensins (63). Furthermore, we have tested the antimicrobial activities of LL-37 against *P. aeruginosa* at various NaCl concentrations (Fig. 4B). Our data illustrates synergistic effects between NaCl concentration and LL-37 antimicrobial activity that peak between 300–450 mM. Based on this we conclude that the antimicrobial activity of LL-37 is not suppressed by NaCl concentrations needed to solubilize LL-37 (Fig. 4A).

In addition to its antimicrobial effect, LL-37 has been shown to exhibit strong immunomodulatory effects, including chemotaxis (13–15) and induction of chemokine responses in monocytes and airway epithelia (19, 21). The immunomodulatory effects of LL-37 bound to anionic matrices such as GAGs have not yet been studied. However, should nebulized hypertonic saline induce a proinflammatory response due to release of LL-37, this effect is likely to be suppressed by degradation of LL-37 by cathepsin D and neutrophil elastase. Such degradation could possibly give rise to truncated peptidic fragments of LL-37 that may display altered inflammatory and antimicrobial behavior as has previously been demonstrated for peptic fragments of LL-37 in the skin (22). In contrast, should LL-37 be proinflammatory when bound to anionic matrices the exposure of hypertonic saline may be of major benefit as it simply facilitates the degradation of the peptide. Furthermore, the pathologic consequence of molecular adsorption to anionic matrices in the airways of individuals with CF is not restricted to those involving LL-37 and not always with inhibitory consequences. For example, IL-8 interacts with heparan sulfate proteoglycans facilitating binding of the cytokine to its specific receptor, protecting it from proteolysis and prolonging its activities (46). Further work is necessary to evaluate the effects of hypertonic saline or GAG lyses on other highly charged antimicrobial peptides of the lung, such as SLPI, lactoferrin, and lysozyme.

Once released from GAGs, LL-37 is now exposed to the intensely proteolytic milieu of the CF lung. Previously, we have shown that cathepsins B, L, and S inactivate the antiprotease SLPI (25) and \(\beta\)-defensins 2 and 3 (26) by proteolytic degradation. In this study, we observed the antimicrobial activity of synthetic LL-37 eliminated by cathepsin D and neutrophil elastase. Neutrophil elastase is well characterized in the CF lung and has been implicated in many deleterious effects on host defense and in causing direct tissue damage. Cathepsin D is less well characterized and not previously shown to be responsible for the degradation of LL-37 in the setting of CF. It is induced by *P. aeruginosa* (64) in CF individuals (65) and is up-regulated in the lung tissue of bleomycin-treated rats. It is also found in the lungs of patients with idiopathic pulmonary fibrosis and is expressed by alveolar macrophages post cigarette smoke exposure (66–68). In this study, we ascribe the proteolytic degradation of LL-37 to cathepsin D. As cathepsin D is one of the lysosomal enzymes that requires a more acidic pH to be proteolytically active it is possible that cathepsin D cleaves substrates such as LL-37 with greater efficiency under the conditions of low pH prevailing within the CF lung milieu (69).

In summary, the data obtained by this study elucidate a novel mechanism by which LL-37 although present in large quantities is essentially inactive against microbes in the CF lung. This is due mainly to complex formation with GAGs. GAG lyses and hypertonic saline can break these complexes, liberating LL-37 and restoring a window of antimicrobial opportunity which however can be closed by proteolytic inactivation of liberated LL-37 by cathepsin D and neutrophil elastase. The fact that a currently used and readily available therapy such as hypertonic saline can, even temporarily, restore the antimicrobial effects of LL-37 in the CF lung is potentially important particularly if this effect can be prolonged by the judicious use of anti-proteases.

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### Disclosures
The authors have no financial conflict of interest.

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