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Inactivation of Human β-Defensins 2 and 3 by Elastolytic Cathepsins[1]

Clifford C. Taggart,[2]* Catherine M. Greene,* Stephen G. Smith,[†] Rodney L. Levine,[‡] Paul B. McCray, Jr.,[§] Shane O'Neill,* and Noel G. McElvaney*

β-Defensins are antimicrobial peptides that contribute to the innate immune responses of eukaryotes. At least three defensins, human β-defensins 1, 2, and 3 (HBD-1, -2, and -3), are produced by epithelial cells lining the respiratory tract and are active toward Gram-positive (HBD-3) and Gram-negative (HBD-1, -2, and -3) bacteria. It has been postulated that the antimicrobial activity of defensins is compromised by changes in airway surface liquid composition in lungs of patients with cystic fibrosis (CF), therefore contributing to the bacterial colonization of the lung by *Pseudomonas* and other bacteria in CF. In this report we demonstrate that HBD-2 and HBD-3 are susceptible to degradation and inactivation by the cysteine proteases cathepsins B, L, and S. In addition, we show that all three cathepsins are present and active in CF bronchoalveolar lavage. Incubation of HBD-2 and -3 with CF bronchoalveolar lavage leads to their degradation, which can be completely (HBD-2) or partially (HBD-3) inhibited by a cathepsin inhibitor. These results suggest that β-defensins are susceptible to degradation and inactivation by host proteases, which may be important in the regulation of β-defensin activity. In chronic lung diseases associated with infection, overexpression of cathepsins may lead to increased degradation of HBD-2 and -3, thereby favoring bacterial infection and colonization. *The Journal of Immunology,* 2003, 170: 931–937.

D efensins are antimicrobial peptides that possess a high positive charge and three intramolecular disulfide bridges, the main subfamilies of which are the α- and β-defensins (1). Defensins possess a wide range of microbicidal activity toward Gram-positive and Gram-negative bacteria as well as against some fungi and enveloped viruses (2). The α-defensins are produced by intestinal Paneth cells and neutrophils; in the latter they are stored as mature peptides in the azurophilic granules (3). Four have been identified in neutrophils, human neutrophil peptides-1, -2, -3, and -4 (HNP-1 to -4),[3] and two, HD-5 and HD-6, have been identified in the secretory granules of Paneth cells. (4). β-Defensins are expressed by epithelial cells of the kidneys, urogenital tract, respiratory epithelium, and other mucosal sites. To date four β-defensins of epithelial origin have been identified, human β-defensins 1 (HBD-1), -2, -3, and -4 (5–8). In addition, recent evidence indicates that there is a much larger family of β-defensin genes in humans (9).

HBD-1 is constitutively expressed and is not up-regulated by bacterial or cytokine stimuli (10, 11). However, it has been shown that HBD-1 can be up-regulated by IFN-γ and LPS in monocyte-derived-macrophages (12). In contrast, HBD-2 and -3 expression is induced by a variety of proinflammatory stimuli, such as LPS, IL-1β, and TNF-α (13–15), with HBD-3 being particularly strongly up-regulated by IFN-γ (16). Although all four HBDs share some similar bactericidal activities, differences exist. HBD-3 has microbicidal activity toward *Staphylococcus aureus*, whereas HBD-1 and -2 have little or no activity toward this microorganism (7).

Defensins are part of the innate immune system, which keeps the airways sterile and free of bacteria. However, in cystic fibrosis (CF) the pulmonary mucosal defenses are impaired, leading to bacterial colonization, initially by *Haemophilus influenzae* and *S. aureus* and later on by *Pseudomonas aeruginosa* (17). This bacterial colonization of the lung is chronic and is associated with increased morbidity and mortality in CF. Changes in β-defensin activity, specifically HBD-1, have been linked to the lung pathogenesis of CF. It has been proposed that the high salt concentration present in the airway surface liquid (ASL) of the lung in CF inhibits the microbicidal activity of HBD-1 (18). Indeed, CF airway epithelia fail to kill apically applied *P. aeruginosa*, contrasting with the bacterial properties of normal airway epithelia (19). It has since been demonstrated that a number of antimicrobial proteins and peptides present in respiratory tract secretions are salt-sensitive, and it has been theorized that a high salt concentration in the CF airways may compromise the microbicidal activity of many innate immune factors (20).

Proteases present in CF ASL have previously been demonstrated to cleave and inactivate antimicrobial proteins. Lactoferrin and transferrin are degraded by neutrophil elastase and *Pseudomonas* elastase, and *Pseudomonas* elastase cleaves lysozyme (21, 22). However, due to the small size of members of the defensin family as well as their positive charge and the constrained structure adopted as a result of disulfide bridging, it has been assumed that these peptides are resistant to proteolysis. We have previously

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[3] Abbreviations used in this paper: HNP, human neutrophil peptide; ASL, airway surface liquid; BAL, bronchoalveolar lavage; CF, cystic fibrosis; HBD, human β-defensin; ILD, interstitial lung disease; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; SLPI, secretory leukoprotease inhibitor.

demonstrated that another antiprotease/antimicrobial protein, secretory leukoprotease inhibitor (SLPI), a small m.w. protein, with a similar charge and function as the defensins, is susceptible to cleavage and antiprotease inactivation by members of the elastolytic cathepsin family of proteases (cathepsins B, L, and S) (23). Cathepsins are cysteine proteases produced predominantly by macrophages and are involved in matrix remodeling and Ag processing (24). Elevated levels of active cathepsin L have been shown in the bronchoalveolar lavage (BAL) of smokers and individuals with emphysema due to the increased numbers of macrophages present, and they are thought to play an important role in the lung destruction observed in the emphysematous lung (23, 25). The observation of SLPI proteolysis by cathepsins B, L, and S has raised the question of whether members of the defensin family are also susceptible to proteolytic cleavage by the cathepsins. We present evidence that HBD-2 and -3 are degraded by cathepsins B, L, and S, which affect the microbicidal activity of both β-defensins toward *P. aeruginosa*. In addition, we show that members of the elastolytic cathepsin family are present and active in CF BAL. Incubation of recombinant HBD-2 and -3 with CF BAL resulted in their degradation, which was completely (HBD-2) or partially (HBD-3) inhibited using a cathepsin inhibitor. These observations provide evidence that small antimicrobial peptides are susceptible to inactivation by cathepsins. The inactivation of β-defensins by proteases may contribute to bacterial infection and colonization of diseased airways.

Materials and Methods

Reagents

HBD-2 and -3 were recombinantly produced in *Escherichia coli* using a proprietary fusion protein expression system from PeproTech (Rocky Hill, NJ). Following purification, both β-defensins were refolded, and the disulfide bonds were oxidized. Peptide purity was confirmed by mass spectrometry and capillary electrophoresis. Goat polyclonal anti-HBD-2 antisera were raised by immunizing animals with keyhole limpet hemocyanin-conjugated peptide. Rabbit anti-HBD-3 antisera were obtained from Abcam (Cambridge, U.K.). Purified cathepsins B, L, and S and Ab to cathepsin B were purchased from CN-Biosciences (Nottingham, U.K.). Neutrophil elastase was purchased from Elastin Products (Owensville, MO). Abs for cathepsins L and S were obtained from Sigma-Aldrich (Poole, U.K.) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively.

Cathepsin incubation with HBD-2 and HBD-3

HBD-2 (100 ng) and HBD-3 were incubated with cathepsins B, L, and S (100 ng), either separately or all three cathepsins combined in 0.1 M sodium acetate, 0.15 M NaCl, 1 mM EDTA, and 1 mM DTT, pH 5.5. Incubations were conducted for 15 and 60 min (HBD-2) or for 5 and 30 min (HBD-3) at 37°C. Samples were boiled for 5 min in SDS-PAGE sample treatment buffer and separated on a 17.5% Tricine/SDS-PAGE.

Following electrophoresis, the gel was blotted onto nitrocellulose. The blot was incubated in blocking buffer (5% dried skimmed milk and 0.2% gelatin in PBS-Tween (PBST)) for 1 h, followed by incubation in goat anti-HBD-2 IgG (1/500) or rabbit anti-HBD-3 IgG (1/500) overnight at 4°C. The blot was then washed in PBST/0.5% dried skimmed milk/0.2% gelatin, followed by incubation with HRP-labeled anti-goat secondary Ab (Sigma-Aldrich; 1/10,000) or anti-rabbit secondary Ab (New England Biolabs; 1/3,000). The blot was developed using chemiluminescent substrate specific for HRP (LumiGlo; New England Biolabs, Beverly, MA).

Mass spectrometric analysis of cathepsin-HBD-2/HBD-3 incubations

Incubations of HBD-2 and HBD-3 with cathepsin were set up as described above, but the reactions were terminated by being made 6 M in guanidine HCl with the addition of 1 mg/μl solid guanidine HCl at various time points (4, 12, and 24 h for HBD-2 and 30, 60, and 120 min for HBD-3). The samples were lyophilized and then reconstituted with 56 μl of 500 mM sodium phosphate, pH 7.4, and 3 mM [2-carboxyethyl]phosphine at a final concentration of 1 mM to ensure the reduction of all disulfide bonds. Samples were incubated for 15 min at 37°C to ensure the reduction of disulfide

bridges. Twenty-three microliters of 10% trifluoroacetic acid was added to each sample to bring the pH to 2–3, and the samples were analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) spectrometry using a Hewlett-Packard (model G2025A) instrument with α-cyano-4-hydroxycinnamic acid (Hewlett-Packard, Palo Alto, CA) as the matrix. The sample and matrix (0.4 μl each) were mixed on the target and dried under vacuum. The instrument was calibrated with an external standard mixture of peptides (Agilent Technologies, Palo Alto, CA).

Radial diffusion assay

Various concentrations of cathepsins B, L, and S (50–1000 ng) were incubated with a fixed amount of HBD-2 and -3 (1 μg) as outlined above (24 h at 37°C), and the reactions were stopped with the cathepsin inhibitor, Z-Phe-Ala-CHN$_2$ (50 μM). The samples were then tested for microbicidal activity according to the method of Lehrer et al. (26). Following incubation, the samples were lyophilized and reconstituted in 5 μl of 0.01% acetic acid/0.1% human serum albumin. An exponential phase culture of PAO1 was prepared by diluting an overnight culture 1/100 in trypticase soy broth until an A_{600} of between 0.1 and 0.4 was reached. The cells were washed in cold sterile 10 mM sodium phosphate and resuspended at 4×10^6 CFU/ml of molten agar (10 mM sodium phosphate, 1% trypticase soy broth, and 1% agarose), poured onto sterile petri dishes, and allowed to set. Three-micrometer plugs were punched out of the gel and removed with a sterile tip, into which the 5-μl sample incubations and HBD-2 standard were pipetted. The plates were incubated at 37°C for 3 h, after which each plate was overlayed with more agar (8 ml of molten agar plus 2 ml of 5% sterile agarose gel). As soon as the gel solidified, the plates were inverted and placed in an incubator at 37°C overnight. The plate was stained (Coomassie brilliant blue R-250/formaldehyde (37%)/methanol/deionized H$_2$O), and zones of clearance around each 3-mm plug were estimated using a digital camera. Results were plotted on a semilog graph. The inhibition of PAO1 by HBD-2 or -3 standard was given the arbitrary value of 100%, and the inhibition of PAO1 by cathepsin/HBD-2 or -3 samples was compared with that by the HBD-2 or -3 standard.

Presence of cathepsins B, L, and S in CF BAL

BAL fluid was derived from BAL according to standardized conventional guidelines (27) and as approved by the Beaumont Hospital ethics committee. Cathepsin B activity was determined in 100-μl samples of CF BAL using the substrate Z-Arg-Arg-AMC (0.1 mM). Cathepsin L and S activities were determined using the Z-Phe-Arg-AMC substrate in the presence of the cathepsin B inhibitor CA-074 (10 μg/ml). The reaction buffer used for cathepsin B activity estimation was 0.2 M sodium phosphate, 1 mM EDTA, 1 mM DTT, 1 μM pepstatin, and 2 mM Pefabloc, pH 5.5. The reaction buffer for cathepsin L activity was 0.1 M sodium acetate, 1 mM EDTA, 1 mM DTT, 1 μM pepstatin, and 2 mM Pefabloc, pH 5.5. The same buffer was used for cathepsin S activity measurement, except that the pH was set at 7.5. The BAL samples were incubated with substrate for 60 min at 37°C, and fluorescence (substrate turnover) was determined by excitation at 355 nm and emission at 460 nm.

Antigenic analysis of cathepsins B, L, and S was conducted by electrophoresing 10-μl samples of CF BAL, followed by Western transfer and overnight incubation with Abs specific for cathepsin B (1/4000), cathepsin L (1/200) and cathepsin S (1/1000) in 5% dried skimmed milk/PBST. Detection was achieved using appropriate HRP-labeled secondary Abs.

HBD-2 and -3 cleavage activity in CF BAL

Degradation of HBD-2 or -3 by CF BAL was investigated by incubating CF BAL (15 μl) with HBD-2 (20 ng) or HBD-3 (50 ng) in 0.1 M sodium phosphate, 1 mM EDTA, and 1 mM DTT, pH 5.5, for 24 h at 37°C, followed by Western blot analysis for both β-defensins. The effects of various protease inhibitors on HBD-2 or -3 degradation were also examined by incubating CF BAL with either defensin in the presence of the serine protease inhibitor, Pefabloc (2 mM); the *Pseudomonas* elastase inhibitor, phosphoramidon (0.5 mM); and the cathepsin inhibitor, Z-Phe-Ala-CHN$_2$ (50 μM).

Results

Cathepsin incubation with HBD-2 and -3

Equal concentrations of cathepsin and defensin were incubated over short time periods to determine 1) the ability of cathepsins to degrade HBD-2 and -3 over a physiologically relevant timeframe and 2) the relative potency of each cathepsin separately and in combination. Incubation of HBD-2 with cathepsins for 15 min resulted in very little degradation of HBD-2 by each cathepsin

(Fig. 1*a*, *lanes 2–4*). However, all three cathepsins incubated together with HBD-2 resulted in significant degradation of HBD-2 (Fig. 1*a*, *lane 5*), suggesting that the cathepsins act synergistically to degrade HBD-2. At the later time point of 60 min, cathepsins B (Fig, 1*b*, *lane 2*) and S (Fig. 1*b*, *lane 4*) had significantly (up to 50%) degraded HBD-2, whereas cathepsin L had almost completely degraded HBD-2 (Fig. 1*b*, *lane 3*), indicating that cathepsin L is the most active cathepsin toward HBD-2 over the 60-min period. Once again, the combined action of all three cathepsins had completely degraded HBD-2 (Fig. 1*b*, *lane 5*).

Incubation of HBD-3 with each cathepsin resulted in more rapid degradation than that seen with HBD-2. After 5-min incubation, cathepsin L had significantly degraded HBD-3 (Fig. 1*c*, *lane 3*). Once again, all three cathepsins acted synergistically to completely degrade HBD-3 (Fig. 1*c*, *lane 5*). At the later time point of 30 min, cathepsins L and S had completely degraded HBD-3 (Fig. 1*d*, *lanes 3* and *4*) with almost complete degradation by cathepsin B (Fig. 1*d*, *lane 2*).

Mass spectrometry of cathepsin-HBD-2 reactions

Incubation of HBD-2 with cathepsin S for 4 h resulted in the generation of two main species (Fig. 2*a*, peaks 1 and 2). The calculated mass of peak 1 was 1003.3 Da, identifying it as HBD-2 residues 1–10 (expected mass, 1002.19 Da). Likewise, the calculated mass of peak 2 was 3351.1 Da, identifying it as HBD-2 residues 11–41 (expected mass, 3350.06 Da). The other lesser peak present in the chromatogram (peak 3) had a mass of 4334.0 Da, identifying it as intact HBD-2 residues 1–41 (expected mass,

FIGURE 1. Time-course incubation of HBD-2 and -3 with cathepsins B, L, and S. HBD-2 (100 ng) was incubated with equal concentrations of cathepsin B (*lane 2*), cathepsin L (*lane 3*), cathepsin S (*lane 4*), or cathepsins B, L, and S (*lane 5*) for 15 min (*a*) and 60 min (*b*) at 37°C. HBD-3 (100 ng) was incubated with equal concentrations of cathepsin B (*lane 2*), cathepsin L (*lane 3*), cathepsin S (*lane 4*), or cathepsin B, L, and S (*lane 5*) for 5 min (*c*) and 30 min (*d*) at 37°C. Samples were electrophoresed on a 17.5% Tricine/SDS-PAGE, followed by Western analysis with goat anti-HBD-2 or rabbit anti-HBD-3 Ab.

4334.24 Da). This result indicated that cathepsin S cleaved HBD-2 at the $Lys^{10}-Ser^{11}$ bond. Incubation of HBD-2 with cathepsins B and L did not generate interpretable fragments, and therefore, a primary cleavage site could not be determined for these cathepsins. This was most likely due to cleavage of HBD-2 by cathepsins B and L, followed by immediate degradation of the cleavage products generated.

Incubation of HBD-3 with cathepsin S for 120 min resulted in the generation of two main species (Fig. 2*b*, peaks 2 and 3) and a less significant third species (Fig. 2*b*, peak 1). The calculated mass of peak 2 species was 4419 Da, identifying it as residues 8–45 (expected mass, 4421.4 Da). Peak 3 had an observed mass of 4662.6 Da, identifying it as residues 6–45 (expected mass, 4662.6). The third peak (peak 1) had an observed mass of 2795.3 Da, identifying it as residues 22–45 (observed mass, 2797 Da). The intact HBD-3 peptide was found to have a mass of 5161.3 Da (data not shown; observed mass, 5161.2 Da). This result indicated that cleavage of HBD-3 occurred at the Thr^5-Leu^6 and Gln^7-Lys^8 bonds, with trimming back of the peptide to generate the 22–45 HBD-3 fragment. Cathepsin L incubation with HBD-3 also resulted in cleavage of the Gln^7-Lys^8 bond, with subsequent trimming to generate the 22–45 HBD-3 fragment (data not shown). As with HBD-2, the cathepsin B cleavage site of HBD-3 could not be accurately determined, although generation of the 22–45 fragment was detected. It is possible that cleavage occurred earlier at the 7–8 bond, but despite carrying out the cathepsin B-HBD3 incubation at earlier time points, no other cleavage fragments were identified, possibly due to their rapid degradation.

Radial diffusion assays

Due to the potent microbicidal activity of HBD-2 and HBD-3, we wished to investigate the effect of cathepsin cleavage on the antimicrobial activity of both defensins and chose as the candidate microorganism, *P. aeruginosa* (PAO1 strain) toward which HBD-2 and -3 had previously been demonstrated to have activity (7, 20). Once again, HBD-2 and -3 were incubated with cathepsins B, L, and S, and degradation was confirmed by Western analysis (not shown). Incubation of cathepsin (0.5 or 1 μg) with HBD-2 (1 μg) revealed that the percentage of microbicidal activity of HBD-2 toward PAO1 was dramatically decreased, with <5% activity remaining following incubation with 1 μg of cathepsin B, L, or S compared with HBD-2 standard (Fig. 3*a*).

Incubation of HBD-3 (1 μg) with cathepsin required less of each cathepsin (250 ng) to completely diminish the microbicidal activity of HBD-3 to <5% (Fig. 3*b*). Even smaller concentrations of each cathepsin (50 ng) partially decreased the microbicidal activity of HBD-3 (1 μg; Fig. 3*b*).

Presence of cathepsin in CF BAL

To confirm that cathepsins B, L, and S are present in CF BAL, we incubated 10 CF BAL samples with synthetic substrates specific for cathepsin activity. These assays revealed the presence of cathepsin B, L, and S activity in all 10 CF BAL samples (Fig. 4*a*). We have previously shown that there is very little cathepsin B or L activity in healthy control BAL samples (23). Due to the difficulty in differentiating cathepsin L and S activity (they both turn over the Z-Phe-Arg-AMC substrate at pH 5.5), we examined CF BAL for antigenic cathepsins B, L, and S. Using Abs specific for all three cathepsins, we confirmed the presence of mature forms of cathepsin B, L, and S in CF BAL (Fig. 4*b*). From densitometric analysis of Western blots there was between 500-1000 ng of each cathepsin/ml CF BAL. Analysis of antigenic cathepsins B, L, and S in the 10 control BAL samples revealed that mature cathepsins
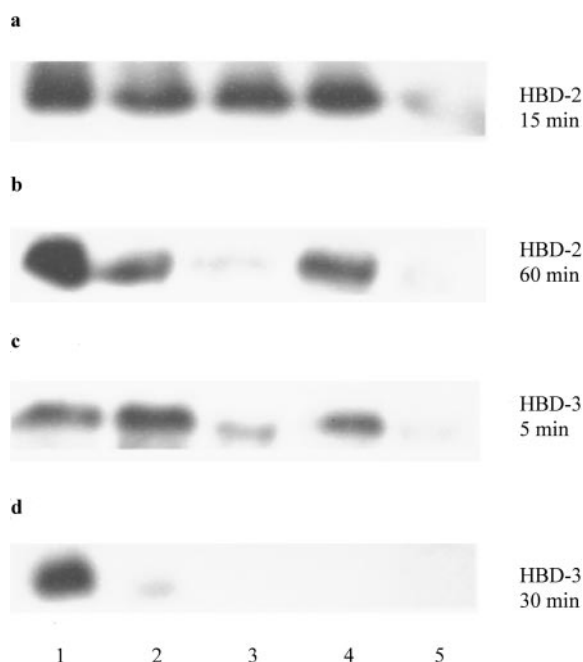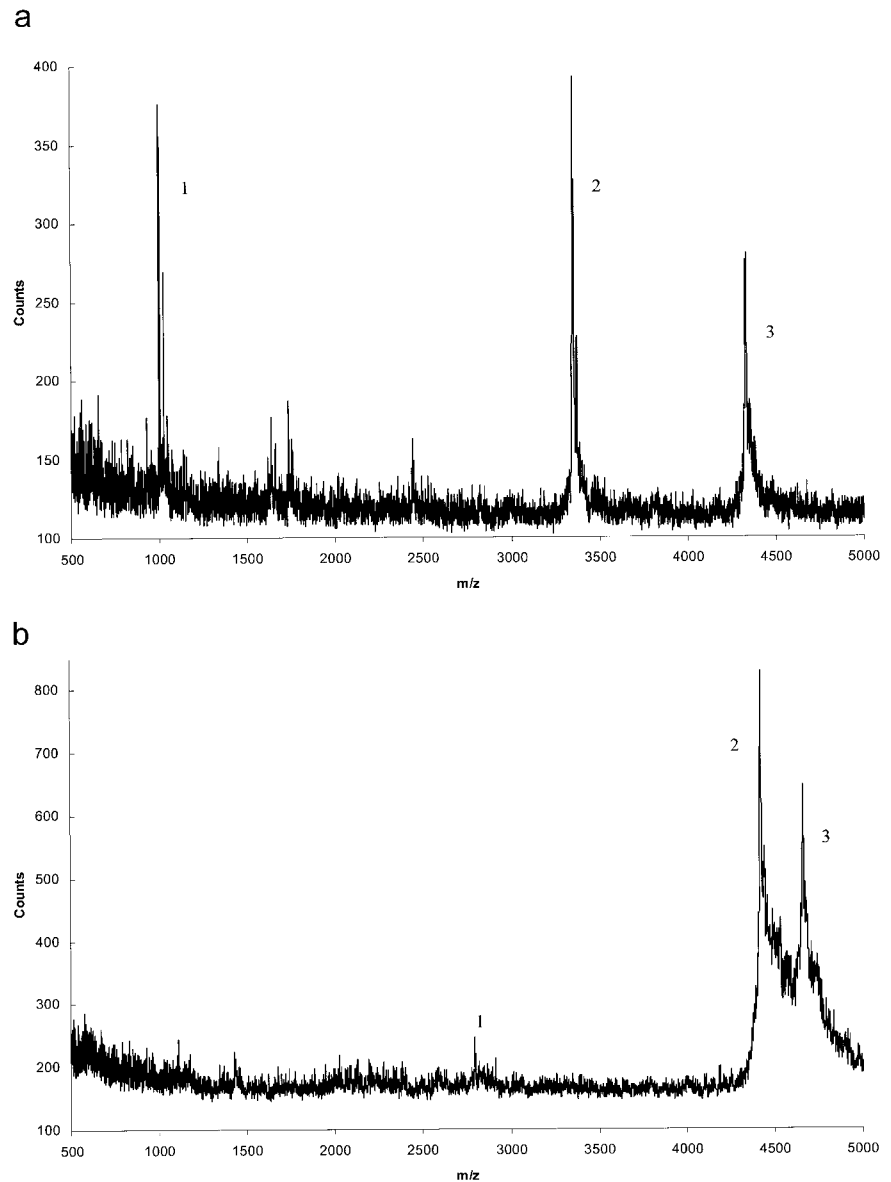
FIGURE 2. MALDI-TOF analysis of cathepsin S-HBD-2/HBD-3 incubation. *a*, HBD-2 (1 μg) was incubated with cathepsin S (1 μg) for 4 h at 37°C, and the HBD-2 fragments were analyzed by MALDI-TOF. The HBD-2 fragments generated allowed identification of the cathepsin S cleavage site in HBD-2. Peak 1, HBD-2 residues 1–10; peak 2, HBD-2 residues 11–41; peak 3, intact HBD-2. *b*, HBD-3 (1 μg) was incubated with cathepsin S (1 μg) for 120 min at 37°C, and the HBD-3 fragments were analyzed by MALDI-TOF. The HBD-3 fragments generated allowed identification of the cathepsin S cleavage site in HBD-2. Peak 1, HBD-2 residues 22–45; peak 2, HBD-2 residues 8–45; peak 3, HBD-2 residues 6–45.

B and L were present in two and one BAL samples, respectively, and cathepsin S could not be detected in control BAL (data not shown).

Effects of protease inhibitors on CF BAL degradation of HBD-2 and -3

We examined the effect of CF BAL on the degradation of HBD-2 and -3 added to CF BAL samples in the presence of protease inhibitors. Western analysis demonstrated that CF BAL could degrade HBD-2 peptide (Fig. 5*a*, *lane 2*). This degradation could not be prevented by a serine protease inhibitor or a *Pseudomonas* elastase inhibitor (Fig. 5, *lanes 3* and *4*, respectively). However, degradation of HBD-2 by CF BAL was inhibited using a cathepsin inhibitor (Fig. 5*a*, *lane 5*). In the case of HBD-3, CF BAL could degrade HBD-3 (Fig. 5*b*, *lane 2*). This degradation could not be prevented by a *Pseudomonas* elastase inhibitor (Fig. 5*b*, *lane 4*), but was partially inhibited by a serine protease inhibitor (Fig. 5*b*, *lane 3*) and a cathepsin inhibitor (Fig. 5*b*, *lane 5*). This result suggests that as well as cathepsins, other serine proteases, such as neutrophil elastase, cathepsin G, and proteinase 3, may also degrade HBD-3.

Discussion

HBD-2 and -3 are inducible defensins with broad-spectrum antimicrobial activity toward Gram-negative bacteria and, in the case of HBD-3, significant activity toward Gram-positive *S. aureus* bacteria as well. It has previously been demonstrated that the microbicidal activity of defensins is inactivated in high ionic strength solutions. One of the theories advanced to explain *Pseudomonas* colonization in the lungs of individuals with CF is that increased NaCl levels present in the CF ASL (due to the cystic fibrosis transmembrane conductance regulator mutation) inactivate defensins and other antimicrobials (18). In this report we show that the microbicidal effects of HBD-2 and -3 are inactivated following proteolytic degradation by elastolytic cathepsins. Incubation of HBD-2 and -3 with CF BAL also resulted in degradation that could be completely inhibited by a cathepsin inhibitor (HBD-2) or partially inhibited (HBD-3).

Four β-defensins have been isolated to date and have a similar broad range activity against Gram-negative bacteria, with the notable exception that HBD-3 is bactericidal toward *S. aureus*, whereas HBD-2 is only bacteriostatic against *S. aureus* (7). However, both
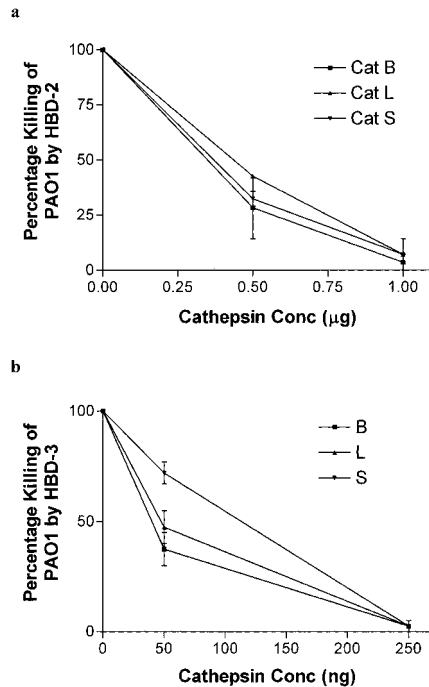
FIGURE 3. Effect of cathepsins on the antimicrobial activity of HBD-2 and HBD-3. *a*, HBD-2 (1 μg) was incubated with cathepsin B, L, and S (0.5 and 1 μg) for 24 h at 37°C. *b*, HBD-3 (1 μg) was incubated with cathepsins B, L, and S (50 and 250 ng) for 24 h at 37°C. The samples were lyophilized and reconstituted in 0.01% acetic acid/0/1% human serum albumin and added to wells in agar impregnated with *P. aeruginosa* (PAO1). After 24-h incubation at 37°C, the agar was stained, and the zones of clearance around each well were determined. The zones of clearance for HBD-2 and -3 standards were given the arbitrary value of 100%, and the cathepsin-incubated HBD-2 and -3 samples were ascribed percentage values of inhibition compared with HBD-2 and -3 alone.

FIGURE 4. Presence of cathepsins B, L, and S in CF BAL. *a*, Cathepsin activity was determined in 10 CF BAL (CF) and 10 healthy control (N) BAL samples using the Z-Arg-Arg-AMC and Z-Phe-Arg-AMC substrates. Fluorescence was determined by excitation at 355 nm and emission at 460 nm, and results are plotted as fluorescence units per minute per milliliter of BAL. *b*, Cathepsins B, L, and S in CF BAL were analyzed by Western blot using Abs specific for each cathepsin. Blots of cathepsin B, L, and S from three representative CF BAL samples are shown.

HBD-2 and -3 have potent activity against *P. aeruginosa*, the principal colonizing microorganism of the CF lung (7, 28). Similar to HBD-1, HBD-2 is salt sensitive and therefore could lose activity toward *P. aeruginosa* and other organisms in the CF lung. HBD-3 has been shown to retain its microbicidal activity against *S. aureus* at physiological salt concentrations (7). It was previously demonstrated that HBD-2 is found at elevated levels in CF BAL compared with BAL from healthy individuals (28). However, comparison of HBD-2 levels in CF BAL with BAL from individuals with interstitial lung disease (ILD) reveals that HBD-2 levels are lower in CF BAL (0.1–10 ng/ml in CF compared with 10–100 ng/ml in ILD). This decrease in HBD-2 in CF may reflect variations in inducible stimuli such as LPS and IL-1β in the CF lung compared with the ILD lung or modifications that occur in the epithelium as the disease progresses. However, HBD-2 activity in CF could be further compromised by degradation of the peptide by cathepsins present in CF BAL. The resultant loss of HBD-2 activity may be partly responsible for the decreased antibacterial activity in the CF lung and could predispose to bacterial colonization. HBD-3 levels in normal or diseased BAL have not been reported, possibly due to the recent discovery of HBD-3. However, HBD-3 expression is induced by treatment of primary tracheal epithelial cells with TNF-α and *P. aeruginosa*, indicating that this defensin may be present on the respiratory tract during inflammation.

In addition to their antimicrobial activity, β-defensins exhibit chemoattractant properties for memory T cells and immature dendritic cells via the chemokine receptor CCR6 (29). Such contrasting activities has stimulated an interest in structural analysis of
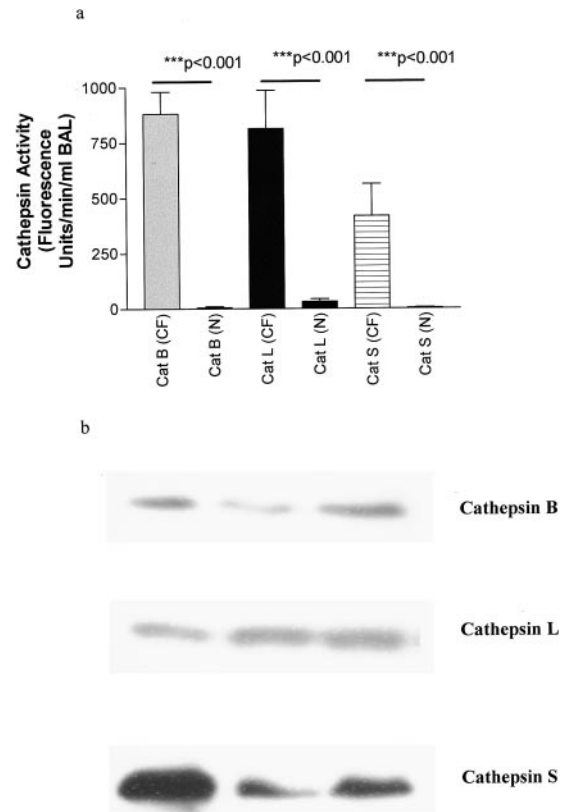
HBD-1 to -3 to identify the residue(s) responsible for the microbicidal and chemoattractant properties of β-defensins. To this end, nuclear magnetic resonance- and x-ray-derived structures of all three β-defensins were determined (30–33).

FIGURE 5. Effect of CF BAL on HBD-2 and HBD-3 degradation. HBD-2 (20 ng; *a*) and HBD-3 (50 ng; *b*) were incubated with 15 μl of CF BAL for 24 h at 37°C alone (*lane 2*) or in combination with Pefabloc (2 mM; *lane 3*), plus phosphoramidon (0.5 mM; *lane 4*), or plus Z-Phe-Ala-CHN$_2$ (*lane 5*). *a*, *Lane 1*, Twenty nanograms of HBD-2 standard; *b*, *lane 1*, 50 ng HBD-3 standard. Samples were run on 17.5% Tricine/SDS-PAGE, followed by Western analysis with goat anti-HBD-2 IgG or rabbit anti-HBD-3 IgG.

We demonstrated that the HBD-2 cleavage site for cathepsin S occurs at the Lys^{10}-Ser^{11} bond. Lys^{10} is one of the residues present in the α-helical loop described to occur between residues Pro^5-Lys^{10} at the N-terminus of the HBD-2 protein (30, 31). Crystal structures of HBD-2 indicate the existence of an octameric arrangement of HBD-2 that may be important in binding to and disrupting the cell membrane surface of bacteria (30). Lys^{10} is one of the residues involved in the contact between HBD-2 monomers and contributes to the formation of an HBD-2 octamer. From the nuclear magnetic resonance and crystal structural information, HBD-2 has been proposed to form an octameric structure, postulated to bind to the bacterial cell surface and increase cell membrane permeabilization. Therefore, cleavage of the Lys^{10}-Ser^{11} bond may destabilize the α-helical loop of HBD-2, leading to disruption of the octameric structure of HBD-2 required for bacterial cell membrane disruption. This may help explain the inhibition of microbicidal function toward *Pseudomonas* that we observed following incubation of HBD-2 with cathepsin. Unlike HBD-1 and -2, HBD-3 has been demonstrated to form a dimer in solution, but has a tertiary structure similar to that of HBD-1 and -2 (33). However, whereas the cathepsin cleavage site in HBD-2 resides within an α-helical region, the cathepsin cleavage sites in HBD-3 (Thr^5-Leu^6 and Gln^7-Lys^8) appear to occur within a more disordered N-terminal region (33). This lack of structure at the N-terminal region of HBD-3 may render it more susceptible to cleavage by cathepsins than HBD-2 and may explain our observation that lower concentrations of cathepsin are required to degrade HBD-3 compared with HBD-2. The cathepsin cleavage sites in HBD-2 and HBD-3 occur at N-terminal residues, and it will be interesting to discover whether other members of the β-defensin family are also susceptible to cleavage at this site.

The question arises of why a group of host proteases, cathepsins B, L, and S, should degrade and inactivate a host microbicidal protein. We have demonstrated previously that the cathepsins also degrade and inactivate another microbicidal protein, SLPI, in individuals with emphysema (23). Cathepsins are not present in the healthy lung, but can be secreted in response to certain stimuli, e.g., IFN-γ and IL-13 (34, 35). The presence of secreted cathepsins in the lung may be necessary to regulate the activities of inducible microbicidal proteins and peptides. A naturally occurring inhibitor of cathepsins, cystatin C, has been detected in the BAL of smokers (36). Interestingly, increased levels of cathepsin L and cystatin C were observed in the BAL of smokers with emphysema compared with smokers without emphysema (36); however, the mere presence of active cathepsin L suggests that cystatin C levels were not sufficient to inhibit cathepsin L activity in smokers BAL. Cystatin C levels have not been determined in CF BAL, but the presence of active cathepsins B, L, and S, as shown in this report, would also indicate that any cystatin C present in the CF lung is overwhelmed.

In chronic lung diseases such as CF and emphysema, it is possible that cathepsin expression and secretion may be dysregulated to the extent that continuous production of cathepsin compromises the antimicrobial screen. Therefore, modulation of cathepsin expression and/or secretion in chronic lung disease may be important in controlling bacterial infection. Alternatively, the design of cathepsin-resistant antimicrobials may be a therapeutic option in the future treatment of CF and other chronic lung diseases.

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