Antimicrobial and Protease Inhibitory Functions of the Human Cathelicidin (hCAP18/LL-37) Prosequence

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Cathelicidins are a class of small cationic peptide antibiotics that are expressed in skin and in other epithelial cells and are an active component of mammalian innate immunity. Human cathelicidin (hCAP18/LL-37) consists of a conserved prosequence called the cathelin-like domain and a C-terminal peptide named LL-37. To date, our understanding of the cathelin-like domain was very limited. To bring insight into the function of this evolutionarily conserved prosequence, we produced recombinant human cathelin-like protein and full-length hCAP18/LL-37 in *Escherichia coli*. As the cathelin-like protein shares homology with the cystatin family of cysteine protease inhibitors, we first analyzed the effect of the cathelin-like recombinant protein on the cysteine protease cathepsin L. We found that the cathelin-like protein inhibited protease activity. Next, we tested the cathelin-like protein for antimicrobial activity using solid phase radial diffusion and liquid phase killing assays. The cathelin-like prosequence, but not full-length hCAP18/LL-37, killed human pathogens including *E. coli* and methicillin-resistant *Staphylococcus aureus* at concentrations ranging from 16 to 32 μM. Together these findings suggest that after proteolytic cleavage the cathelin-like domain can contribute to innate host defense through inhibition of bacterial growth and limitation of cysteine-proteinase-mediated tissue damage. As these dual functions are complementary to the LL-37 peptide released from the C-terminus of full-length hCAP18/LL-37, human cathelicidin represents an elegant multifunctional effector molecule for innate immune defense of the skin. ***Key words: Innate immunity/protease/infection/skin/staphylococcus. J Invest Dermatol 120:810–816, 2003***

Small, cationic antimicrobial peptides (AMPs) are naturally occurring antibiotics of the innate immune system. AMPs are widely distributed in animals and plants and are among the most ancient host defense factors (Hoffmann *et al*, 1999). Their spectrum of activity includes Gram-positive and Gram-negative bacteria as well as fungi and certain viruses (Boman, 2000; Zasloff, 2002). As resistance of pathogenic microbes to conventional antibiotics increases, researchers are exploring these endogenous antibiotics as a potential source for new therapies against a variety of infectious diseases. In humans, there are several classes of known AMPs including α-defensins, β-defensins, and cathelicidins. Cathelicidins are found in several mammalian species and accumulating evidence supports a key role for this class of AMPs in innate immune defense (for review see Gennaro and Zanetti, 2000; Ramanathan *et al*, 2002; Zaiou and Gallo, 2002). Recently, development and characterization of a cathelicidin knockout mouse provided evidence that endogenous expression of an AMP protects the host against invasive bacterial infection of the skin (Nizet *et al*, 2001). Additional investigations have identified roles for cathelicidins in specific clinical disease states. Production of cathelicidins is induced in response to epithelial wounding or infectious challenge (Dorschner *et al*, 2001; Schaller-Bals *et al*, 2002), or suppressed by the virulence mechanisms of certain bacterial pathogens, e.g., *Shigella dysenteriae* (Islam *et al*, 2001). Cathelicidin expression is also differentially affected in certain chronic inflammatory disorders. In psoriasis, cathelicidin levels are elevated and secondary infection is rare (Frohm *et al*, 1997), whereas in atopic dermatitis cathelicidin expression is deficient and bacterial or viral superinfection is common (Ong *et al*, 2002). Therapeutic benefits of cathelicidin have been demonstrated experimentally, including decreased bacterial colonization of skin wounds following topical administration (Cole *et al*, 2001) and improved pulmonary bacterial clearance with cathelicidin overexpression through viral gene transfer (Bals *et al*, 1999a,b).

Cathelicidin proteins are composed of two distinct domains: an N-terminal "cathelin-like" or "prosequence" domain and the C-terminal domain of the mature AMP. The highly variable C-terminal domains of cathelicidins were among the earliest mammalian AMPs to show potent, rapid, and broad-spectrum killing activity (Zanetti *et al*, 1990; Gennaro *et al*, 1998). In contrast, the function of the conserved prosequence domain of cathelicidins is less well understood. The term "cathelin-like" derives from the similarity of this sequence with that of cathelin, a 12 kDa protein isolated from porcine neutrophils that shares similarity with the cystatin superfamily of cysteine protease inhibitors (Ritonja *et al*, 1989; Storici *et al*, 1996; Scocchi *et al*, 1997). Cathelicidins are expressed in neutrophils and myeloid bone marrow cells (Zanetti *et al*, 1990; Gallo *et al*, 1997; Ganz and Lehrer, 1997; Sorensen *et al*, 1997) and at most epithelial surfaces (Frohm *et al*, 1997; Harder *et al*, 1997; Bals *et al*, 1998; Dorschner

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Abbreviations: AMC, 7-amido-4-methylcoumarin; AMP, antimicrobial peptide; CFU, colony-forming unit; MRSA, methicillin-resistant *Staphylococcus aureus*; TSB, trypticase soy broth.

et al, 2001), and were the first AMPs discovered in mammalian skin due to their presence in wound fluid (Gallo *et al*, 1994). In the neutrophil, cathelicidins are synthesized as a full-length precursor and targeted to the secondary granules where they are stored. Upon stimulation, the full-length cathelicidin protein is proteolytically processed to unleash the microbicidal activity of the C-terminal peptide from the cathelin-like domain (Zanetti *et al*, 1991; Sorensen *et al*, 2001).

Although the killing activities and biologic functions of mature cathelicidin antimicrobial peptides have been investigated extensively, a satisfactory description and evidence of the function of the conserved cathelin-like prosequence are still unavailable. The structure of this 96–104 residue protein domain is believed to be stabilized by four cysteines engaged in two disulfide bonds (Storici *et al*, 1996; Sanchez *et al*, 2002a,b). These four cysteines as well as their relative positions are well conserved in all species. The strict evolutionary conservation of this domain and its similarity to cystatins, a family of proteinase inhibitors, suggests it plays a specific and independent biologic function in host defense (Gennaro and Zanetti, 2000).

To investigate possible function(s) of the cathelin-like domain, we selected the sole human cathelicidin, hCAP18/LL-37 (cationic antibacterial protein of 18 kDa). The C-terminal mature AMP of 37 amino acids (LL-37) of CAP18/LL-37 has been well characterized (Agerberth *et al*, 1995). Here we report prokaryotic expression of recombinant full-length hCAP18/LL-37 and its cathelin-like prosequence. We show that the human cathelin-like domain acts as a cysteine proteinase inhibitor and discover that it exhibits antibacterial activity against pathogens including *Escherichia coli* and methicillin-resistant *Staphylococcus aureus* (MRSA). This antimicrobial activity is distinct from that of the LL-37 peptide. These findings suggest that the cathelin-like domain of hCAP18/LL-37 is a distinct contributor to skin innate host defense through inhibition of both bacterial growth and cysteine-proteinase-mediated tissue damage.

MATERIALS AND METHODS

Construction of human cathelicidin expression vectors Expression plasmids containing the human full-length cathelicidin cDNA (hCAP18/LL-37) residues (31–170) or the cathelin-like domain residues (31–131) were constructed as fusion proteins in the pET-28a vector (Novagen, Madison, WI) using standard methods (Sambrook *et al*, 1989). High-fidelity polymerase chain reaction was used to amplify the coding sequence of hCAP18/LL-37 with primers designed from the published sequence (Gudmundsson *et al*, 1996): forward primer P1, 5′-TCCGAGCTCGACGATGACGATAAGCTGCTGGGTGATTTCTTCCGG-3′, containing a *Sac*I recognition site and enterokinase cleavage site (underlined), and reverse primer P2, 5′-CCGCTCGAGCTAGGACTCTGTCCTGGGTACAAGATTCCG-3′. For plasmid pET-Cath, we used primer P1 (above) and reverse primer P3, 5′-CCGCTCGAGCTACTAGGCAAATCTCTTGTTATCCTT-3′. P2 and P3 both contain stop codons and *Xho*I restriction site extensions. *Sac*I and *Xho*I digested polymerase chain reaction amplicons were used for unidirectional ligation into pET-28 vector. The pET-hCAP18 and pET-Cath constructs were confirmed by plasmid purification and direct DNA sequencing.

Expression of cathelicidin proteins pET-hCAP18 or pET-Cath were transformed into protease-deficient *E. coli* strain BL21 (DE3). Overnight cultures of each in Luria–Bertani broth (LB) (1% bactotryptone, 0.5% yeast extract, 1% NaCl) supplemented with kanamycin (50 μg per ml) were used to inoculate 1 l LB broth and then grown at 37°C with agitation to 0.6 OD at 600 nm. Expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a concentration of 0.5 mM. Cells were harvested by centrifugation ($6500 \times g$) for 10 min at 4°C and then resuspended in 50 ml ice-cold sonication buffer (0.1 M Tris–HCl pH 8.0; 0.01 M Na_2HPO_4, 0.1 M NaCl; 0.05 M ethylenediamine tetraacetic acid (EDTA); 0.005 M β-mercaptoethanol) supplemented with the protease inhibitors 0.1% aprotinin and 2 mM phenylmethylsulfonyl fluoride. Cells were disrupted by sonication on ice and the mixture was centrifuged at $20{,}000 \times g$ for 30 min to separate the insoluble material. Recombinant protein solubility was assessed by comparative sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) separation of both the supernatant and the pellet fractions. The supernatant fraction containing soluble recombinant proteins was collected and guanidine–HCl and β-mercaptoethanol were added to final concentrations of 6 M and 0.1%, respectively. The pellet resulting from the centrifugation was washed with 50 mM Tris–HCl and 5 mM EDTA. Insoluble full-length hCAP18/LL-37 and cathelin-like proteins were extracted overnight at 4°C with lysis buffer supplemented with 6 M guanidine–HCl and β-mercaptoethanol with a yield of 70%–90% without degradation. The suspension was then centrifuged at $20{,}000 \times g$ for 30 min to remove the remaining insoluble material. The supernatants of both extractions were dialyzed against 200 mM NaCl, 200 mM L-arginine, 10 mM β-mercaptoethanol, and 50 mM Tris–HCl pH 8.0, followed by extensive dialysis against 10 mM Tris–HCl pH 7.5. The solution was centrifuged for 10 min at $15{,}000 \times g$ to remove any precipitate. Proteins were pooled with the soluble fractions obtained earlier.

Purification of recombinant proteins Immobilized metal affinity chromatography was employed. The supernatant was dialyzed against buffer A consisting of 0.5 M NaCl, 0.02 M Na_2HPO_4 pH 7.5. Proteins were loaded onto an Ni^{2+}–NTA 5 ml His-Trap column (Pharmacia Biotech, Piscataway, NJ) previously equilibrated with buffer A to which 40 mM imidazole was added (flow rate 1 ml per min). The column was washed with 50 volumes of buffer A containing 40 mM imidazole to remove unspecific bound materials and bound proteins were eluted with 500 mM imidazole in buffer A, collecting 1 ml fractions. The elution profile was monitored by separation of samples by SDS-PAGE. Fractions containing proteins of interest were pooled and dialyzed against 0.5 M NaCl, 0.02 M Na_2HPO_4 pH 7.5 and the purification step was repeated at least twice. Eluted fractions were pooled and were dialyzed against enterokinase buffer (50 mM Tris–HCl pH 8.0, 1 mM $CaCl_2$, 0.1% Tween-20).

Enterokinase digestion The upstream 43-residue N-terminal fusion sequence of pET containing the enterokinase recognition sequence DDDDK was cleaved from the recombinant hCAP18/LL-37 and Cath proteins by digestion with enterokinase (obtained as a 1 unit per μl solution from Invitrogen, Carlsbad, CA). The reaction was incubated at 37°C overnight with an enzyme:protein substrate ratio of 1:25. Digested proteins were dialyzed against 10 mM Tris–HCl pH 7.5 and then proteins were applied onto a Superdex-75 column (2.6×100 cm) and eluted using 10 mM phosphate buffer (pH 7.5) containing 150 mM NaCl at room temperature. Fractions containing proteins of interest were collected and dialyzed against 10 mM Tris–HCl pH 7.5.

SDS-PAGE and immunoblotting The purity of cathelin-like or hCAP18/LL-37 protein was first confirmed by SDS-PAGE followed by Coomassie blue and silver staining. For Western blot, protein was separated by SDS-PAGE and was then transferred to nitrocellulose membranes using the Bio-Rad system. Membranes were blocked for 1 h at room temperature with 0.1% low fat milk in 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.2% Tween-20, and were probed overnight at 4°C with chicken polyclonal anticathelin-like antibodies (1:15,000) to detect cathelin-like protein or with rabbit anti-LL-37 antibodies (1:6000) to detect full-length hCAP18/LL-37, followed by extensive washing. Immunoreactive materials were detected by enhanced chemiluminescence using horseradish peroxidase conjugated antichicken antibodies (1:20,000) or horseradish peroxidase conjugated antirabbit antibodies (1:5000).

Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry A MALDI-TOF mass spectrometer (Applied Biosystems, Framingham, MA) was used to analyze purified proteins and further confirm identity and purity. Protein samples were prepared for analysis by mixing in a 1:1 ratio with sinapinic acid matrix (3,5-dimethoxy-4-hydroxycinnamic acid). Calibration was performed using internal standards: bovine insulin, apomyoglobin, and thioredoxin (Core facility at the University of California, San Diego).

Proteinase activity inhibition assay Proteinase inhibitory activity of recombinant cathelin-like protein was assayed spectrofluorometrically by measuring its inhibitory action against human liver cathepsin L (Calbiochem, CA). For the reaction assay, cathepsin L (0.1 mU) in the assay buffer (340 nM sodium acetate, 60 mM acetic acid, 8 mM dithiothreitol, and 4 mM EDTA, pH 5.5, supplemented with 0.1% BRIJ 35) was preincubated for 2 min at 30°C with 10^{-6} M cathelin-like protein before adding 20 μM of substrate Z-Phe-Arg-7-amido-4-methylcoumarin (Calbiochem). When hydrolyzed by cathepsin L, this substrate releases highly fluorescent 7-amido-4-methylcoumarin (AMC). AMC intensity was determined using a spectrophotometer at 370 nm excitation and an emission wavelength of 460 nm. One unit was defined as the amount of

enzyme that will hydrolyze 1.0 μmol of Z-Phe-Arg-AMC per min at 25°C, pH 5.5.

Antimicrobial assays *E. coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 8427), *Salmonella typhimurium* (ATCC 13311), *Proteus vulgaris* (ATCC 8427), *Staphylococcus epidermidis* (ATCC 12228), and MRSA (ATCC 33591) isolates were maintained on trypticase soy broth (TSB) agar plates. Individual colonies were selected and cultured overnight in TSB, subcultured once at 1:50 in fresh TSB, and then grown to stationary phase for use in all experiments. The radial diffusion assay was performed as described previously (Steinberg and Lehrer, 1997) in 0.5% agarose and 0.75% tryptone brought to ebullition and cooled to 43°C, and then mixed with 100 μl of bacterial suspension and poured into a 10 cm Petri dish. A series of small wells (diameter, 3 mm) were punched in the plate after the agarose solidified. Two microliters of test samples were applied in each well. Plates were incubated at 37°C overnight to allow visible growth of bacteria. Antibacterial activity was indicated by the clear zone (no bacterial growth) around the well.

The colony-forming assay (CFU) was performed as described previously (Valore *et al*, 1996). Briefly, bacterial cultures (*E. coli* or MRSA) were collected at the logarithmic phase of growth in TSB, washed twice with phosphate-buffered saline, pH 7.4, and diluted to 10^4 CFU per ml in 10 mM phosphate buffer, pH 7.4, Na_2HPO_4/NaH_2PO_4–1% TSB (g per l). Forty-five microliters of bacterial suspension were mixed with 5 μl of H_2O (control) or with 5 μl of different concentrations of cathelin-like proteins, and the mixture was incubated at 37°C. Every 30 min, a 10 μl aliquot of the reaction mixture was plated directly onto a TSB agar plate and then incubated at 37°C overnight for enumeration of CFU. Data are reported as growth index = final CFU/CFU in initial inoculum.

Processing of full-length hCAP18/LL-37 to cathelin-like protein and LL-37 Full-length hCAP18/LL-37 recombinant protein (10 ng) was incubated with 10 mU of human neutrophil elastase (Calbiochem) at 37°C for 30 min. The sample was subsequently boiled in Laemmli sample buffer and run by SDS-PAGE followed by immmunoblot analysis with anticathelin-like and anti-LL37 antibodies.

RESULTS

Expression and purification of cathelicidin proteins in *E. coli* In this study, cathelin-like protein and full-length cathelicidin hCAP18/LL-37 proteins were required for functional analysis. In order to achieve efficient expression of these proteins in *E. coli*, cDNA encoding full-length hCAP18/LL-37 or cathelin-like domain alone were cloned into the pET 28a(+) expression vector system (**Fig 1*a***). This system generates fusion proteins with an N-terminal peptide of 5 kDa containing a $His_{(6)}$ purification tag. Through primer design, a sequence corresponding to an enterokinase cleavage site, pentapeptide (Asp)4-Lys, was inserted after the fusion domain and before the cDNA of interest. Cultures of BL21 (DE3) bacteria transformed with either pET-hCAP18 or pET-Cath were then used for expression following IPTG induction. Over 70% of recombinant cathelin-like proteins were found in the soluble fraction. Only about 40% of full-length cathelicidin proteins were soluble, however. The remaining protein was found in the insoluble fraction. The expression of cathelicidin proteins with His-tag sequence at their N-terminus allowed for convenient purification from other soluble bacterial proteins using immobilized metal affinity chromatography. Proteins were further purified to homogeneity using size exclusion chromatography. The purity of recombinant cathelin-like protein after elution was first checked by SDS-PAGE (**Fig 1*b***). A band of approximately 16 kDa, which corresponds to the cathelin-like region and the fusion sequence (5 kDa), was detected by Coomassie blue staining (**Fig 1*b***). The identity of the bands of expected size was confirmed by Western blot using antibodies against the cathelin-like domain (**Fig 1*c***). A single band of approximately 32 kDa was also detected that probably corresponds to a homodimer of cathelin-like protein. These results demonstrated the effectiveness of the expression system used and recovery after purification (10–15 mg per l). Full-length hCAP18/LL-37 was identically purified and confirmed by identical techniques (data not shown). Cathelicidin proteins were

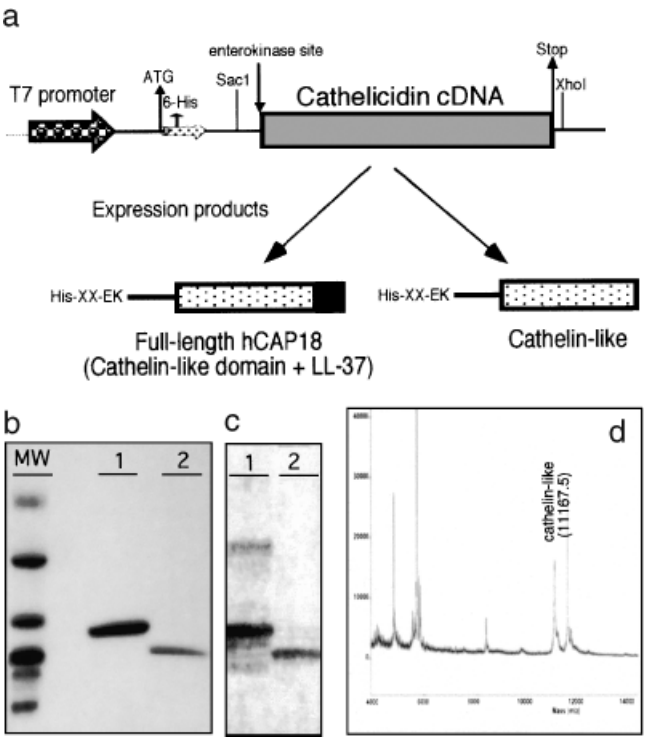

Figure 1. Expression and characterization of recombinant cathelicidin proteins. (*a*) Schematic representation of cathelicidin expression system in *E. coli*. (*b*) SDS-PAGE of cathelin-like protein by Coomassie blue staining; MW, molecular weight markers (from top to bottom: 45K; 30K; 20.1K; 14.3K; 6.5K; 3.5K; 2.5K); *lanes 1* and *2* are cathelin-like protein before and after digestion with enterokinase. (*c*) Western blot analysis using anti-cathelin-like antibodies; *lanes 1* and *2*, cathelin-like protein before and after digestion with enterokinase. (*d*) MALDI-TOF mass spectrometry. Molecular mass of pure human cathelin-like protein is a single peak at 11167.5 Da. Internal standards were included and are seen as accompanying peaks.

removed from the N-terminal tag by cleavage with enterokinase. Optimization studies found that enterokinase treatment yielded complete cleavage when digestion was carried out at 37°C overnight. No nonspecific cleavage or degradation was observed during this period as confirmed by Coomassie blue staining of the gel (**Fig 1*c***, *lane 2*). Following enterokinase cleavage, cathelicidin proteins were further purified by size exclusion chromatography, fractions were analyzed by SDS-PAGE, and then identity and purity were confirmed by mass spectrometry (**Fig 1*d***). Approximately 50% of the preparations of recombinant cathelin-like protein displayed the expected peak at 11167.5 mass units by matrix-assisted laser desorption/ionization in agreement with theoretical mass and full disulfide bond formation of this protein. To further confirm the identity of hCAP18/LL-37, the recombinant protein was treated with elastase to observe if this protein was processed similarly to the native cathelicidin. Elastase treatment generated a band migrating at the same size as synthetic LL-37, and a band at 14 kDa as seen by Western blot using anti-LL-37 antibodies (data not shown). This profile is similar to that observed with the previously reported experiments on native human cathelicidins isolated from neutrophils and treated with elastase or proteinase 3 (Sorensen *et al*, 2001). These experiments do not provide evidence of correct assembly of the disulfide bridges of recombinant hCAP18, however.

Proteinase inhibitory activity To evaluate the possible protease inhibitory activity action of cathelin-like protein, the activity of human cathepsin L was measured in the presence or absence of recombinant cathelin-like protein using the fluorescent substrate Z-Phe-Arg-AMC. As shown in **Fig 2**,

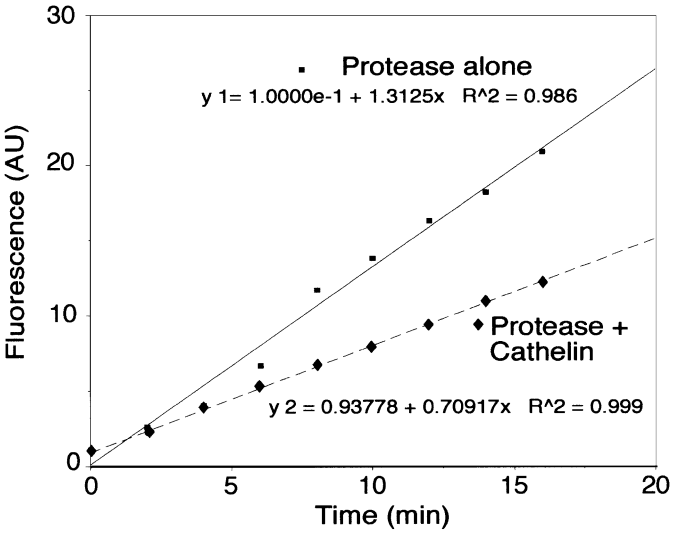

Figure 2. Proteinase inhibitory activity of recombinant cathelin-like protein. Hydrolysis of the fluorogenic substrate Z-Phe-Arg-AMC by cathepsin L (protease alone), and in the presence of cathelin-like protein (protease + cathelin). Fluorescence measurements were made as described in *Materials and Methods*. Data demonstrate inhibition of cathepsin L by the addition of cathelin-like recombinant protein. Slopes, intercepts, and correlation coefficients were calculated by linear regression. Data were recorded 10 times over 30 min and are representative of two experiments.

there was a linear relationship ($R^2 = 0.98$–99) between the control (cathepsin L alone) and the test (cathepsin L + cathelin-like protein). The generation of fluorescence was significantly decreased (slope = 1.31 *vs* 0.71, respectively) when cathelin-like protein was added to the reaction. The hydrolytic activity of the cysteine proteinase cathepsin L was inhibited by 46% when cathelin-like protein was added at a final concentration of 10^{-6} M. Parallel experiments with recombinant full-length hCAP18/LL-37 did not demonstrate inhibitory activity (data not shown).

Antimicrobial activity of recombinant cathelin-like protein

Recombinant cathelicidins were tested for antimicrobial activity using a standard radial diffusion assay. Different concentrations of the cathelin-like protein were loaded into wells on assay plates containing Gram-positive MRSA, *S. epidermidis,* and strains of the Gram-negative bacterial species *E. coli*, *S. enteritidis*, *P. vulgaris*, and *P. aeruginosa.* Cathelin-like protein had minimal inhibitory activity against growth of MRSA at 32 μM and *E. coli* at 16 μM, but did not inhibit growth of *P. aeruginosa* (**Fig 3*a***). Similarly, cathelin-like protein inhibited growth of *S. epidermidis* (32 μM) but not *S. enteritidis* or *P. vulgaris*. The full-length recombinant cathelicidin hCAP18/LL-37 did not demonstrate inhibitory activity against any of the bacteria tested, even at concentrations greater than 64 μM. In contrast to cathelin-like protein, the C-terminal peptide domains of the human and mouse cathelicidins (LL-37 and CRAMP, respectively) were inactive against the Gram-positive bacteria tested in this assay system, but were highly active against *E. coli*, *P. aeruginosa*, and *S. enteritidis* as previously described (Agerberth *et al*, 1995; Gallo *et al*, 1997; Turner *et al*, 1998; Bals *et al*, 1999).

Next we investigated cathelin-like protein in a standard liquid phase AMP testing assay to validate the observed activities on agar (**Fig 3*b*, *c***). As LL-37 was active against *E. coli* but inactive against MRSA in the radial diffusion assay, whereas cathelin-like protein was active against both, we chose these two bacteria for comparison of antimicrobial activity of full-length cathelicidin and its two distinct domains. With *E. coli*, addition of cathelin-like protein (16 μM) resulted in marked growth inhibition compared to the untreated controls, whereas LL-37 at 3 μM was bactericidal (**Fig 3*c***). With MRSA, an inverse pattern of activity

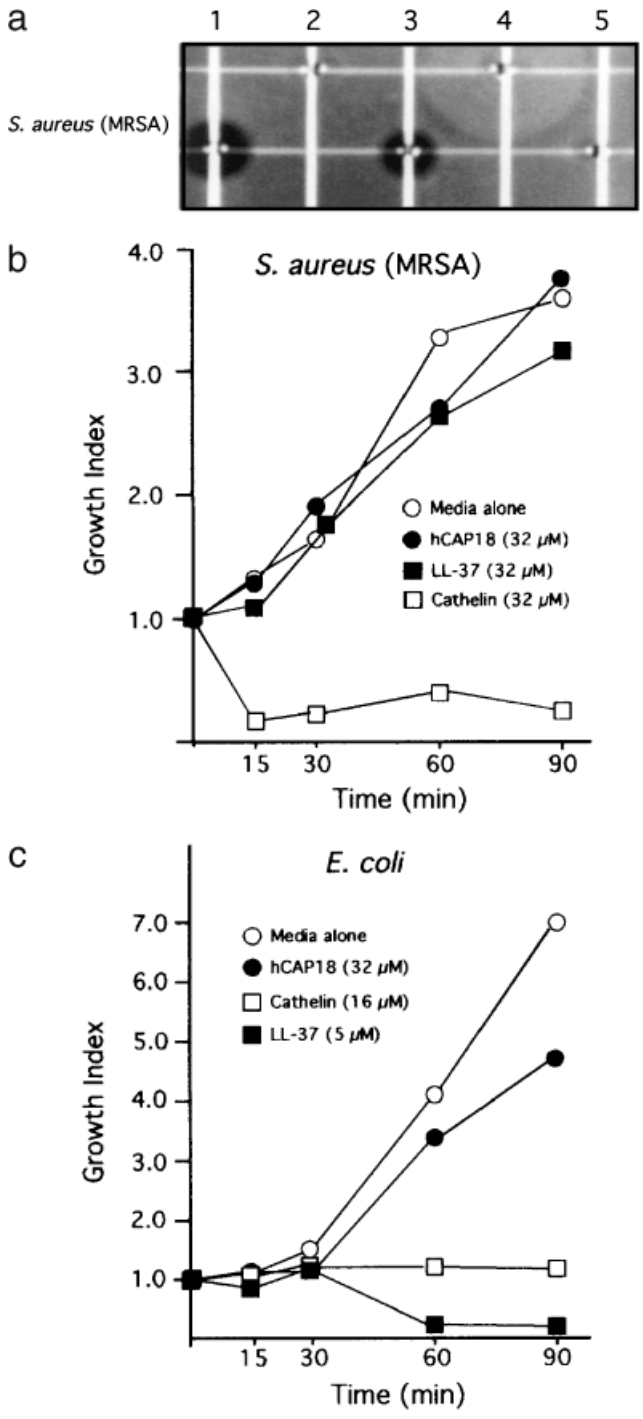

Figure 3. Growth inhibition of MRSA, *E. coli*, and *P. aeruginosa* by cathelin-like protein. (*a*) Inhibition zone assay on tryptone/agarose plates with MRSA. Clear circles show lack of bacterial growth. *Lanes 1, 3, 5, bottom row*: cathelin-like protein at 64 μM, 32 μM, 16 μM, respectively. *Lane 2, top row*: 32 μM of His-tagged cathelin-like protein before cleavage. *Lane 4, top*: enterokinase enzyme alone. All assays were performed in duplicate and repeated three times. (*b*), (*c*) CFU assay: growth inhibition of (*b*) MRSA and (*c*) *E. coli,* cultured in 10 mM phosphate and 1% TSB (pH 7.4) and then supplemented with different concentrations of human cathelin-like protein.

was seen with cathelin-like protein and LL-37. Cathelin-like protein decreased MRSA CFU for the first 15 min and then was bacteriostatic, whereas LL-37 had no effect even at 32 μM. Full-length hCAP18/LL-37 (32 μM) did not show any activity against *E. coli* or MRSA (**Fig 3*b*, *c***). These results are consistent with those obtained in the radial diffusion plate assay. Taken together, our results demonstrate that cathelin-like protein possesses

inherent antimicrobial activity and suggest a novel role for this protein in host defense.

DISCUSSION

The expression of the cathelicidin gene product is an important determinant of resistance to infection in mammals (Nizet *et al*, 2001). Heretofore, the antimicrobial activity of this molecule has been attributed to the action of the C-terminal peptide (in humans known as LL-37) after proteolytic processing of the precursor protein (in humans known as hCAP18/LL-37) to "remove" the N-terminal cathelin-like domain. The major goal of this study was to explore potential biologic function(s) of the evolutionarily highly conserved cathelin-like domain of human cathelicidin. We produced recombinant human cathelin-like protein and unprocessed full-length cathelicidin proteins in bacteria. These proteins were purified and characterized immunologically and physicochemically. Due to the complex structure of the cathelin-like domain, it was difficult to obtain protein that was soluble. When soluble, however, functional analysis demonstrated that the recombinant cathelin-like domain possessed both antimicrobial and cysteine proteinase inhibitory activities. The cathelin-like protein caused inhibition of growth of some Gram-negative (*E. coli*) and Gram-positive (*S. aureus*) bacterial species. This activity was distinct from the known antimicrobial spectrum of the mature C-terminal peptide. Human cathelin-like protein also inhibited the action of the lysosomal cysteine proteinase cathepsin L. The recombinant full-length hCAP18/LL-37 cathelicidin lacked both activities before processing. Our findings suggest that the human cathelicidin gene encodes an inactive precursor (full-length hCAP18/LL-37) that, once processed by enzymes such as elastase or proteinase-3, generates two distinct antimicrobial molecules: cathelin-like protein and the mature LL-37 AMP. These data are distinct from an earlier study showing that native bovine proBac 5 isolated from bovine neutrophils can inhibit the activity of cathepsin L (Verbanac *et al*, 1993). Bovine pro-Bac5 closely resembles the full-length hCAP18/LL-37 that was inactive in our experiments. Our data suggest that recombinant human cathelin-like protein alone is an inhibitor of cysteine proteinase cathepsin L. Lack of activity of the full-length protein may reflect incorrect folding of our recombinant protein. Alternatively, the purified native pro-Bac5 previously reported may have contained undetectable amounts of cleaved cathelin-like protein that imparted activity.

Studies have reported that the lysosomal cysteine proteinase cathepsin L is present in inflammatory cells, and may contribute substantially to tissue injury at inflammatory lesions due to its potent elastinolytic and collagenolytic activities (Mason *et al*, 1986). As cathelin-like protein is similar to cystatins, which are known to have diverse functions including the ability to inhibit cysteine proteinases, we tested recombinant cathelin-like protein for proteinase inhibitory action. Indeed, recombinant human cathelin-like protein was able to inhibit the proteolytic activity of the human cysteine proteinase cathepsin L. This phenomenon could be an evolutionary system designed to mitigate tissue injury and other potentially deleterious effects of cathepsin L, and potentially other proteinases, released from lysosomes during inflammatory responses. Moreover, several human pathogens secrete cysteine proteases that have been shown to play a role in disease pathogenesis, including SpeB and IdeS of *Streptococcus pyogenes* (Lukomski *et al*, 1999; von Pawel-Rammingen *et al*, 2002), Lys-gingipain of *Porphyromonas gingivalis* (Kuboniwa *et al,* 2001), and the extracellular cysteine proteases Ehcp1–6 of *Entamoeba histolytica* (Zhang *et al*, 2000). Theoretically, the inhibition of such microbial virulence determinants may be another adaptive function of the cathelin-like domain in innate immune defense. Studies are under way to express sufficient quantities of recombinant cathelin-like protein in eukaryotic expression systems to determine the affinity and inhibitory constant (K_i) with cathepsin L and a range of other cysteine proteases.

Mammalian cathelicidin is produced as an inactive precursor that is proteolytically cleaved inside activated neutrophils and at epithelial sites of inflammation. Following this activation step, the active AMP and the cathelin-like domain with antimicrobial and cysteine protease inhibitor activities become available. Recent research has shown that granule proteases such as elastase and cathepsin each play important roles in neutrophil function and resistance to infection (Reeves *et al*, 2002). It is possible that protease cleavage of full-length cathelicidin into two active antimicrobial agents is a critical step in the pathway to microbial killing (Cole *et al*, 2001). The prosequence inhibition of cathepsin activity may represent a feedback loop to control the magnitude of local tissue degradation during the inflammatory process. It remains to be determined whether cathelicidin prosequence inhibition of proteases in itself represents an antimicrobial property. Motifs involved in the interaction of cystatins with cysteine proteinases are not fully conserved in the cathelin-like domain, however. For example, glycine 9 (chicken cystatin numbering), a conserved residue found in all known sequences of inhibitory cystatins (Abrahamson *et al*, 1987; Ritonja *et al*, 1989), is not present in cathelin-like protein. Another highly conserved sequence, QXVXG (residues 53–57), is also not present (Ritonja *et al*, 1989), indicating that the interaction with cysteine proteinases is different. Overall, the proteinase inhibition activity of cathelin-like protein may be an alternative strategy of the host to control the regulation of its own enzymes and also to defend itself against microbe proteinases.

Next, we investigated the antimicrobial activity of the recombinant cathelin-like protein. Surprisingly, the results from these studies displayed antimicrobial activity at concentrations ranging from 16 to 32 μM against bacteria resistant to LL-37. Cathelin-like protein caused growth inhibition of both Gram-negative (*E. coli*) and Gram-positive (*S. aureus*) bacteria. We also observed that the parent hCAP18/LL-37 was inactive even at higher concentrations (>64 μM), which is consistent with the previously reported data on rabbit and bovine cathelicidin precursors purified from tissue (Zanetti *et al*, 1991; Zarember *et al*, 2002). In rabbit CAP-18, however, the unprocessed protein has shown synergistic antimicrobial activity when combined with the antimicrobial and lipopolysaccharide-binding protein BPI (Zarember *et al*, 2002). The mechanism of this synergistic interaction is unknown. Further studies are required to determine the spectrum activity of cathelin-like protein and also to assess the minimal inhibitory activity required to eliminate these pathogens *in vivo*. In addition, experiments combining both cathelin-like proteins and LL-37 peptides are under way to test for the synergistic effect of these two domains.

How the cathelin-like domain carries out its two distinct functions, antimicrobial and proteinase inhibition, is at present unknown. Many cationic and amphipathic peptides such as indolicidin (Falla *et al*, 1996) and magainins (Westerhoff *et al*, 1989) exert their antimicrobial activity through membrane disruption and pore forming (Hancock, 1997). Cathelin-like protein is not a basic protein, however, and therefore the mechanism of antimicrobial action is probably different from this class of peptides. One possibility is that cathelin-like protein has structural features that interact with the microbial membrane. Based on the proposed three-dimensional structure of the prosequence of the porcine protegrin-type cathelicidin (Sanchez *et al*, 2002a), the N-terminus of this molecule presents an α-helical structure. This structure could be involved in the disruption of the normal function of microbial membrane. Furthermore, cystatins are similar to cathelin-like proteins (reviewed in Zaiou and Gallo, 2002), are not basic proteins, and also show antibacterial and antiviral activity against a range of organisms (Takahashi *et al*, 1994; Blankenvoorde *et al*, 1998). A synthetic peptide mimicking the cysteine proteinase inhibitory site of human cystatin C was found to kill *S. pyogenes* (Bjorck *et al*, 1989). Secretory leukocyte protease inhibitor is a 12 kDa protein that also contains both serine protease inhibitory and antimicrobial activities (Ashcroft *et al*, 2000). Recently, a cysteine proteinase inhibitor, designated L-cystatin, which is stored in the large granules of horseshoe crab hemocytes,

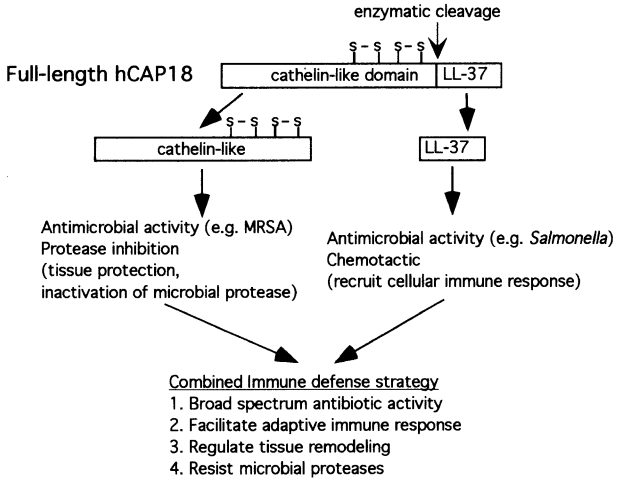

Figure 4. Schematic representation of the potential actions of human cathelicidin in innate immune defense. An inactive full-length precursor is cleaved by enzymes released in the inflammatory process to yield two distinct antimicrobial peptides with complementary actions.

was identified (Agarwala *et al*, 1996). This molecule is 12,600 kDa, is similar to cystatin superfamily members, contains two disulfide bridges at positions corresponding to the cathelin-like domain, and has shown antimicrobial activity against Gram-negative bacteria. These structural similarities support the function proposed for the cathelin-like protein. Furthermore, a recent investigation has solved the crystal structure of a homologous domain from the porcine cathelicidin protegrin-3 (Sanchez *et al*, 2002a). These data confirm the similarity between cathelin-like proteins and cystatins, and also demonstrate potential homodimer formation for this protein as we observed in **Fig 1**(*c*).

In summary, the findings of this study highlight a novel biologic function of cathelin-like protein as an antimicrobial protein in addition to its proteinase inhibitory activity. Recently, observations of cathelicidin in human sweat have shown the existence of cathelicidin processed to the expected size of the cathelin-like domain (Murakami *et al*, 2002). Our data suggest that cathelin-like protein may play important roles not only in the protection of cells from unfavorable proteolysis by host and microbial cysteine proteases, but also in the direct killing or inhibition of invading pathogens. Several functions for the peptide C-terminal domain of the cathelicidin gene product have been discovered ranging from influencing proteoglycan expression, angiogenesis, and chemotaxsis to its antimicrobial effects. This study now shows that the cathelin-like domain has multiple functions that can have complementary actions with the mature AMP in tissue defense (illustrated schematically in **Fig 4**). A full knowledge of the function of the different domains of cathelicidin would be a valuable asset in understanding normal innate immune defense strategies in mammals and in attempts to design new antibiotics for use in treatment or prevention of infectious diseases.

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