# Kallikrein-mediated proteolysis regulates the antimicrobial effects of cathelicidins in skin

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ABSTRACT **The presence of cathelicidin antimicrobial peptides provides an important mechanism for prevention of infection against a wide variety of microbial pathogens. The activity of cathelicidin is controlled by enzymatic processing of the proform (hCAP18 in humans) to a mature peptide (LL-37 in human neutrophils). In this study, elements important to the processing of cathelicidin in the skin were examined. Unique cathelicidin peptides distinct from LL-37 were identified in normal skin. Through the use of selective inhibitors, SELDI-TOF-MS, Western blot, and siRNA, the serine proteases stratum corneum tryptic enzyme (SCTE, kallikrein 5) and stratum corneum chymotryptic protease (SCCE, kallikrein 7) were shown to control activation of the human cathelicidin precursor protein hCAP18 and also influence further processing to smaller peptides with alternate biological activity. The importance of this serine protease activity to antimicrobial activity *in vivo* was illustrated in *SPINK5*-deficent mice that lack the serine protease inhibitor LEKTI. Epidermal extracts of these animals show a significant increase in antimicrobial activity compared with controls, and immunoabsorption of cathelicidin diminished antimicrobial activity. These observations demonstrate that the balance of proteolytic activity at an epithelial interface will control innate immune defense.—Yamasaki, K., Schauber, J., Coda, A., Lin, H., Dorschner, R. A., Schechter, N. M., Bonnart, C., Descargues, P., Hovnanian, A., Gallo, R. L. Kallikrein-mediated proteolysis regulates the antimicrobial effects of cathelicidins in skin. *FASEB J.* 20, 2068–2080 (2006)**

*Key Words: antimicrobial peptide · stratum corneum tryptic enzyme · stratum corneum chymotryptic protease · lympho-epithelial Kazal-type related inhibitor · SELDI-TOF-MS*

Cathelicidins are an antimicrobial gene family identified in mammals (1), birds (2), and fish (3). They are best known for their action as innate antimicrobials that protect the host against infection by Gram-positive bacteria (4), Gram-negative bacteria (5, 6), and some viruses (7). Analysis of several animal models (4), and human clinical conditions (8), have demonstrated that the presence of cathelicidin correlates with the ability of the host to effectively mount a defense against infection. Cathelicidin deficiency has also been reported in Kostmann syndrome and is associated with severe congenial neutropenia and frequent oral infection (9). Although antimicrobial peptides such as the cathelicidins represent an evolutionarily ancient form of immune response, current evidence strongly supports the conclusion that these molecules are an integral element of human immunity (10). Antimicrobial peptides such as cathelicidins act both to kill microbes and to initiate or modify other cellular immune events. As such, these molecules have been alternatively referred to as "alarmins" (11). Due to this activity on the host, strict control of cathelicidin expression and function is necessary to restrict activity of the peptide to conditions where maximal defense against microbial invasion is required.

The nascent cathelicidin protein is inactive and consists of an N-terminal cathelin domain that is conserved among mammalian species and a C-terminal domain encoding the mature cathelicidin peptide. A comparison of cathelicidin peptides purified from various species or predicted by their respective cDNA sequences has revealed that these potent antimicrobial molecules are variable sequences of 20 to 40 amino acids in the C-terminal domain. In humans and mice the cathelicidin peptides are alpha-helical, cationic, and amphipathic. These properties enable association and integration with negatively charged cell membranes.

Expression of cathelicidins, such as the human LL-37 released from neutrophils, is regulated by transcriptional and post-transcriptional processing. Cathelicidin transcript and total protein abundance *in vivo* is induced by infection (12), inflammation (13), wounding (14), and differentiation (15, 16). In humans, 1,25-dihydroxyvitamin $D_3$ is a direct inducer of cathelicidin expression (17) whereas in mice the cathelicidin

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mCRAMP (1) (mouse cathelin-related antimicrobial peptide) expression is dependent on HIF-1a (hypoxia-inducible factor 1, alpha) (18). Although these stimuli modify cathelicidin mRNA abundance, factors that regulate the translation and final activation of cathelicidin are unclear. Recent observations of cathelicidins *in vivo* suggest that the final proteolytic processing of cathelicidin dictates antimicrobial or alarmin activity (19).

The nascent human cathelicidin gene product is designated hCAP18 (human 18-kDa cationic antimicrobial protein) (20). hCAP18 has been shown to be processed to the antimicrobial peptide LL-37 by proteinase-3 in neutrophils (21). The prostate-derived protease gastricsin can also process hCAP18 to ALL-38 (22). At the skin surface, unidentified proteases present in human sweat can cleave LL-37 to smaller peptides such as RK-31, KS-30, and KR-20 (23). The native cathelicidin peptides at the skin surface are unknown, but these smaller peptides generated *in vitro* have more potent antimicrobial activity than LL-37. Analysis of alternatively processed human cathelicidin peptides has further shown that processing alters the immunostimulatory capacity of cathelicidins, eliminating their ability to stimulate interleukin (IL)-8 release (19). These observations demonstrate the importance of proteolytic processing to the activity of cathelicidins and, by extension, their importance to immune defense in general.

Since the presence of cathelicidin in skin is critical for normal microbial defense and innate immunity, we sought in the present study to identify the cathelicidins in human skin and define the proteases responsible for cathelicidin activation. We show that novel cathelicidin peptide forms are present at the skin surface and that kallikreins, a family of serine proteases known for their influence on the development of the epidermis, are responsible for their generation. Moreover, we show that cathelicidin processing is altered *in vivo* in the absence of the serine protease inhibitor LEKTI. These data suggest that the function of kallikreins in the skin is in part to regulate the immune barrier, both by modifying the physical structure of the epidermis and by determining innate antimicrobial peptide function.

## MATERIALS AND METHODS

### Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry

To define the cathelicidin peptides in small samples of normal human skin, we used a surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) system. Normal human skin was obtained after informed consent. Five to 10 mm of normal skin at the surgical margin was removed during routine surgical excisions of nonmelanoma skin lesions. This was embedded in Tissue-Tek™ O.C.T. compound (Electron Microscopy Sciences, Fort Washington, PA, USA) and freshly frozen. Twenty sections (10 μm thickness) were cut from frozen skin in O.C.T. compound and collected in tubes. Proteins were extracted with 100 μl of 1 × radio-immunoprecipitation assay (RIPA) buffer (50 mM HEPES, 150 mM NaCl, 0.05% SDS, 0.25% deoxycholate, 0.5% Nonidet P-40, pH 7.4) with proteinase inhibitor mixture (complete EDTA-free; Roche, Indianapolis, IN, USA). Samples were kept in –20°C until further analysis. All sample acquisitions, including the skin biopsies, were approved by the Committee on Investigations Involving Human Subjects of the University of California, San Diego.

Protein chips (RS100 protein chip array, Ciphergen Biosystems, Fremont, CA, USA) were coated with 4 μl of rabbit anti-LL-37 antibody (Ab) (0.73 mg/ml) for 2 h at room temperature, followed by blocking with 0.5 M etahnolamine in PBS (pH 8.0). After washing three times with PBS/0.5% Triton X, protein chips were assembled in the Bioprocessor™ reservoir, and samples (50 μl) were applied and incubated for 2 h at room temperature. Protein chips were washed three times with 1 × RIPA buffer, twice with PBS/0.5% Triton X-100, and three times with PBS, followed by soaking in 10 mM HEPES buffer, then air dried. A half microliter of energy absorbance molecule (50%-saturated alpha-cyano-4-hydroxy cinnamic acid in 50% acetonitrile, 0.5% trifluoric acid) was applied twice, and all spots were completely dried up. Samples were analyzed on a SELDI mass analyzer PBS II with a linear time-of-flight mass spectrometer (Ciphergen Biosystems) using time-lag focusing. Specificity and accuracy in this system were confirmed by several synthetic cathelicidin peptides as standards (19, 23). Synthetic LL-37 and KR-20 peptides were used as an internal reference. Skin extracts from three individuals were analyzed and showed similar patterns of cathelicidin peptides.

### Collection and assay of skin proteases

Protease activity at the skin surface was evaluated in sweat collected as described for collection of human sweat (24). Activity was monitored by EnzCheck® Protease Assay Kit green fluorescence (Molecular Probes, Inc., Eugene, OR, USA) according to the manufacturer's instructions. Briefly, 100 μl of the aqueous solution collected from the skin surface was mixed with 100 μl of BODIPY FL casein substrate and incubated at 37°C for designated periods. Protease activity was monitored as increased fluorescence with SpectraMax GEMINI EM (Molecular Devices Corp., Sunnyvale, CA, USA). In some experiments protease inhibitors were added, including mixed protease inhibitor mixture (complete EDTA-free, 1 tablet/50 ml; Roche, Indianapolis, IN, USA), 200 μg/ml bestatin, 20 μg/ml E-64, and 20 μg/ml aprotinin (Sigma-Aldrich, St. Louis, MO, USA), 200 μM 4-(2-aminoethyl)-benzenesulfonylfluoride (AEBSF), 200 μM human neutrophil elastase inhibitor (methoxysuccinyl-Ala-Ala-Pro-Ala-chloromethyl ketone, Calbiochem, San Diego, CA, USA), 200 μM human leukocyte elastase inhibitor (methoxysuccinyl-Ala-Ala-Pro-Val-chloromethylketone, Calbiochem), 100 μM chymostatin (Roche), or 10 μM leupeptin (Roche).

### Purification of protease activity

Sweat (240 ml) collected from the skin surface was concentrated using Macrosep™ Centrifugal Devices (3 kDa cutoff; Pall Life Science, Ann Arbor, MI, USA). Separation of enzyme activity was performed using an ΔKTA purification system (Amersham Pharmacia Biotech, Piscataway, NJ, USA) on a reverse phase HPLC (μRPC C2/C18 ST 4.6/100 column; Amersham Pharmacia Biotech). Columns were equilibrated in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min and eluted using gradients of 0–60% acetonitrile for 91 min. Column effluent was monitored at 214, 230, and 280 nm. All

fractions collected (1 ml) were lyophilized and suspended in 50 μl of distilled water for protease assay and immunoassay.

Skin surface fractions purified by HPLC were evaluated by quantitative dot blot. Five microliters of each fraction was compared with a standard curve of recombinant SCTE (25) applied onto NitroBond nitrocellulose membrane (GE Osmonics Labstore, Minnetonka, MN, USA). For immunoblot, membranes were blocked with 5% nonfat milk and 3% BSA in 0.1% TTBS (0.1% Tween 20/TBS (150 mM NaCl and 10 mM Tris base, pH 7.4) for 60 min at room temperature, then incubated with mouse anti-human SCTE monoclonal antibody (mAb) (1/1000 in the blocking solution; R&D Systems Inc., Minneapolis, MN, USA) overnight at 4°C. After washing three times with 0.1% TTBS, the membrane was incubated with HRP-conjugated goat antimouse Ab (1/5000 in the blocking solution; DAKO, Carpinteria, CA, USA) for 60 min at room temperature. After washing the membrane again with 0.1% TTBS, the membrane was immersed in enhanced chemiluminescence (ECL) solution (Western Lightning™ Chemiluminescence Reagents Plus; Perkin-Elmer Life Sciences, Boston, MA, USA) for 60 s, then exposed to X-ray film (X-Omat™; Eastman Kodak, Rochester, NY, USA).

### Analysis of cathelicidin processing

LL-37 was synthesized and prepared as described previously (23). For analysis of LL-37 processing, 32 nmol of LL-37 synthetic peptide was incubated with proteases in 100 μl for 0, 1, 6, and 24 h at 37°C. The buffer used was 0.2 M NaCl, 0.1 M MOPS, pH 7.0 for SCTE and 2.0 M NaCl, 0.045 M Tris-HCl, pH 8.0 for SCCE. After incubation, peptides were separated by reverse phase HPLC (Sephasil peptide C18 12 μm, ST 4.6/250 column; Amersham Pharmacia Biotech). Column was equilibrated in 10% acetonitrile with 0.1% trifluoroacetic acid at a flow rate of 2 ml/min and cleaved peptides were eluted using gradients of 10–20 and 20–70% acetonitrile for 2 and 31 min, respectively. Column effluent was monitored at 214, 230, and 280 nm. All collected fractions (1 ml) were lyophilized and suspended in 10 μl of distilled water for antimicrobial radial diffusion assay or directly analyzed by mass spectrometry.

For Western blot, recombinant hCAP18 (26) was incubated with proteases and separated by 16% Tris-tricine gel (Gene-Mate® Express gels; ISC BioExpress, Kaysville, UT, USA), then transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Billerica, MA, USA). For the positive control, 5 pmol of LL-37 synthetic peptide was applied. Membranes were blocked with 5% nonfat milk and 3% BSA in 0.1% TTBS, incubated with anticathelin domain chicken Ab (1/5000 in the blocking solution) or anti-LL37 rabbit Ab (1/5000 in the blocking solution), and developed as described above.

### Mass spectrometry and protein sequence analysis

Mass spectrometry was performed by the Mass Spectrometry Facility in the Department of Chemistry and Biochemistry at University of California, San Diego. The matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) spectra were obtained with a Finnigan LCQ DECA mass spectrometer (Thermo Electron Corporation, Waltham, MA, USA). The instrument has unit mass resolution over a mass range of 50–2000 m/z with a typical mass accuracy of 100 ppm (0.01%). Protein sequence analysis for target HPLC fractions was performed by the Division of Biology Protein Sequencer Facility, University of California, San Diego. Amino acid sequencing was performed on Applied Biosystems Procise model 494 sequencer (Foster City, CA, USA) using the pulsed liquid program supplied by the manufacturer.

### Antimicrobial assays

For screening of antimicrobial activity, radial diffusion assay was used as described previously (24). Lyophilized HPLC fractions were dissolved in 10 μl of MOPS buffer (pH 7.0) and tested against *Staphylococcus aureus mprF* (gift from A. Peschel, Microbial Genetics, University of Tubingen, Tubingen, Germany). This strain of *S. aureus* was selected for screening due to its increased sensitivity to cationic peptides. Thin plates (1 mm) of 1% SeaKem® GTG® Agarose (Cambrex Corp., East Rutherford, NJ, USA) and 1% LB Broth (EM Science, Gibbstown, NJ, USA) in 10 mM phosphate buffer (pH 7.2) containing $5 \times 10^6$ cells/ml of *S. aureus mprF* were used. One-millimeter wells were punched in the plates, and 2 μl of samples were loaded in each well. As a positive control, synthetic LL-37 was applied to separate wells. After incubation at 37°C overnight, the inhibition zone diameters were measured.

### Fluorescence immunohistochemistry

Frozen sections of normal skin (6 μm) were fixed with paraformaldehyde, sections were blocked with 5% goat serum, then incubated simultaneously with polyclonal rabbit anti-LL-37 and monoclonal mouse anti-SCTE primary antibodies. Goat anti-rabbit IgG conjugated to AlexaFluor488 (Molecular Probes) and goat antimouse IgG conjugated to tetramethylrhodamine isothiocyanate (TRITC) were used as secondary antibodies, respectively. Nuclei were stained with 4′,6′-diam idino-2-phenylidole (DAPI) and sections were mounted in ProLong Anti-Fade reagent (Molecular Probes). Images were obtained using an Olympus BX41 fluorescent microscope (Scientific Instrument Company, Temecula, CA, USA).

### siRNA transfection

siRNA for SCTE and SCCE and transfection reagents were obtained from Darmacon, Inc. (Chicago, IL, USA). The human keratinocyte cell line HaCaT, a generous gift from Dr. Norbert Fusenig (Krebsforschungszentrum, Heidelberg, Germany), was cultured in Dulbecco's modified Eagle medium (DMEM) plus 10% fetal calf serum and 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. After cells reached 70% confluence they were treated with 1 μg of siRNA with transfection reagent or with transfection reagent only. Cultured media and cells were collected 48 h after transfection. Buffer with supplements at final concentrations of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, and 1 μg/ml pepstatin were added and samples were sonicated on ice for 5 min. After centrifuge at 14,000 rpm for 10 min, protease activities of supernatants was monitored by EnzCheck® Protease Assay Kit as described above. The suppression of SCTE and SCCE mRNA were confirmed by the quantitative RT-polymerase chain reaction (RT-PCR) with specific primers and probes (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase primers and probe (Applied Biosystems) were used as an endogenous control. Results of the quantitative polymerase chain reaction (PCR) were plotted as the relative expression to the control using the Comparative Ct Method (User Bulletin #2, Applied Biosystems).

### Cathelicidin peptide extraction from mouse skin

Neonatal mouse skin was excised by 8 mm punch biopsy, homogenized in 1 ml of 1 M acetic acid, and incubated at 4°C overnight. After centrifugation for 15 min at 14,000 rpm, soluble fractions were transferred to new tubes and lyophilized. Pellets were dissolved in 50 μl of MOPS buffer (5 mM, pH 7.0). Antimicrobial activity against *Staphylococcus aureus mprf* was examined by radial diffusion assay as described previously. Antimicrobial activities were determined in solution assay against wild-type (WT) *S. aureus* [Rosenbach American Type Culture Collection (ATCC) 25923; ATCC, Manassas, VA, USA], methycilin-resistance *S. aureus* strain (81025 and 82056, and clinical isolate generously provided by Dr. Joshua Fierer, Veterans Affairs Healthcare Systems (San Diego, CA, USA), group A streptococcus (M49 strian NZ131 and M1 strain), and *Pseudomonas aeruginosa* (clinical isolate generously provided by Dr. Joshua Fierer). Bacteria ($1\times10^6$-$10^9$ CFU) in log-phase growth were suspended in 20 μl of RPMI1640 with 10% FCS, and 5 μl of the skin extracts was added and incubated at 37°C for 3 h. Bacteria were then serially diluted in PBS and plated on TSB agar (tryptic soy broth; 30 g/l, Sigma-Aldrich, Bacto™agar; 10 g/l, BD Bioscience, Sparks, MD, USA) for direct colony count and determination of CFU. Amount of mCRAMP was determined by immuno-dot blot using synthetic CRAMP peptides as references, and mean and SE of three mice were plotted on the graph. Processing of mCRAMP was analyzed by SELDI-TOF-MS as described previously with rabbit anti-CRAMP Ab (14) (1.55 mg/ml) instead of antihCAP18/LL-37 Ab. To delete cathelicidin activity from skin extracts, skin extracts were incubated with immobilized anti-CRAMP Ab, and supernatants were analyzed as cathelicidin-immunoabsorbed skin extracts. Depletion of cathelicidin was confirmed by a second SELDI-TOF analysis. Serine protease activity with the presence of 5 mM EDTA and 1 μg/ml pepstatin was determined using EnzCheck® as described previously.

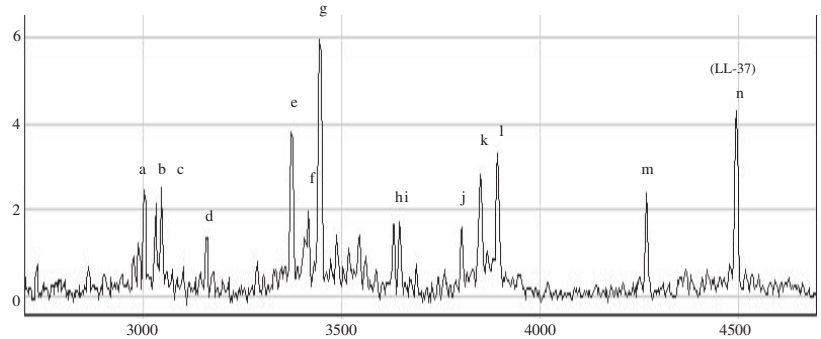
## RESULTS

### Unique cathelicidin peptides are constitutively present on human skin

Enzymatic processing of cathelicidin peptides dictates their function as an antimicrobial or immune modifying molecule. These observations compelled us to first directly identity the human cathelicidins in normal skin, a site where the presence of cathelicidin is important for defense against bacterial and viral infections. We hypothesized that LL-37, the form of cathelicidin first identified from human neutrophils, may not be the major form of cathelicidin normally present in the skin. To identify the native cathelicidin peptides, normal skin was extracted and peptide mass determined by SELDI-TOF-MS. Multiple cathelicidin peptides were detected in human skin samples in this system, with LL-37 representing only 13.7% of the total peptides detected (peak "n" in **Fig. 1**). The presence of this spectrum of cathelicidin peptides suggested that local proteolysis has a major influence on the identity of cathelicidin. Furthermore, these findings show that prior conclusions were inaccurate when LL-37 was assumed to be the form of cathelicidin in skin.

### Identification of cathelicidin processing enzymes at the skin surface

To identify enzymes at the skin surface that cleave human cathelicidin, protease activity collected from the skin surface of healthy volunteers was first detected by a fluorescence-conjugated casein-based assay. Protease activity was detected by the increase in fluorescence over time compared with that seen when substrate was incubated in a buffer solution of similar ionic composition (**Fig. 2*A***). Enzyme activity was detectable for up to 4 days after incubation. To determine the nature of these proteases, a panel of inhibitors that distinguish between the various major protease families was included. The only effective inhibitors were aprotinin and AEBSF, which are inhibitors of serine protease (Fig. 2*B*). Bestatin (amino peptidase inhibitor), E-64 (cystein protease inhibitor), N.E.I. (neutrophil elastase inhibitor), L.E.I. (leukocyte elastase inhibitor), EDTA (metalloprotease inhibitor), and pepstatin (asparate protease inhibitor) were ineffective. Chymostatin and leupeptin, which specifically inhibit chymotrypsin-like and trypsin-like serine proteases, respectively, were used to characterize this more precisely. Under these conditions, chymostatin suppressed protease activity whereas leupeptin had a minimum effect, suggesting a chymotrypsin-like serine protease was most active (Fig. 2*C*).




| peak | a | b | c | d | e | f | g | h | I | j | k | l | m | n | total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| % | 3.7 | 3.3 | 4.4 | 2.4 | 11.5 | 3.2 | 22.6 | 3.1 | 5.1 | 3.0 | 7.9 | 9.9 | 6.2 | 13.7 | 100 |

**Figure 1.** Processing of cathelicidin peptides in human skin. Cathelicidin peptides in human skin extracts were captured with anti-LL-37 Ab, and mass sizes were examined with SELDI-TOF-MS. One representative data set from three individuals is shown. The ratio of each peptide was determined by the area of the peaks and is listed. LL-37, the major cathelicidin peptide in neutrophils, was detected in peak marked as n.

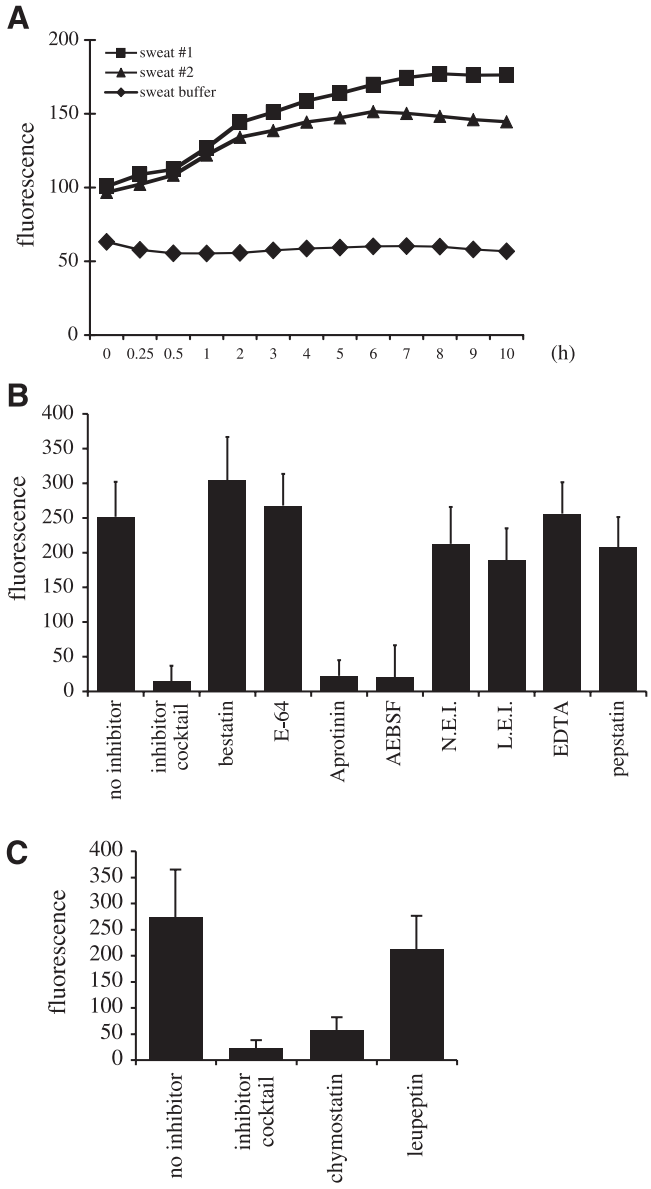


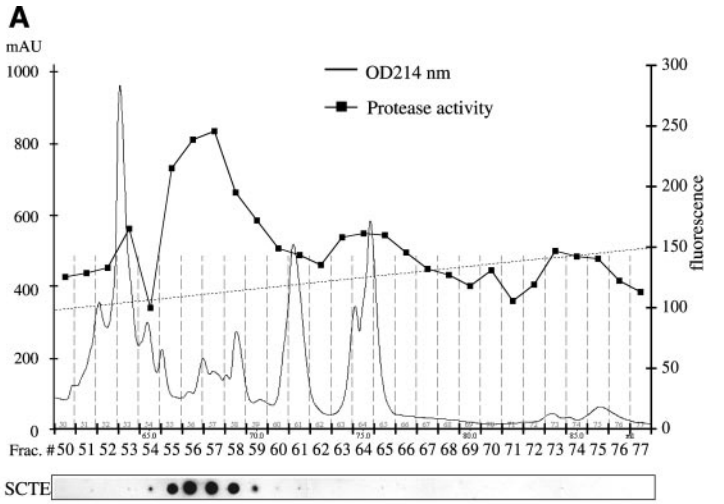

**Figure 2.** Protease activity at the human skin surface. *A)* Soluble material recovered from the skin surface was incubated with fluorescence-conjugated casein substrate, and activity was estimated based on the generation of fluorescent product as described in Materials and Methods. *B)* Surface protease activity was examined as in panel *A* at 37°C for 24 h in the presence of inhibitor cocktail (nonspecific protease inhibitor mixture), aprotinin and AEBSF (serine protease inhibitors), bestatin (amino peptidase inhibitor), E-64 (cystein protease inhibitor), N.E.I (neutrophil elastase inhibitor), L.E.I (leukocyte elastase inhibitor), EDTA (metalloprotease inhibitor), and pepstatin (asparate protease inhibitor). Data are presented as the mean and SE of three independent samples. *C)* Soluble protease activity was examined in the presence of serine protease inhibitors: chymostatin (specifically inhibits chymotrypsin-like proteases), leupeptin (specifically inhibits tryspsin-like proteases). Data are presented as the mean and SE of three independent samples.

Surface enzyme activity was next concentrated by centrifugal dialysis and fractionated by reverse-phase chromatography (**Fig. 3*A***). Fractions numbered 55 to 59 showed the greatest protease activity against a casein substrate and could cleave LL-37 to several smaller peptides (Fig. 3*B*). The products cleaved from LL-37 were evaluated by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and their protein sequences were deduced from the molecular weights determined in the analysis (Fig. 3*C*). Radial diffusion assay revealed that some of these fragments, such as KS-30, KS-27, KR-20, and LL-23, showed antimicrobial activity against *S. aureus mprF*, whereas other shorter peptides lost antimicrobial activity (data not shown). The deduced cleavage sites producing the peptide patterns in Fig. 3*C* suggested that two different serine proteases were involved: one with trypsin-like substrate specificity that hydrolyze after Arg residues and the other with chymotrypsin-like substrate specificity that cleaves at after Phe residues. Therefore, tryptic and chymotryptic serine proteases were considered candidates for skin surface cathelicidin proteases.

Members of the kallikrein family of serine proteases have been detected in the stratum corneum. Two proteases well established as human skin are stratum corneum tryptic enzyme (SCTE, kallikrein 5/KLK5, hK5) and stratum corneum chymotryptic protease (SCCE, kallikrein 7/KLK7, hK7) (27, 28). To determine whether these enzymes were present in the skin surface preparations and whether they colocalized with cathelicidin in skin, antiserum to each protease was used. SCTE immunoreactivity coeluted with fractions that showed protease activity (Fig. 3*A*). Immunohistochemistry of normal human skin demonstrated that SCTE and cathelicidin are colocalized in the granular to spinous layer, and LL-37 alone is detected at the uppermost layer of stratum corneum (**Fig. 4*A***).

Next, to determine what proportion of serine protease activity from keratinocytes could be attributed to kallikrein activity, we examined the influence of siRNA targeting of SCTE and SCCE mRNA on the level of caseinolytic activity secreted from keratinocytes. SCTE and SCCE siRNA suppressed SCTE mRNA expression to 56% of control and SCCE mRNA to 72%, respectively (**Fig. 5*A***), and both SCTE and SCCE siRNA significantly reduced human keratinocytes protease activity (Fig. 5*B*).

Human cathelicidin is initially synthesized as a precursor protein hCAP18 (26). To determine whether SCTE and SCCE could cleave hCAP18, recombinant hCAP18 was incubated with recombinant SCTE or SCCE (25), and cleavage was examined by Western blot. Polyclonal antibody (pAb) against the cathelin domain (recognizing the N-terminal cathelin domain, but not detecting cleaved C-terminal peptides, ref. 26) detected bands of ~12 kDa and 10 kDa after SCTE treatment and a ~14 kDa band after SCCE treatment (**Fig. 6*A***). Ab against LL-37 (recognizing the C-terminal 37 amino acid mature peptide domain) revealed that




C

| peak | Peptide sequence | abbreviation | MW |
|---|---|---|---|
| *a* | NLVPRTES | NL-8 | 914.4 |
| *b* | LRNLVPRTES | LR-10 | 1184.7 |
| *c* | KRIVQRIKDF | KR-10 | 1302.7 |
| *d* | IKDFLRNLVPRTES | IK-14 | 1686.7 |
| *e* | LLGDFFRKSKEKIGKEF | LL-17 | 2042 |
| *f* | LLGDFFRKSKEKIGKEFKRIVQR | LL-23 * | 2823 |
| *g* | LLGDFFR | LL-7 | 867.5 |
| *h* | KRIVQRIKDFLRNLVPRTES | KR-20 * | 2468 |
| *i* | KSKEKIGKEFKRIVQRIKDFLRNLVPR | KS-27 * | 3326 |
| *j* | KSKEKIGKEFKRIVQRIKDFLRNLVPRTES | KS-30 * | 3643 |
| *k* | LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES | LL-37 * | 4491 |

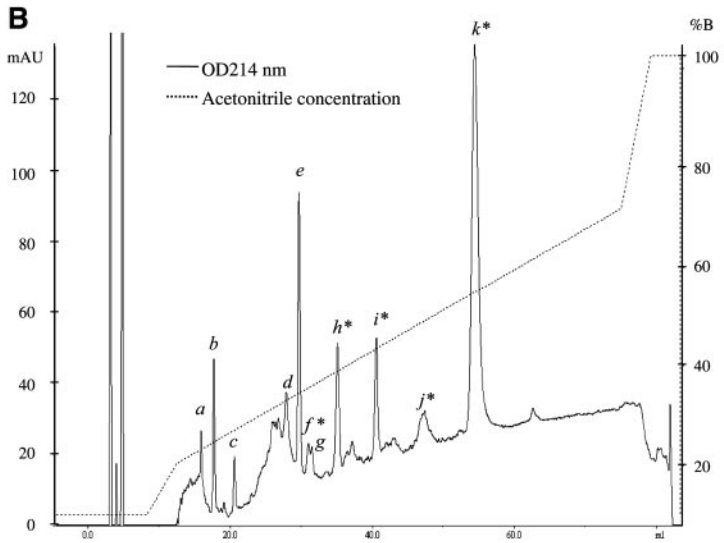



**Figure 3.** Skin production of SCTE cleaves LL-37 to shorter peptides. *A)* Concentrated surface protease preparations were separated on C2/C18 reverse phase chromatography and the activity of each fraction was measured by incubating with fluorescence-conjugated casein substrate. Immunoreactivity of each fraction to SCTE Ab was examined by dot blot. Fraction #55 to #59 had strong protease activity and immunoreactivity to SCTE Ab. *B)* Synthetic LL-37 peptide was incubated with fraction #55, and digested peptides were separated on C18 reverse phase chromatography. Antimicrobial activity of each fraction was examined by radial diffusion assay, and peptides that have antimicrobial activities were indicated by an asterisk. *C)* Molecular mass of each peak indicated in panel *B* by small characters was determined by MALDI-TOF-MS and listed with its peptide sequence. Asterisks indicate the peptide with antibacterial activity.

SCTE generated a small fragment of ~6 kDa, and SCCE generated an ~8 kDa band. To determine the sequence of these small fragments directly, SCTE-treated hCAP18 peptides were isolated by chromatography and the molecular mass were determined by MALDI-MS. The small peptide cleaved by SCTE had a molecular mass of 4491, identifying it as LL-37 (Fig. 6*B*). On the other hand, treatment of hCAP18 with SCCE generated multiple peptides whose abundance was insufficient to identify by this approach (data not shown).

### SCTE and SCCE cleave LL-37 to shorter peptides

Unlike the *in vitro* conditions described in Fig. 6, the cleaved cathelin domain is rarely detected *in vivo*. This protein has activity as a protease inhibitor and antimicrobial (26), and structural studies suggest the cathelin domain has a pocket in which the C-terminal LL-37 peptide may bind (29). As this may protect the peptide from further proteolysis, we next examined conditions that reflect those observed in skin. LL-37 was incubated with recombinant SCTE and SCEE, and the peptide products were determined. As shown in the time course of **Fig. 7*A***, the major products of SCTE digestion of LL-37 after 1 h of incubation were KS-29, KS-30 (peak g), and KS-22 (peak f). KS-22 was derived from KS-30 by second cleavage eight residues from the C terminus. These three peptides have antimicrobial activity, indicating that at early times of digestion SCTE will generate biologically functional peptides. However, after 3 h the peaks with antimicrobial activity (peaks f, g) began to decline, indicating further processing to small nonfunctional peptides.

Cleavage of LL-37 by SCCE was less efficient than by SCTE, as the LL-37 peak disappeared much more slowly in the incubation containing SCCE than in that containing SCTE. Consistent with its predicted specificity, SCCE cleaved LL-37 after Phe residues (Fig. 7*D*). Although SCCE generated peptides RK-31 and KR-20 (peaks o and n in Fig. 7*C*, respectively), which had antimicrobial activity and were seen on the surface of skin, the existence of these peptides appeared to be short-lived. Their quantities showed a detectable change with time, and were no longer present after 24 h of incubation. Thus, their production appears highly transient, with digestion to

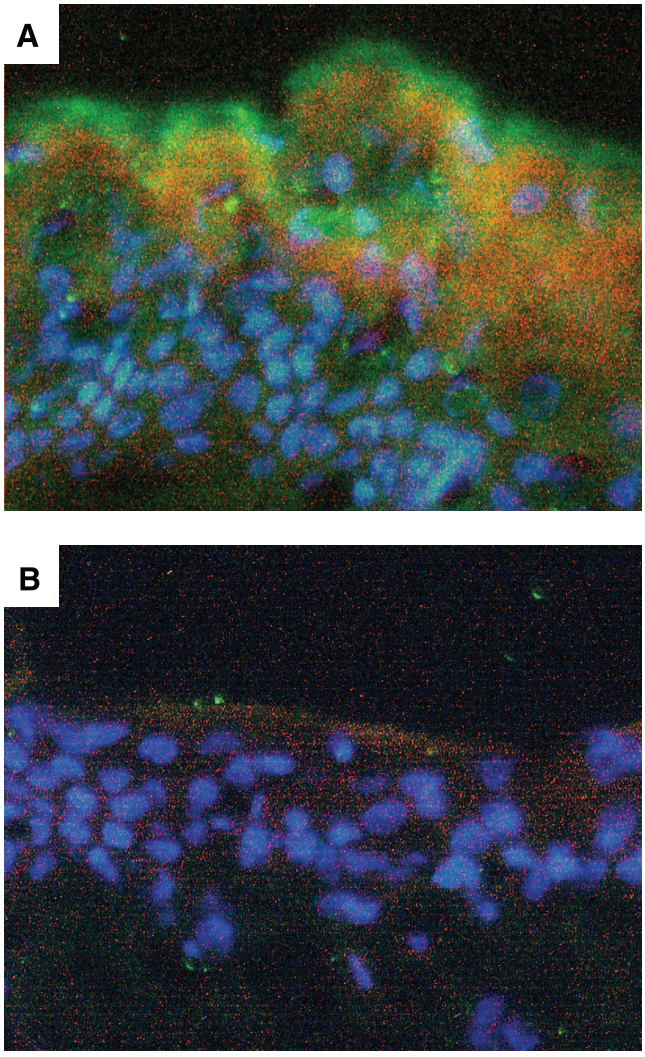



**Figure 4.** Cathelicidin and SCTE are colocalized in human skin. *A, B)* Localization of cathelicidin and SCTE in normal human skin were examined by immunofluorescence. *A)* Red-TRIC represents SCTE and green-FITC represents cathelicidin. *B)* control IgG. Nuclei were stained with blue-4′,6′-diam idino-2-phenylidole in both. Original magnification, ×400.

smaller fragment proceeding faster than their generation.

### Serine protease activity controls antimicrobial function in skin

We next tested cathelicidin proteolysis in an *in vivo* model. Lympho-epithelial Kazal-type related inhibitor (LEKTI) is a serine protease inhibitor encoded by *SPINK5* (serine protease inhibitor Kazal-type 5) gene (30). LEKTI is expressed in the epidermis, and mutations of *SPINK5* gene are found in the human condition, Netherton syndrome, leading to a disruption in skin barrier function (31). The activities of SCTE and SCCE are increased in *SPINK5*-deficient mice, and these mice mimic the epidermal dysfunctions in Netherton syndrome (32). We extracted skin of neonatal *SPINK5*-deficient mice and of WT littermates and confirmed that protease activity in skin extracts from *SPINK5*-deficient mice was higher than WT littermates (**Fig. 8*A***). Direct analysis of the effect of recombinant SCTE and SCCE on bacterial growth showed that these enzymes are not inherently antimicrobial. Furthermore, the abundance of total immunoreactive cathelicidin expressed in *SPINK5*-deficient mice was not significantly different from WT littermates (Fig. 8*B*). Despite the similarity in the expression of cathelicidin, skin extracts from *SPINK5*-deficient mice inhibited growth of *S. aureus mprF* whereas skin extracts from WT littermates did not (Fig. 8*C*). Other Gram-positive and negative bacterial species were similarly inhibited by *SPINK5*-deficient, but not WT mice including WT *S. aureus*, methycillin-resistant *S. aureus*, group A *Streptococcus*, and *Pseudomonas aeruginosa* (data not shown). Immunoabsorption of skin extracts with anti-CRAMP Ab effectively removed mCRAMP and precursor protein based on HPLC and SELDI-TOF analysis (data not

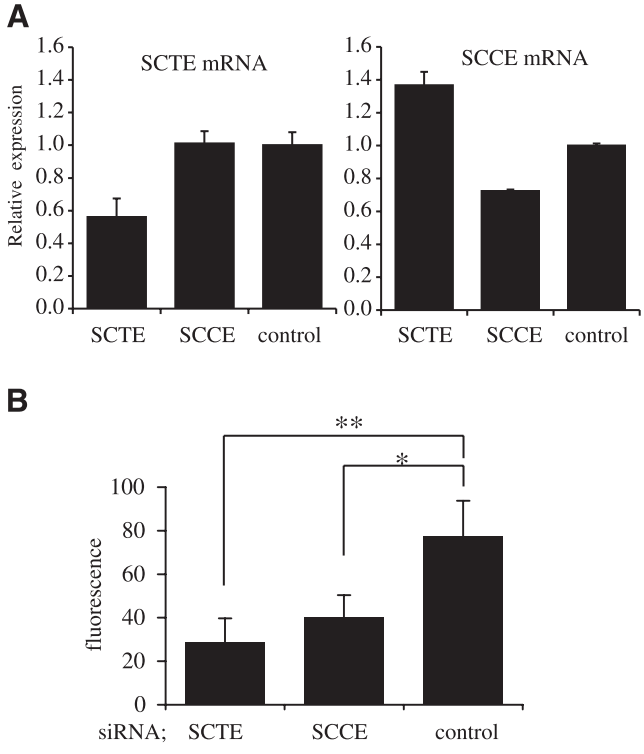



**Figure 5.** SCTE and SCCE are major serine proteases in human keratinocytes. *A)* Expression of SCTE and SCCE mRNA in HaCat cells treated with siRNA to either SCTE or SCCE for 72 h were analyzed by quantitative RT-PCR. X-axis labels indicate siRNA preparation used or transfection control (control). The mean and SE of three independent experiments are plotted. Data demonstrate selectivity of siRNA treatment for each enzyme. *B)* HaCat cells were treated with SCTE or SCCE siRNA for 72 h. Culture media was collected and protease activities in media were measured by incubating with fluorescence-conjugated casein substrate at 37°C for 24 h. The mean and of three independent experiments were plotted. Data show significant (*$P<0.05$, **$P<0.01$) contribution of both enzymes to total protease activity.

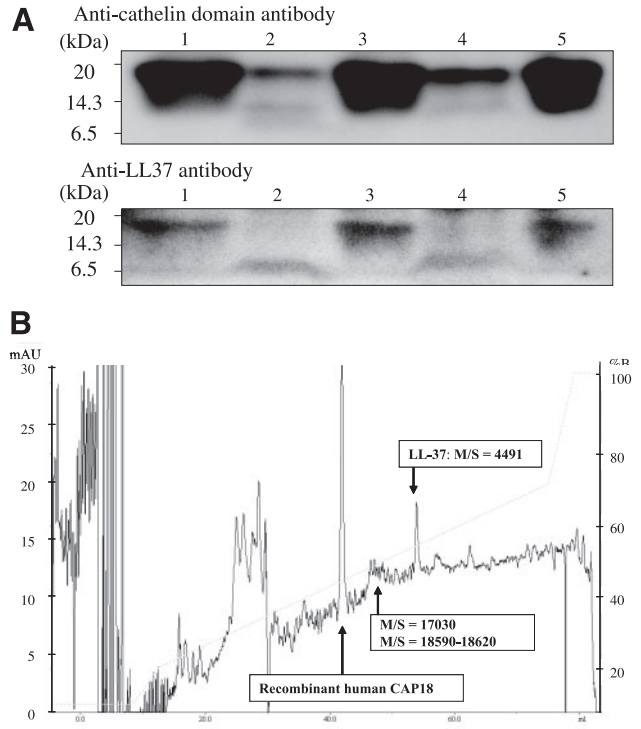



**Figure 6.** SCTE and SCCE cleave hCAP18 to shorter peptides. *A)* Recombinant hCAP18 was incubated with recombinant SCTE (1 μM) or SCCE (1 μM) at 37°C for 2 h. Cleavage of hCAP18 was monitored by Western blot with Ab against cathelin domain (upper panel) or Ab against LL-37 peptide domain (lower panel). Lane 1, hCAP18 +SCTE buffer; 2, hCAP18 +SCTE; 3, hCAP18 +SCCE buffer; 4, hCAP18 +SCCE; 5, hCAP18 alone. *B)* Recombinant hCAP18 was incubated with recombinant SCTE at 37°C for 2 h. Digested peptides were separated on C18 reverse phase chromatography. Molecular mass of each peak was determined by MALDI-TOF-MS and indicated on chromatograph.

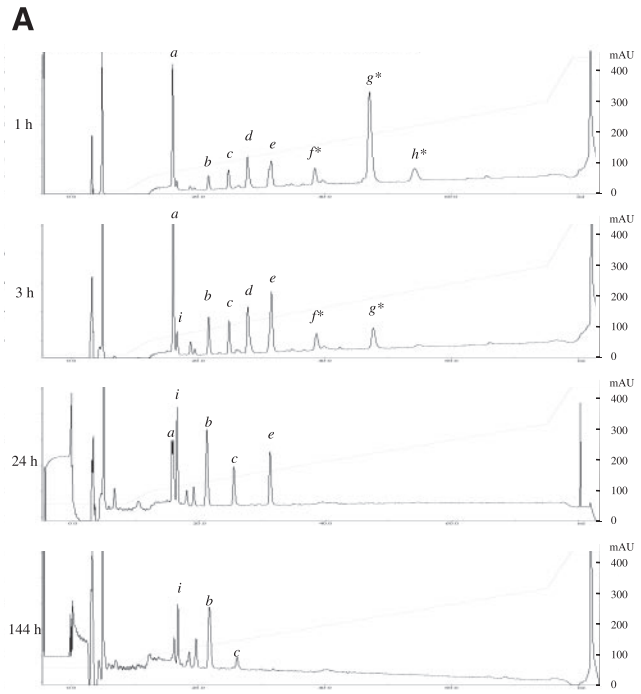
shown) and eliminated antimicrobial activity detected in the in *SPINK5*-deficient mice (Fig. 8*A*). SELDI-TOF-MS analysis confirmed that mCRAMP in *SPINK5*-deficient mouse skin was processed to shorter peptides (data not shown). The mass of the major peak in *SPINK5*-deficient mouse was 3878 Da and matched with the mCRAMP1 (GLL-34) peptide (33). In WT littermates, major peaks of ~17 and 20 kDa matched the size of mCRAMP proprotein without and with signal peptide, respectively. These data suggested that processing of cathelicidin in SPINK5-deficient mice skin augmented antimicrobial activity.

## DISCUSSION

Cathelicidin antimicrobial peptides such as human LL-37 and mouse mCRAMP are essential molecules in innate immune defense. Prior work has suggested that they play dual roles in protecting the host: they act as natural antibiotics and also modify leukocyte recruitment (10). In the present study we show that cathelicidin, in either the proform hCAP18 or the peptide form LL-37 released upon neutrophil recruitment, is susceptible to proteolytic processing by serine proteases belonging to the tissue kallikrein family. This processing generates novel peptides with antimicrobial activity and suggests that cathelicidins in skin are more diverse than previously reported. Their function ultimately depends on the activity of proteases at the epithelial surface. Inhibitors of serine proteases such as LEKTI (25) and anti-leukoprotease/SLPI (34) regulate this process (**Fig. 9**). Although not directly evaluated here, these findings also suggest that other serine protease inhibitors such as SKALP/elafin, a potent proteinase 3 inhibitor expressed in human epidermis (35), may affect hCAP18 proteolysis through the regulation of proteinase 3 activity.

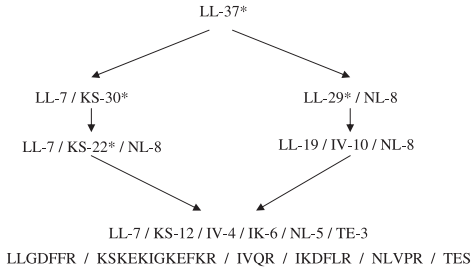
Evidence to support the role of serine proteases to modify the proinflammatory activity of cathelicidin comes from observations of their distribution in the epidermis and their biochemical activity. SCTE and cathelicidin are colocalized in the granular layer, but cathelicidin is dominant in the cornified layer of normal human skin. Since cathelicidin and kallikreins are both stored in lamellar granules and secreted from keratinocytes (36, 37), their localization in skin suggests that after secretion, hCAP18 can be efficiently cleaved by kallikreins and subsequently released at the surface to form an antimicrobial barrier. If processed to forms that are not inherently chemotactic or capable of stimulating host cells to release chemotactic factors such as IL-8, this mechanism explains why the constitutive presence of cathelicidins on the skin is not inflammatory under normal conditions or in transgenic models (38) despite the potent capacity of LL-37 or mCRAMP to induce cytokine release. Kallikrein in the normal human epidermis has the potential to both activate and degrade cathelicidin to inactive peptides.

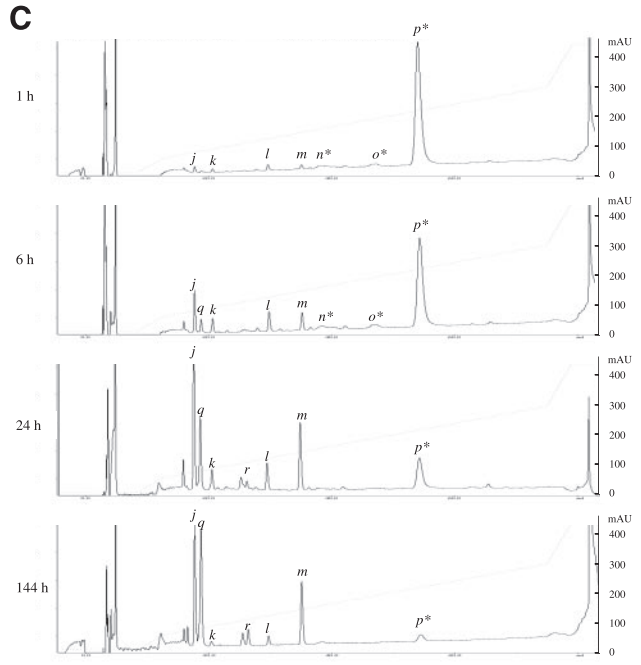
Analysis of the enzymatic processing of cathelicidin suggests that two steps are required: activation from the precursor hCAP18 and subsequent processing to modify activity. We found that two cleavage patterns were generated in the skin from cathelicidin precursor proteins: cleavage C-terminal to arginine (R), and cleavage C-terminal to phenylalanine (F). SCTE was effective in generating LL-37 from the precursor hCAP18 and can subsequently produce KS-30, KS-22, and LL-29 from LL-37. RK-31 and KR-20 peptides are cleaved by SCCE from LL-37. The ability to generate these antimicrobial peptides quickly and in high amounts relative to other peptides suggests that the cleavage sites producing these peptides are preferred by SCTE. SCTE has trypsin-like activity, and the preference of SCTE for arginine over lysine has been noted in model substrates (39). Thus, SCTE prefers to cleave C-terminal to arginine residues in LL-37, although LL-37 has several arginine and lysine residues in its sequence and may best be considered a generator of antimicrobial activity rather than a degrader of LL-37. Conversely, data




B

| Peak | sequence | abbreviation |
|---|---|---|
| *a* | NLVPRTES | NV-8 |
| *b* | IKDFLR | IK-6 |
| *c* | IVQRIKDFLR | IV-10 |
| *d* | LLGDFFRKSKEKIGKEFKR | LL-19 |
| *e* | LLGDFFR | LL-7 |
| *f* | KSKEKIGKEFKRIVQRIKDFLR | KS-22* |
| *g* | KSKEKIGKEFKRIVQRIKDFLRNLVPRTES<br>LLGDFFRKSKEKIGKEFKRIVQRIKDFLR | KS-30*<br>LL-29* |
| *h* | LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES | LL-37* |
| *i* | NLVPR | NL-5 |




D

| Peak | sequence | abbreviation |
|---|---|---|
| *j* | RKSKEKIGKEF | RK-11 |
| *k* | KRIVQRIKDF | KR-10 |
| *l* | LLGDFFRKSKEKIGKEF | LL-17 |
| *m* | LLGDFF | LL-6 |
| *n* | KRIVQRIKDFLRNLVPRTES | KR-20* |
| *o* | RKSKEKIGKEFKRIVQRIKDFLRNLVPRTES | RK-31* |
| *p* | LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES | LL-37* |
| *q* | LRNLVPRTES | LR-10 |
| *r* | LLGDF | LL-5 |

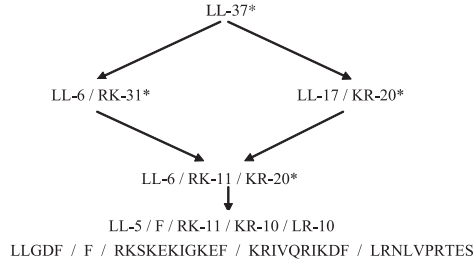



**Figure 7.** SCTE and SCCE cleave synthetic LL-37 peptide to shorter peptides. *A, C)* Synthetic LL-37 was incubated with 10 nM of SCTE (*A*) or SCCE (*C*) at 37°C for the periods indicated. Digested peptides were separated on C18 reverse phase chromatography. Antimicrobial activity of each fraction was examined by radial diffusion assay, and peptides that have antimicrobial activities were indicated by an asterisk. *B, D)* Molecular mass size of each peak indicated by alphabet in chromatography was determined by MALDI-TOF-MS and listed with its peptide sequence (*B*: peptides generated by SCTE, *D*: peptides generated by SCCE). Asterisks indicate the peptide with antibacterial activity.

obtained with SCCE suggested this enzyme might be considered a degrader of LL-37 rather than a generator of antimicrobial peptides. SCCE has been shown to prefer tyrosine over phenylalanine and to be less able to cleave C-terminal to phenylalanine in model substrates (34). Therefore, SCCE is less effective than SCTE in cleaving LL-37 to generate smaller peptides, and smaller peptides may be easier to be accessed and degraded by SCCE. Thus, SCCE may serve as an inactivator, an important role when the chemotactic activity of LL-37 is no longer beneficial to the defense process.

It is debatable whether only SCTE and SCCE digest

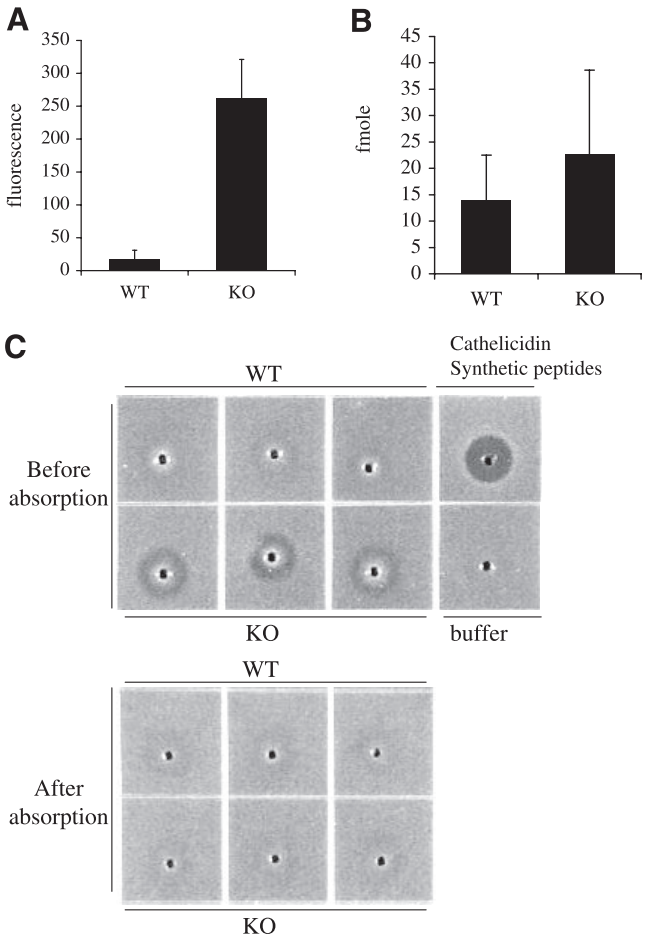



**Figure 8.** Altered processing of cathelicidin in *SPINK5*-deficient mice. *A)* Serine protease activity in the skin of *SPINK5*-deficient mice and WT littermates using casein-based protease assay with the presence of protease inhibitors (EDTA and pepstatin). The mean and SE of three individuals are plotted. *B)* The amount of mCRAMP in the skin extracts from 8 mm punch biopsy was determined by immuno-dot blot with synthetic CRAMP peptide as a reference. The mean and SE of three individuals are plotted. *C)* Antimicrobial activity against *S. aureus mprF* were examined from skin extracts by radial diffusion assay. Skin extracts (2 μl) from three *SPINK5*-deficiency mice (KO) and three WT littermates (wild-type) are shown. Upper two rows show the inhibitory area of total extract (before absorption) and lower two rows show extracts treated with anti-CRAMP Ab (after absorption). Synthetic cathelicidin peptide (LL-37, 32 μM, 1 μl) and buffer were spotted as control.

hCAP18 and generate cathelicidin antimicrobial peptides at the complex human skin surface. Human skin epidermis and appendages express several kallikrein proteases, and these proteases may act together (40). Treatment of human keratinocytes with siRNA for SCTE and SCCE showed a decrease of serine protease activity, suggesting that SCTE and SCCE are major serine proteases in culture. Purification of crude skin extracts was detected SCTE in fractions that cleaved LL-37. The chymotryptic serine protease SCCE is also a candidate because protease activity in the skin surface was suppressed the most by the chymotrypsin-specific inhibitor chymostatin, and both chymotrypsin-like cleavage patterns and trypsin-like cleavage patterns were observed with LL-37 as substrate. However, unlike SCTE, we did not detect SCCE in protease-active fractions with commercially available anti-SCCE antibodies (data not shown). This may reflect a relative lack in the sensitivity of this Ab, as our other data based on RNAi and uses of recombinant enzymes suggest that SCTE and SCCE can both cleave cathelicidin. Alternatively, assays of total proteolytic activity from the skin surface based on casein substrate may not detect either SCCE or SCTE, and may actually detect another chymotrypsin. Furthermore, in the current study SELDI-TOF-MS revealed that diverse cathelicidin peptides exist in skin, but these were not identical to the peptides isolated earlier by standard HPLC approaches from human sweat (24). Because the current samples were extracted from whole skin, these may have been generated by proteases from various cells in skin such as keratinocytes, endothelial cells, fibroblast, dendritic cells, and some inflammatory cells, and not only surface proteases such as SCTE and SCCE. Alternatively, the purification methods may have influenced these results. However, taken together with results derived from tissues and recombinant enzymes, the current observations strongly support a major role for both SCTE and SCCE in processing cathelicidin in skin.

The actual identity (size and sequence) of cathelicidin peptides at various locations has only recently come into question. Subsequent to the initial cloning of human cathelicidin from a human bone marrow library, a 39 aa peptide designated FALL-39 was predicted (41). Subsequent purification and direct sequencing from human neutrophils identified the actual peptide as a 37aa peptide, LL-37, and proteinase 3, an elastase-like serine protease stored in neutrophil granules, was suggested to be the enzyme responsible for the processing of precursor protein hCAP18 to gener-

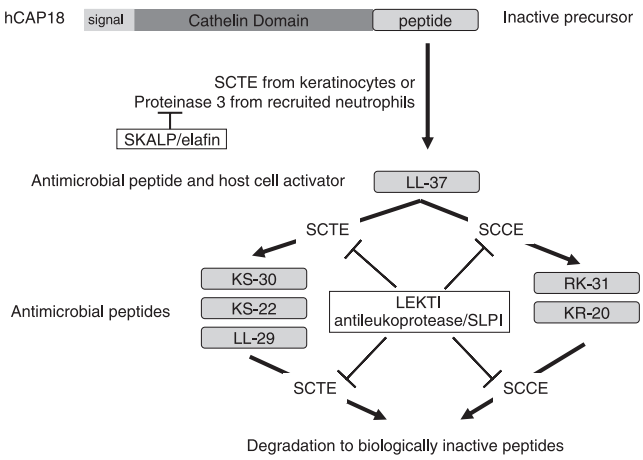



**Figure 9.** Schematic model of cathelicidin processing by skin surface proteases. Multiple enzymatic activation events are required for control of cathelicidin activity. Initial processing of inactive hCAP18 precursor protein is followed by further processing to peptides with alternative antimicrobial and immunostimulatory activities. LEKTI regulates activation and final degradation.

ate LL-37 (21). More recently, urogenital epithelium was found to process hCAP18 to ALL-38 by the prostate-derived protease gastricsin (22). In contrast to these longer peptides, several alternate cathelicidin antimicrobial peptides were detected at the skin surface (23), an interface that has been shown to rely on the expression of cathelicidin for defense against microbial invasion (4). These observations suggested that previous conclusions claiming LL-37 was the sole human cathelicidin were an oversimplification.

The significance of variable processing of cathelicidin to peptides other than LL-37 lies in the observations that LL-37 induces chemokine release and can function in host cell stimulation through direct receptor-ligand interactions such as the stimulation of formyl peptide receptor-like 1 (42) or through transactivation of the epidermal growth factor (EGF) receptor (43). A comparison of the ability of LL-37 and shorter peptides to induce CXCL8 secretion has shown that shorter peptides are less able to induce inflammatory reactions but are more potent antimicrobials (19). LL-37 acts as a chemoattractant of neutrophils, monocytes, T cells, and mast cells (42, 44, 45). Human cathelicidin peptide LL-37 and mouse cathelicidin CRAMP also show angiogenic properties (46). However, smaller cathelicidin peptides such as KR-20, RK-31, and KS-30 have greater antimicrobial activity and are less able to induce CXCL8 (19, 23). Additional cathelicidin peptides were found at the skin surface in the present work (LL-23, LL-29, and KS-27), confirming the antimicrobial activity of such shorter cathelicidins.

Based on these observations, it is reasonable to hypothesize that after infection or wounding, cathelicidin expression in skin will shift from the short form cathelicidins generated and inactivated by the kallikreins toward a predominance of the LL-37 form released from neutrophils. This would act to amplify the recruitment of neutrophils through its action as a chemoattractant and host cell stimulant. Upon resolution of the microbial challenge or injury, epidermal protease activity will process LL-37 to forms lacking proinflammatory activity and thus re-establish an effective microbial shield without further increasing tissue damage as a result of inflammation.

The importance of maintaining normal activity of serine proteases for control of antimicrobial activity was shown directly in the LEKTI-deficient mice. A lack of this serine protease inhibitor increased the balance of serine protease activity, increased antimicrobial activity, and increased the proportion of mature cathelicidin in the skin. The increase of cathelicidin was not due to an increase in leukocyte recruitment or keratinocyte synthesis since these newborn mice were euthanized within 8 h of birth, and there was neither infection nor inflammatory cell infiltration at that stage. However, the increase in the antimicrobial properties of the skin of SPINK5-deficient mice is not sufficient to compensate for the increased susceptibility to infection due to the severe disruption in barrier properties of the epidermis. Similarly, patients with Netherton syndrome develop frequent and sometimes severe skin infections (47).

Several antimicobial molecules in addition to cathelicidin are produced in skin and may be subject to similar enzymatic regulation. These include defensins and SKALP/elafin (48). Human β-defensin 2 (HBD-2) and SKALP/elafin have been reported to be strongly expressed in the epidermis of Netherton syndrome (49). Like cathelicidins, HBD-2 and SKALP/elafin are inducible by bacterial infection and inflammatory reactions, but low in normal skin (48, 50). Reports of high HBD-2 and SKALP/elafin in Netherton syndrome may reflect the consequence of repeated skin infection and inflammation due to barrier disruption (51). In the current mouse model, the use of neonatal mice minimized this effect and permitted evaluation of antimicrobial function as regulated by enzymatic processing distinct from the quantity of the gene expressed.

Taken together, these data show that the balance of serine protease activity in the skin leads directly to the control of innate antimicrobial activity. All components of this system, their triggers, and individual modifying agents remain to be elucidated. Our findings show that kallikreins are at least one important part of this system, and demonstrate that several cathelicidin antimicrobial peptides are generated at the barrier between the host and the external environment. The balance of proteolytic activity will modulate cathelicidin function to direct diverse antimicrobial and stimulatory activities; this is a previously unrecognized mechanism for regulation of immune defense. Furthermore, these findings show that control of microbial growth and an inflammatory response may rest in the balance of epithelial proteolytic activity. Fj

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