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# Postsecretory Processing Generates Multiple Cathelicidins for Enhanced Topical Antimicrobial Defense<sup>1</sup>

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The production of antimicrobial peptides and proteins is essential for defense against infection. Many of the known human antimicrobial peptides are multifunctional, with stimulatory activities such as chemotaxis while simultaneously acting as natural antibiotics. In humans, eccrine appendages express DCD and CAMP, genes encoding proteins processed into the antimicrobial peptides dermcidin and LL-37. In this study we show that after secretion onto the skin surface, the CAMP gene product is processed by a serine protease-dependent mechanism into multiple novel antimicrobial peptides distinct from the cathelicidin LL-37. These peptides show enhanced antimicrobial action, acquiring the ability to kill skin pathogens such as *Staphylococcus aureus* and *Candida albicans*. Furthermore, although LL-37 may influence the host inflammatory response by stimulating IL-8 release from keratinocytes, this activity is lost in subsequently processed peptides. Thus, a single gene product encoding an important defense molecule alters structure and function in the topical environment to shift the balance of activity toward direct inhibition of microbial colonization. *The Journal of Immunology*, 2004, 172: 3070–3077.

Peptides with antimicrobial activity are found throughout nature and are known to be important for the immune defense of plants, insects, and animals (1). Many of these peptides exhibit a broad spectrum of antimicrobial activity, inhibiting or killing Gram-positive and Gram-negative bacteria, fungi, and viruses. In mammals, antimicrobial peptides belonging to the cathelicidin family have been shown to be important for the antimicrobial efficacy of neutrophils, macrophages, and mast cells (2–6). In addition, epithelia of lung, gut, urinary bladder, oral mucosa, and skin produce antimicrobial peptides of the defensin and cathelicidin families constitutively or in response to injury (7–9). At the epithelial interface with the external environment, these molecules are thought to serve as a rapid first line defense for inhibition of microbial proliferation and invasion.

In mammalian skin, cathelicidins have been directly shown to be essential for defense against invasive bacterial infection by group A streptococcus (10). Found in abundance in neutrophils and mast cells, the cathelicidins are expressed at relatively low levels in normal keratinocytes, but are rapidly induced during inflammation (11). The recruitment of cathelicidin-rich cells and the increase in expression by the epithelial keratinocytes lead to accumulation of cathelicidins in wound fluid and in the overlying crust (12). Recently, cathelicidins and dermcidin have also been found constitutively produced by the eccrine apparatus and secreted constitutively into human sweat (13, 14). In this scenario, antimicrobial

activity becomes available at the most external interface, providing an inhibitory barrier to infection.

The clinical consequences of antimicrobial peptide expression are demonstrated by observations that patients with atopic dermatitis lack the ability to increase cathelicidins and defensins in response to inflammatory stimuli (15). As patients with atopic dermatitis are uniquely susceptible to infection compared with normal individuals or those with elevated expression, as seen in psoriasis, this first-line immune defense mechanism appears to have an essential function for resistance against skin infections. Similarly, patients with Kostmann syndrome, a rare inherited disorder characterized by frequent infections and neutrophil dysfunction, have a deficiency in production and processing of cathelicidin (16). Such emerging clinical associations support the need to further explore the function and regulation of this evolutionarily ancient aspect of the human immune system.

Most antimicrobial peptides are synthesized as inactive prepro-peptides that require enzymatic processing for release of active peptides. Lack of processing, such as seen in  $\alpha$ -defensins from Panath cells of matrilysin-deficient mice (17), leads to impaired clearance of infection in the gut. Similarly, inhibition of processing of porcine cathelicidins by elastase impairs bacterial clearance in wounds (18). The enzymatic processing of cathelicidins from a pro-protein to a two-component solution consisting of the cathelin prodomain and the C-terminal cationic antimicrobial peptide, LL-37, can have multiple consequences for immune defense. The cathelin domain is itself antimicrobial and functions to inhibit cysteine proteases such as cathepsin L (19). LL-37, although functional as a broad spectrum antimicrobial, can also stimulate chemotaxis and angiogenesis by binding formyl peptide receptor-like-1 (20, 21) and improve re-epithelialization to enhance wound repair (22).

In this study we determined whether additional antimicrobial peptides are present in the most superficial barrier topically provided by human sweat. Our findings suggest that human cathelicidin is further processed and enables a shift in biological activity toward antimicrobial function and away from the ability to stimulate a host response.

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## Materials and Methods

### Sweat collection and processing

Sweat was collected on paper tissues (Kimwipes; Kimberly-Clark, Neenah, WI) from healthy volunteers after exercise as previously described (13). After collection, 20 ml of sweat was centrifuged at  $2000 \times g$  for 15 min at  $4^{\circ}\text{C}$ , filtered through a 0.20- $\mu\text{l}$  filter (Acrodisc syringe filter, 0.2  $\mu\text{m}$ , low protein binding; Fisher Scientific, Tustin, CA), and frozen at  $-80^{\circ}\text{C}$ . For some experiments sweat was lyophilized to dryness, then suspended in 400  $\mu\text{l}$  of distilled water (DW<sup>3</sup>; cell culture grade, endotoxin free; Life Technologies, Grand Island, NY). For analysis of LL37 processing by sweat, 1.6 nmol of LL-37 synthetic peptide was incubated in 50  $\mu\text{l}$  of sweat for 0, 1, 6, and 24 h at  $37^{\circ}\text{C}$  or  $4^{\circ}\text{C}$ . In some experiments proteinase inhibitors including mixed protease inhibitor mixture (1 tablet/10 ml; Roche, Indianapolis, IN), 100  $\mu\text{g}/\text{ml}$  bestatin, 10  $\mu\text{g}/\text{ml}$  E-64, and 10  $\mu\text{g}/\text{ml}$  aprotinin, (Sigma-Aldrich, St. Louis, MO); 100  $\mu\text{M}$  4-(2-aminoethyl)-benzenesulfonylfluoride (AEBSF), 100  $\mu\text{M}$  neutrophil elastase inhibitor, or 100  $\mu\text{M}$  leukocyte elastase inhibitor (Calbiochem, San Diego, CA) were added during incubation. After incubation, 2  $\mu\text{l}$  was assayed by radial diffusion assay to determine antibacterial activity. For analysis by HPLC, 32 nmol of LL-37 was incubated in 100  $\mu\text{l}$ . To control for potential contamination eluted from paper tissues, parallel processing was performed on tissues soaked in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM  $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$ , and 1.4 mM  $\text{KH}_2\text{PO}_4$ , pH 7.4). No antimicrobial activity was detectable in these preparations. Protein concentrations were evaluated by bicinchoninic acid assay (protein assay reagent; Pierce, Rockford, IL) or Bradford protein assay (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Human tissue and blood collection was approved by the University of California-San Diego human research protection program.

### Peptide synthesis

Dermcidin, LL-37, RK-31, KS-30, and KR-20 peptides were commercially prepared by Synpep (Dublin, OR). Peptide amino acid sequences were LLGDFRKSKEKIGKEFKRIVQRIKDFLRNLPRTES (LL-37), RKSK EKIGKEFKRIVQRIKDFLRNLPRTES (RK-31), KSKEKIGKEFKRIV QRIKDFLRNLPRTES (KS-30), KRIVQRIKDFLRNLPRTES (KR-20), and SSLEKGLDGAKKAVGGLGKLGKDAVEDLESVGKGAVHDVKD VLDSV (dermcidin). All synthetic peptides were purified by HPLC, and identity was confirmed by mass spectrometry.

### HPLC

Peptide separation was performed using an AKTA purification system (Amersham Pharmacia Biotech, Piscataway, NJ) on a Sephasil peptide  $\text{C}_{18}$  column (12  $\mu\text{m}$ , ST 4.6/250; Amersham Pharmacia Biotech). Concentrated human sweat or LL-37 incubated in sweat was separated by reverse phase HPLC after column equilibration in 0.1% trifluoroacetic acid at a flow rate of 0.5 ml/min and eluted using gradients of 0–35 and 35–60% acetonitrile for 16 or 67 min or 0–60% acetonitrile for 120 min. Column effluent was monitored at 214, 230, and 280 nm. All collected fractions (1 ml) were lyophilized and suspended in 10  $\mu\text{l}$  of DW for antimicrobial radial diffusion assay.

### Western and immunoblot analyses

Fractions purified by HPLC as described above were evaluated by quantitative dot blot and Western blot. Two microliters of each fraction was compared with a standard curve of synthetic LL37 peptide applied onto polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA). The Ab used was rabbit anti-LL-37 polyclonal Ab derived and was affinity purified against the entire LL-37 peptide. For immunoblot, membranes were blocked (0.1% TTBS: 5% nonfat milk in 0.1% Tween 20/TBS (150 mM NaCl and 10 mM Tris base, pH 7.4)) for 60 min at room temperature, and then rabbit anti-LL-37 polyclonal Ab (1/5000 in blocking solution) was incubated with the membrane overnight at  $4^{\circ}\text{C}$ . After washing three times with 0.1% TTBS, HRP-labeled goat anti-rabbit Ab (1/5000 in the blocking solution; DAKO, Carpinteria, CA) was incubated with the membrane for 60 min at room temperature. After washing the membrane again with 0.1% TTBS, the membrane was immersed in ECL solution (Western Lightning Chemiluminescence Reagents Plus; NEN, Boston, MA) for 60 s, then exposed to x-ray film (Eastman Kodak, Rochester, NY). For Western blot analysis, sweat samples (10  $\mu\text{l}$ ) were separated by 16.5% Tris-tricine/peptide gel (Bio-Rad, Hercules, CA) and then transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore). For the positive control, 5 pmol of LL-37 synthetic peptide was applied.

### Mass spectrometry and protein sequence analysis

Mass spectrometry was performed by Center for Mass Spectrometry, The Scripps Research Institute (La Jolla, CA). MALDI-MS spectra were obtained with a Voyager DE-RP MALDI-TOF mass spectrometer (PerSeptive Biosystems, Framingham, MA) equipped with a nitrogen laser (337 nm, 3-ns pulse). Spectra were collected in the reflector mode. The accelerating voltage in the ion source was 20 kV. Data were acquired with a transient recorder with 2-ns resolution. The matrix used in this work was  $\alpha$ -cyano-4-hydroxycinnamic acid dissolved in water/acetonitrile (1/1, v/v) to give a saturated solution at room temperature. To prepare the sample for analysis, 1  $\mu\text{l}$  of the peptide solution (containing 1–10 pmol of protein in 0.1% trifluoroacetic acid) was added to 1  $\mu\text{l}$  of the matrix solution and applied to a stainless steel sample plate. The mixture was then allowed to air-dry on the sample plate before being introduced into the mass spectrometer. Each spectrum was produced by accumulating data using 128 laser pulses. Mass assignments were assigned with an accuracy of approximately  $\pm 0.1\%$  ( $\pm 1$  Da/1000 Da). Protein sequence analysis for target HPLC fractions was performed by Division of Biology Protein Sequencer Facility, University of California-San Diego. The amino acid sequencing was performed on a Applied Biosystems Procise model 494 sequencer (Foster City, CA) using the pulsed liquid program supplied by the manufacturer.

### Antimicrobial assays

For screening of antimicrobial activity of HPLC fractions, radial diffusion assay was used as previously described (13). Lyophilized HPLC fractions were dissolved in DW (Life Technologies) and tested against *Staphylococcus aureus mprF* (gift from A. Peschel, Microbial Genetics, University of Tübingen, Tübingen, Germany). This strain of *S. aureus* was selected for screening due to its increased sensitivity to cationic peptides. Thin plates (1 mm) of 1% agarose in 0.5% tryptone containing  $\sim 1 \times 10^6$  cells/ml of *S. aureus mprF* were used. One-millimeter wells were punched in the plates, and 2  $\mu\text{l}$  of samples dissolved in tissue culture grade sterile water were loaded in each well. As a positive control, synthetic LL-37 was applied to separate wells. After incubation at  $37^{\circ}\text{C}$  overnight, the inhibition zone diameters were measured.

To evaluate antimicrobial activity against wild-type *S. aureus* (Rosenbach ATCC 25923; American Type Culture Collection, Manassas, VA) and enteroinvasive *Escherichia coli* O29, both radial diffusion and solution killing assays were performed. Radial diffusion assays were performed as described for *S. aureus mprF*. Solution killing was conducted in 10% TSB in 10 mM PB (TSB = 30 g/L tryptic soy broth; Sigma-Aldrich;  $20 \times \text{PB} = 27.6$  g/L  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  and 53.65 g/L  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ , pH 7.4). Bacteria in log-phase growth were suspended to  $1 \times 10^6$  cells/ml, and peptide was added and incubated at  $37^{\circ}\text{C}$  for 2 h. Bacteria were then plated on TSB agar (TSB; 13 g/L; Bactoagar; BD Biosciences, Sparks, MD) for direct colony count and determination of CFU. Activity against group A streptococcus (NZ131) was determined only in the solution assay as described. To evaluate antimicrobial activity in high salt conditions, the solution assay was performed in 10% TSB/10 mM PBS with several salt concentrations (NaCl concentrations, 10, 50, 100, 150, 300, and 500 mM). Action against the *Candida albicans* was determined in Dixon medium (0.6% peptone, 4% malt extract, 1% glucose, 0.1% ox bile, and 1% Tween 80) in sterile 96-well microtiter plates (Corning Glass, Corning, NY) at a final volume of 50  $\mu\text{l}$ . The assay mixtures contained  $1\text{--}2.5 \times 10^4$  CFU/ml freshly grown *Candida*, 20% Dixon medium, 0.6 mM phosphate buffer (pH 7), and 16  $\mu\text{g}/\text{ml}$  chloramphenicol. Microtiter plates were incubated at  $37^{\circ}\text{C}$  for 24 h with peptides, then plated on Dixon agar to determine the minimum fungicide concentration.

### Hemolysis assay

Hemolytic activity was determined on human whole blood. Freshly obtained whole blood cells were washed three times in PBS and resuspended in PBS at its original volume containing peptides at the indicated concentrations. Samples were incubated at  $37^{\circ}\text{C}$  for 1.5–3 h, and hemolysis was determined by centrifuging at  $300 \times g$  and measurement of absorbance of the supernatant at 578 nm. The hemolytic activity of each peptide was expressed as the percentage of total hemoglobin released compared with that released by incubation with 0.1% Triton X-100.

### Measurement of IL-8 release from keratinocytes

Normal human keratinocytes were cultured in EpiLife cell medium (Cascade Biologics, Portland, OR) containing 0.06 mM  $\text{Ca}^{2+}$ ,  $1 \times$  EpiLife-defined growth supplement, 50 U/ml penicillin, and 50  $\mu\text{g}/\text{ml}$  streptomycin (Invitrogen, Carlsbad, CA). Keratinocytes were seeded in a 96-well plate and were grown to confluence under standard tissue culture conditions. Cells were incubated with 3 or 10  $\mu\text{M}$  LL-37 or LL37-derived peptides for

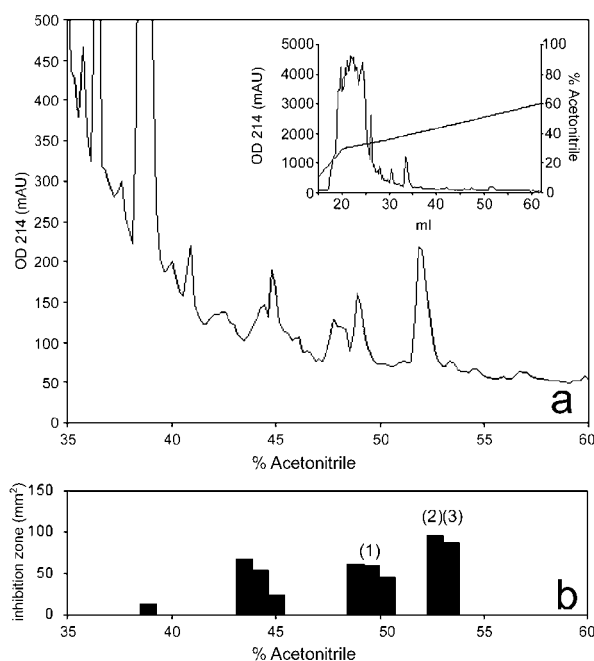
<sup>3</sup> Abbreviation used in this paper: DW, distilled water.

8 h at 37°C. Supernatants were collected and stored at -20°C overnight. The IL-8 ELISA was performed according to the manufacturer's instructions (BD OptEIA; BD Pharmingen, San Diego, CA). Supernatants were diluted 1/10 for assay. Simultaneously, lactate dehydrogenase assays (Roche, Indianapolis, IN) were used to assess the cytotoxicity of peptides to keratinocytes. Lactate dehydrogenase release after peptide exposure was compared with release induced by 1% Triton X-100.

## Results

To identify antimicrobial activity present at the skin surface, human sweat was collected from normal volunteers, concentrated, and separated by HPLC. Fractions of the material eluted between 35–65% acetonitrile were individually evaluated for the ability to inhibit the growth of *S. aureus mprF* by radial diffusion assay (Fig. 1). Multiple distinct fractions were found to be active in this assay. Prior evaluations of human sweat have shown that the antimicrobial peptides LL-37 and dermcidin are produced by the eccrine apparatus and secreted into the topical soluble environment of sweat. As expected, these molecules were detectable in the sweat preparation shown in Fig. 1. The presence of LL-37 was confirmed by immunoblot analysis with Ab specific to LL-37 and by MALDI-TOF mass spectrometry. The dermcidin peptides, DCD and DCD-1L, were identified by MALDI-TOF mass spectrometry and N-terminal amino acid sequencing.

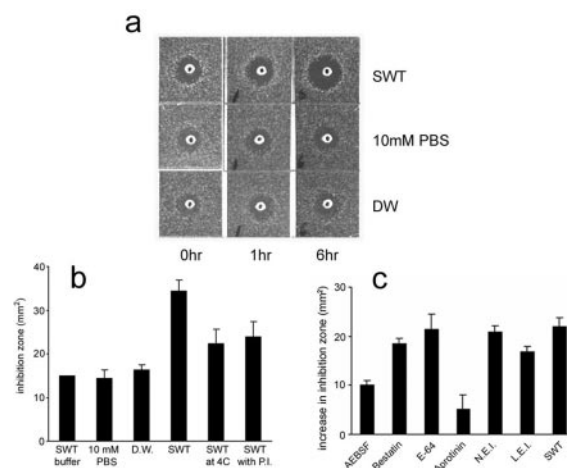
Immunoblot analysis with anti-LL-37 Ab of all fractions isolated by HPLC from human sweat suggested that other molecules related to LL-37 may be present in fractions eluting between 43 and 48% acetonitrile. These fractions were associated with anti-



**FIGURE 1.** Soluble antimicrobial activity on human skin. Human sweat was concentrated 50× and was separated by HPLC on C<sub>18</sub>. *a*, Absorbance profile at 214 nm for eluted material from 35–60% acetonitrile; *inset*, complete absorbance profile of eluted materials. *b*, Ability of material eluted in *a* to inhibit growth of *S. aureus mprF* is shown as the diameter of the zone of *S. aureus mprF* inhibition. Several antimicrobial fractions were detected. Mass spectrometry identified three previously described antimicrobials; fractions labeled (1) and (3) are dermcidin and DCD-1L, respectively (m.w., 4701 and 4818, respectively), confirmed by N-terminal sequencing (1): SSLEKGLDGA; (3): SSLE). Fraction-labeled (2) LL-37 is identified by mass spectrometry (m.w., 4493) and immunoblot. The data shown are representative of single experiment repeated five times with separate sweat preparations.

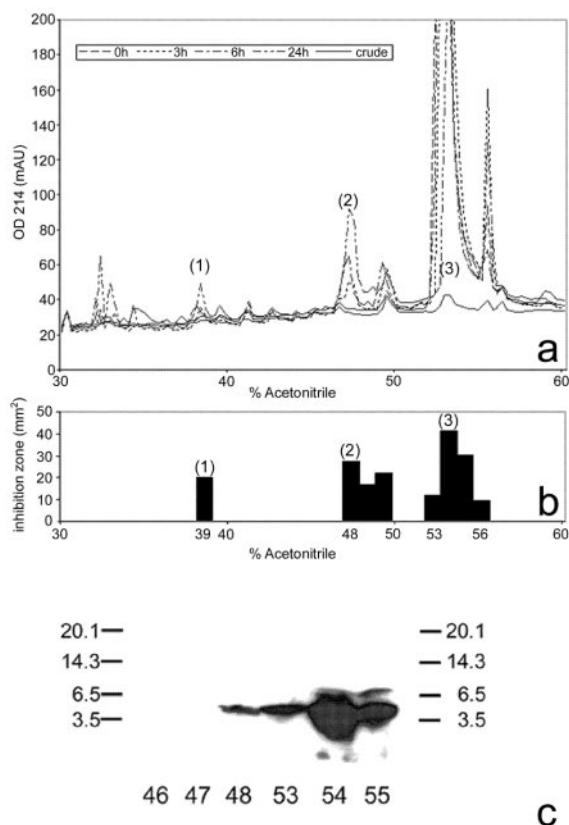
microbial activity, but were not identifiable by MALDI-TOF mass spectrometry and N-terminal amino acid sequencing from the concentrated sweat preparations. Based on the immunoreactivity and elution profile, we hypothesized that these antimicrobial molecules were alternative forms of LL-37 that were further processed in sweat to unique cathelicidin peptides. To test this, human sweat was freshly collected and sterilely filtered, then synthetic LL-37 was added to a final concentration of 32 μM. The relative ability of this solution to inhibit the growth of *S. aureus mprF* was then evaluated. After incubation at 37°C in sweat, LL-37 increased the zone of inhibition and apparent antimicrobial activity (Fig. 2*a*). Incubation of LL-37 under identical conditions in PBS or DW did not affect activity. This increase in apparent antimicrobial activity did not occur at 4°C and was inhibited by addition of the protease inhibitor mixture, suggesting that the gain in antimicrobial function was the consequence of an enzymatic process (Fig. 2*b*). Addition of specific inhibitors of potential processing enzymes demonstrated that the serine protease inhibitors AEBSF and aprotinin were most effective in blocking the increase in antimicrobial activity generated by incubation of LL-37 in sweat (Fig. 2*c*). These protease inhibitors did not show direct antimicrobial activity when applied alone to the radial diffusion assay.

An increase in the relative ability of LL-37 to inhibit the growth of *S. aureus mprF* suggested that the enzymatic processing of this peptide resulted in alternative forms with either increased direct antimicrobial activity or an ability to synergize with the parent peptide. To identify these processed forms of LL-37, synthetic peptide was incubated with the sterile sweat preparation and was separated by HPLC after various periods of incubation (Fig. 3*a*). An increase in the relative abundance of several proteins was seen over time. Correlation of this profile with antimicrobial activity showed that fractions eluting at 39 and 48% acetonitrile gained antimicrobial activity coincident with an increase in the relative abundance of peptides eluting at these positions (Fig. 3*b*). A third



**FIGURE 2.** Serine protease in sweat enhances cathelicidin antimicrobial activity. Antibacterial activity evaluated by radial diffusion assay against *S. aureus mprF* after incubation of LL-37 (32 μM) in sweat. *a*, An increase in the inhibition zone is seen when incubated in sweat, but not when incubated in 10 mM PBS or DW. *b*, Diameters of inhibition zone after 6-h incubation at 37°C in SWT buffer (sweat buffer salts alone), PBS, DW, sterile filtered human sweat (SWT) at 37°C, SWT at 4°C, or SWT with propidium iodide (PI) with protease inhibitor mixture. *c*, Actions of specific protease inhibitors on gain of antimicrobial activity. Data show an increase in the inhibition zone at 37°C after 6 h compared with 0 h. Serine protease inhibitors AEBSF and aprotinin were most effective. N.E.I., neutrophil elastase inhibitor; L.E.I., leukocyte elastase inhibitor. Data are triplicate determinations ± SEM from a single experiment representative of three.

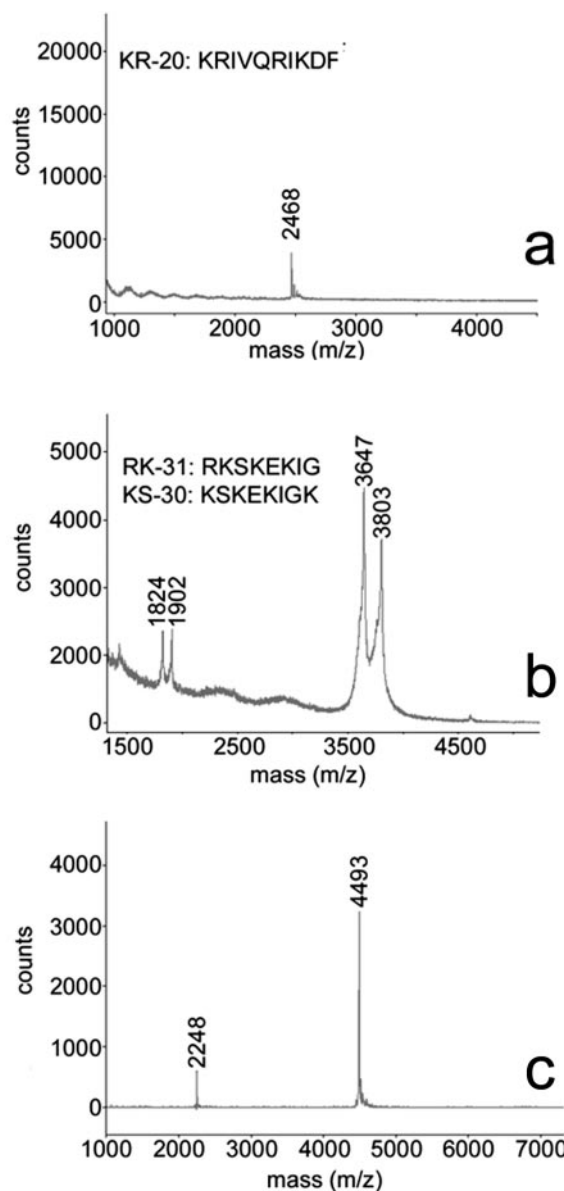




**FIGURE 3.** Purification of novel cathelicidin peptides generated from LL-37. *a*, Human sweat was separated by HPLC on  $C_{18}$ . The absorbance profile at 214 nm is shown for eluted material from 30–60% acetonitrile. Crude is sweat before addition of LL-37; overlay plots show separate runs of sweat after addition of 32 nmol of LL-37 and incubation for 0–24 h at 37°C. *b*, Antibacterial activity with radial diffusion assay against *S. aureus mprF* of fractions eluted from 24-h sample in *a*. Peaks with antimicrobial activity are labeled 1, 2, and 3. *c*, Western blot analysis with anti-LL-37 was performed on all fractions; shown are fractions eluting at acetonitrile concentrations of 46–55%.

major peak of antimicrobial activity seen between 53 and 56% acetonitrile showed a relative decrease in abundance, as estimated by absorbance at 214 nm. Western blots performed with Ab to LL-37 showed immunoreactive peptides <5 kDa in fractions 48–55 (Fig. 3c). MALDI-TOF mass spectrometry and N-terminal sequencing of the peptide eluting at 39% acetonitrile identified this as a 20-aa cathelicidin derivative, KR-20 (Fig. 4a). Similar analysis of peptides eluting at 48% acetonitrile identified two additional cathelicidin peptides, RK-31 and KS-30 (Fig. 4b). Material eluting at 55–56% acetonitrile and decreasing in abundance with incubation was identified as LL-37 by both Western blot and mass spectrometry analysis (Figs. 3c and 4d).

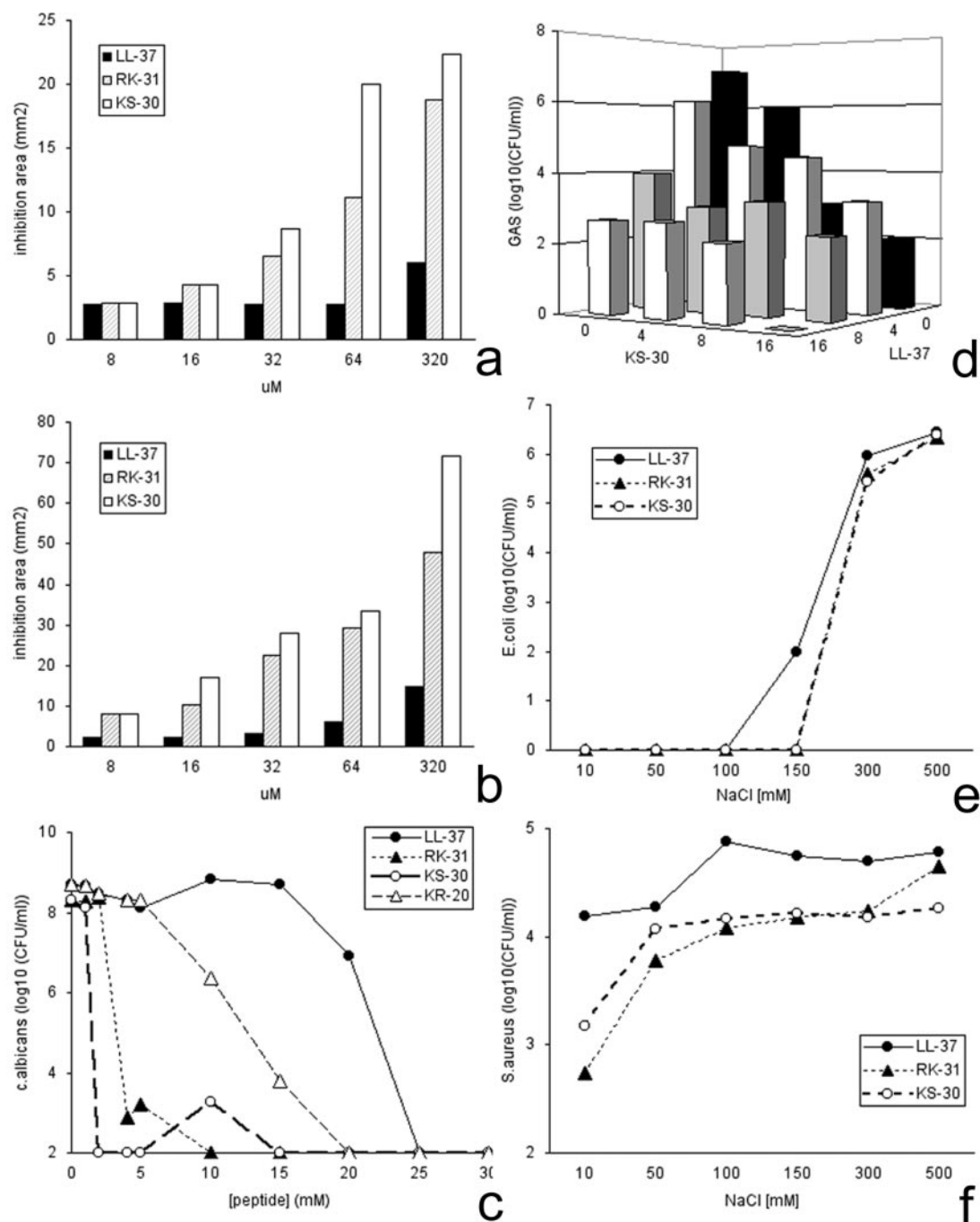
The newly described human cathelicidin peptides KR-20, RK-31, and KS-30 eluted at positions corresponding to unidentified antimicrobial activity seen in crude sweat preparations partially purified in Fig. 1. The low relative abundance of these peptides, yet easily detectable antimicrobial activity, suggested that these peptides might gain antimicrobial activity with processing compared with LL-37. To compare the antimicrobial activities of these cathelicidins, purified synthetic peptides corresponding to LL-37, RK-31, KS-30, and KR-20 were assessed by both radial diffusion assay and solution assay against a variety of microbes (Fig. 5). RK-31 and KS-30 showed greatly increased action against wild-type *S. aureus* and *E. coli*. All three new peptides showed increased fun-



**FIGURE 4.** Identification of novel cathelicidin peptides generated from LL-37. After purification, shown in Fig. 3, major bioactive fractions were identified. *a*, Peak 1 in Fig. 3a identified as KR-20 by mass spectrometry (m.w., 2468) and N-terminal sequence KRIVQRIKDF; *b*, peak 2 detected two peptides, RK-31 and KS-30 (m.w., 3647 and 3803; RKSKEKIG and KSKEKIGK, respectively); *c*, peak 3 identified as LL-37 (m.w., 4493). Data shown are from a single experiment representative of three.

gicidal activity against *C. albicans*. Furthermore, these peptides were synergistic, killing bacteria at lower concentrations when present together (Fig. 5d), and maintained activity at increased salt conditions (Fig. 5, e and f). Hemolytic activity against human erythrocytes was minimal, as seen by assay at a concentration 5–10 times greater than that required for antimicrobial activity (Table I). However, despite gaining antimicrobial activity by processing to shorter forms of the cathelicidin peptide, the hemolytic activity of these antimicrobials decreased relative to that of LL-37.

In addition to the function of antimicrobial peptides as natural antibiotics, many of these molecules have been associated with the ability to stimulate a variety of host responses. To determine whether the secretion and processing of cathelicidin peptides at the



**FIGURE 5.** Processing of LL-37 enhances antimicrobial activity. The antimicrobial activity of cathelicidin peptides evaluated by radial diffusion and solution assays. *a*, Diameter of the zone of inhibition of growth of *S. aureus* after application of various concentrations of LL-37, RK-31, or KS-30. *b*, Diameter of the zone of inhibition of growth of *E. coli* after application of various concentrations of LL-37, RK-31, or KS-30. *c*, Solution assay results of LL-37 and related peptides against *C. albicans*. *d*, Synergistic activity of LL-37 and KS-30 against group A *Streptococcus* in solution assay. *e* and *f*, The effect of salt on the antimicrobial activity of LL-37, RK-31, and KS-30 was evaluated in 10% TSB/10 mM phosphate buffer with several NaCl concentrations against *E. coli* (*e*) and *S. aureus* (*f*). Data shown are representative of triplicate determinations.

surface of the skin could also stimulate a host inflammatory response, human keratinocytes were grown in culture and assayed for the release of IL-8 in response to these peptides. All peptide solutions were endotoxin free. LL-37 had a potent ability to stimulate IL-8 release from keratinocytes, but processing to the shorter form of these cathelicidins decreased the ability to stimulate IL-8 (Fig. 6). At concentrations of LL-37 sufficient to stimulate IL-8 release from cultured keratinocytes, no toxic effects were ob-

served, as evaluated by observations of cell morphology, trypan blue exclusion, and release of lactate dehydrogenase.

## Discussion

The synthesis, processing, and release of antimicrobial peptides are essential elements for defense against infection. In epithelia such as skin, oral mucosa, lung, and gut, the release of antimicrobial peptides appears to play a particularly important defensive

Table I. Antimicrobial and hemolytic activities of human sweat peptides<sup>a</sup>

	Radial Diffusion Assay (μM)			Liquid Assay (μM)		
	<i>S. aureus</i> <i>mprF</i>	<i>S. aureus</i> (ATCC 25923)	<i>E. coli</i> (O29)	GAS (NZ131)	<i>C. albicans</i> ATCC 14053	% Hemolysis
LL-37	32	>64	64	8	20	9
RK-31	8	16	8	8	4	6
KS-30	8	16	8	4	2	.23
KR-20	16	>64	>64	4	10	0
DCD	>64	>64	>64	>64	>32	nd

<sup>a</sup> Radial diffusion assay results represent minimal inhibitory concentrations determined for peptides against the indicated bacteria. Liquid assay results represent the minimal concentration required to kill group A *S. aureus* (GAS) or *C. albicans*. The percent hemolysis is shown for peptides at the following concentrations: LL-37 and KR-20, 100 μM; RK-31, 44 μM; KS-30, 88 μM. nd, not done.

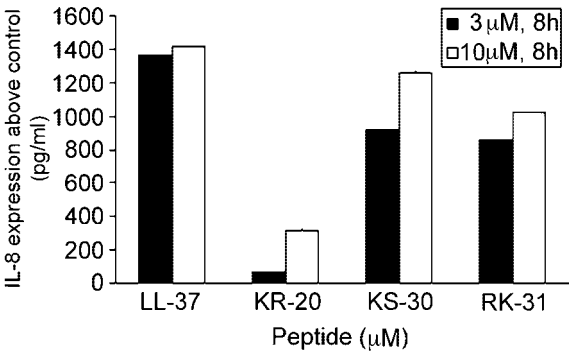
role. However, the expression of antimicrobial peptides by normal skin, an epithelial barrier exposed to constant microbial challenge, is relatively low compared with that by mucosal epithelia of other organs. A partial explanation for this apparent inconsistency is found in recent observations that the antimicrobial peptides hCAP 18/LL-37 and dermcidin are produced by eccrine glands and secreted onto the surface of the skin in sweat. This system would provide a mechanism by which a constitutive antimicrobial barrier may form at the skin surface above the permeability barrier of the stratum corneum and in direct contact with the external environment. A problem with this model is that dermcidin and LL-37 have poor ability to inhibit the growth of important skin pathogens such as *S. aureus* and *C. albicans*. Because of this limitation, we hypothesized that sweat contains additional antimicrobial peptides with increased activity against relevant skin pathogens. In this study we show that enhanced antimicrobial activity exists in normal human sweat and is the consequence of processing of LL-37 to previously unknown, naturally occurring cathelicidin peptides.

HPLC separation of concentrated sweat samples from different individuals showed remarkable consistency in the elution profile of antimicrobial molecules. Screening assays were performed with *S. aureus mprF*, a mutant that lacks the ability to modify anionic membrane lipids with L-lysine (23). This mutation leads to an increase in surface anionic charge and greater binding by cationic host defense molecules. Thus, this strain of *S. aureus* amplifies sensitivity to cationic antimicrobial peptides. Initial purifications confirmed the sensitivity of this approach by detecting peptides already known to occur in human sweat, such as LL-37, DCD, and its variant, DCD-1L. However, antimicrobial activity associated with less abundant peptides in the crude human sweat preparation was difficult to purify using standard biochemical approaches. Detection of these fractions by Ab against LL-37 suggested that some of these less abundant, yet apparently potent, antimicrobial molecules were similar to LL-37. The techniques used for assay and isolation of activity are optimized for cationic peptides such as LL37. Therefore, other antimicrobial molecules, including proteins with antimicrobial activity, may also exist at the skin surface, but may not have been detected in the current study.

Analysis of antimicrobial activity and identification of new peptides generated from synthetic LL-37 showed that further processing occurs by a serine protease present in sweat. These previously unknown peptides eluted at positions similar to the antimicrobial activity detected in native sweat, suggesting that RK-31, KS-30, and KR-20 are naturally occurring, but less abundant in sweat immediately after secretion. Unlike the techniques used in this study to collect sweat for study, under normal conditions sweat remains at the skin surface for further processing and concentration by evaporation. This final enzymatic modification of cathelicidins is thus distinct from that previously studied during neutro-

phil activation. Bovine and porcine precursor cathelicidins in specific granules are acted upon by elastase in the azurophilic granule to release a single C-terminal antimicrobial peptide (24). In humans, the full-length cathelicidin hCAP-18 can be cleaved to LL-37 by proteinase 3, a protease present in human neutrophils (25). In the vagina at low pH, another serum protease, gastricsin, processes a slightly longer C-terminal peptide (ALL-38) from hCAP-18 (26). These systems have not shown further processing of the C-terminal peptide. This may be due to lack of detection of these peptides in prior studies or to the presence of additional serine proteases at the skin surface, such as activated tryptases and kallikrein (27). These and/or other serine proteases of host origin as well as serine proteases potentially released by microflora on the skin may be responsible for the processing observed in the study. As this processing leads to an important gain in antimicrobial function and modification of host stimulatory effects by LL-37, the activity of these enzymes, their expression, and their balance with known skin serine protease inhibitors, such as bikunin (28), elafin (29), anti-leukoprotease (30), and plasminogen activator inhibitor-2 (31), take on new importance for understanding immune defense.

The gain in antimicrobial activity observed after processing of LL-37 to RK-31 and KS-30 has not been previously described. Structural analysis of LL-37 suggests that it is an antipathetic α-helical molecule that probably kills by ionic association with the membrane and subsequent disruption of the lipid bilayer through formation of a toroidal pore. A change in the net charge by processing is an unlikely explanation for the increase in activity, as the estimated PI of LL-37 is 10.4, identical with that of KS-30. Cleavage of the six amino acids at the N terminus representing nonpolar,



**FIGURE 6.** Processing of LL-37 decreases the ability to stimulate keratinocyte IL-8. Cathelicidin peptides were added to culture of normal human keratinocytes at a final concentration of 3 or 10 μM, then IL-8 release was determined after 8 h. All samples were endotoxin free by *Limulus* assay. Data are the mean ± SEM of triplicate determinations.

polar, and acidic residues leads to exposure of a basic residue that may contribute to the increase in activity. Previous analysis of the structurally distinct cathelicidin PR-39 has shown that N-terminal lysines are important to activity and may function by facilitating initial ionic interaction with the anionic microbial surface (32). This explanation is probably incomplete because one of the largest gains in function seen by processing of LL-37 to shorter peptides was in its ability to kill wild-type *S. aureus*, an organism that has developed apparent resistance to cationic antimicrobial peptides such as  $\beta$ -defensins and LL-37 by modification of charge at the cell surface. Furthermore, structural modifications of defensins have major effects on chemotactic activity and lesser influence on antibacterial function (33), a phenomenon similar to that seen in this study with the loss of IL-8 stimulatory capacity. Additional high resolution structural studies of LL-37 and its shorter, more active, related peptides are necessary and may yield important new information relevant to the mechanism of action.

Understanding the role of antimicrobial peptides in mammalian immunity is complicated by many observations that these peptides also act on the host to stimulate a variety of important responses related to defense against injury. Cathelicidins were first found in mammalian skin due to their ability to increase fibroblast proteoglycan synthesis (34). Defensins and cathelicidins have both been shown to have chemotactic activity, possibly functioning through specific receptors, such as CCR6 or formyl peptide receptor-like-1, respectively (21, 35). Consistent with these prior observations, LL-37 was found in the present study to be a potent stimulus for IL-8 release from cultured keratinocytes. This effect was diminished upon processing to RK-31 or KS-30 and was almost completely eliminated in the shortest KR-20 cathelicidin. It is not clear whether topical LL-37 secreted into sweat would have a similar effect in vivo on epidermal keratinocytes when separated by the formidable barrier of the stratum corneum. However, the inhibition of proinflammatory functions by LL-37 in normal epithelia would be a beneficial mechanism to regulate unintended inflammation.

The present findings show that postsecretory processing of LL-37 occurs at the skin surface. The generation of additional potent antimicrobial peptides suggests a model in which a single gene of the innate defense system can generate multiple, differentially active products. Cathelicidins found in mammalian species such as the cow, pig, sheep, rabbit, and horse are found as multiple copies encoding distinct C-terminal peptides. Conversely, in man and rodents only a single cathelicidin gene product is known. After initial processing of the pro-protein into the cathelin-like domain and LL-37, two distinct defense molecules are activated: the cathelin-like domain, which possesses both antimicrobial activity and an ability to act as a protease inhibitor, and LL-37, which has antimicrobial function combined with a range of host stimulatory capacities. The present findings suggest that in humans further cathelicidin diversity is generated by postsecretory processing, tipping the balance of function toward antimicrobial action and away from effects on the host. Thus, the single human cathelicidin gene generates multiple products with a range of biological activities, each relevant to the local environment in which they are released. Taken together these findings suggest stress responses leading to increased production of sweat, such as the febrile response, may contribute to an innate immune response by mobilizing and generating active antimicrobials at the skin surface.

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