

Selective Antimicrobial Action Is Provided by Phenol-Soluble Modulins Derived from *Staphylococcus epidermidis*, a Normal Resident of the Skin

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Antimicrobial peptides serve as a first line of innate immune defense against invading organisms such as bacteria and viruses. In this study, we hypothesized that peptides produced by a normal microbial resident of human skin, *Staphylococcus epidermidis*, might also act as an antimicrobial shield and contribute to normal defense at the epidermal interface. We show by circular dichroism and tryptophan spectroscopy that phenol-soluble modulins (PSMs) γ and δ produced by *S. epidermidis* have an α -helical character and a strong lipid membrane interaction similar to mammalian AMPs such as LL-37. Both PSMs directly induced lipid vesicle leakage and exerted selective antimicrobial action against skin pathogens such as *Staphylococcus aureus*. PSMs functionally cooperated with each other and LL-37 to enhance antimicrobial action. Moreover, PSMs reduced Group A *Streptococcus* (GAS) but not the survival of *S. epidermidis* on mouse skin. Thus, these data suggest that the production of PSM γ and PSM δ by *S. epidermidis* can benefit cutaneous immune defense by selectively inhibiting the survival of skin pathogens while maintaining the normal skin microbiome.

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INTRODUCTION

Infections from organisms such as Group A *Streptococcus* (GAS, *Streptococcus pyogenes*) or *Staphylococcus aureus* range from superficial to invasive, and collectively represent a severe societal burden, only escalating with the increase of resistance to pharmaceutically derived antibiotics (Jones, 2003; Carapetis *et al.*, 2005; McCaig *et al.*, 2006). Increasing our understanding of innate host-derived antimicrobial peptides (AMPs) offers an alternative to the development of treatment of such infections, as AMPs have retained the

capacity to provide protection against infections by GAS, *S. aureus*, and other microbes (Dorschner *et al.*, 2001; Nizet *et al.*, 2001; Di Nardo *et al.*, 2008), and have not lost their antimicrobial relevance as in the case of many pharmaceutical antibiotics.

Although our understanding of AMPs remains incomplete, several classes of these antibiotic peptides have been described. AMPs such as cathelicidin-related AMP (CRAMP) in mice and LL-37 in humans are small cationic α -helical peptides that act through a strong membrane activity. These helical peptides associate with lipid membranes (Yu *et al.*, 2002; Porcelli *et al.*, 2008) and are thought to kill microbes by their capacity to disrupt the normal structure of the lipid membrane (Henzler Wildman *et al.*, 2003). Interestingly, several bacteria have also been shown to produce membrane-disruptive peptides. For example, delta-lysin (delta-toxin or *hld* gene), also known as phenol-soluble modulins- γ (PSM γ), from *S. aureus* has been shown to cause lysis of membranes and displays strong lipid interactions (Alouf *et al.*, 1989). This peptide causes disease through the destruction of red blood cells and neutrophils (Dhople and Nagaraj, 1993) (Wang *et al.*, 2007). The mechanism of action for this member of the PSM group of peptides is similar to some AMPs, as nuclear magnetic resonance studies have shown that *S. aureus* delta-toxin forms an α -helix in lipid micelles (Tappin *et al.*, 1988). A few earlier studies have also found that delta-toxin from *S. aureus* can exert antibacterial activity

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Abbreviations: AMP, antimicrobial peptide; ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid, disodium salt; CD, circular dichroism; CRAMP, cathelicidin-related AMP; dPBS, Dulbecco's phosphate-buffered saline; GAS, Group A Streptococcus; NHEK, normal human epidermal keratinocyte; PI, propidium iodide; PSM, phenol-soluble modulin

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when chemically modified by amino-acid substitution and truncation, but the *S. aureus* native peptide was unable to inhibit *Escherichia coli* or *S. aureus* (Dhople and Nagaraj, 1993, 1995). Despite these hints that PSMs could be antimicrobial, the action of these molecules as AMPs has not been extensively studied.

As the related *Staphylococcal* species, *Staphylococcus epidermidis*, normally resides in abundance on the surface of healthy human skin, in this study, we sought to investigate whether the unique peptides PSM γ and PSM δ found in *S. epidermidis* could be beneficial to the host and thus serve as an additional AMP on normal skin surface. On the basis of the structure, biophysical properties, and antimicrobial activity of PSM γ and PSM δ , this study suggests that *S. epidermidis* has a beneficial role in skin immune defense by producing innate, yet non-host-derived AMPs on the skin surface.

RESULTS

PSMs have structural similarities to AMPs and strongly interact with synthetic lipid membranes

We observed that helical wheel plots of two peptides (PSM γ and PSM δ) produced by *S. epidermidis* predicted the segregation of their hydrophobic and cationic amino acids with a 5-amino-acid periodicity, which resembles that of classic AMPs such as LL-37 (Figure 1a and b). As *S. epidermidis* is a major normal resident microbe on the surface of human skin, a structural similarity to native human AMPs suggested the potential of these peptides to have a similar effect and to contribute to the normal antimicrobial defense of the skin. To evaluate the validity of this structural prediction by wheel plot, and to test the capacity of PSMs to adopt the charge distribution predicted by Figure 1b, circular dichroism (CD) was carried out on synthetically produced PSM γ and PSM δ . CD spectral analysis showed α -helical tendencies for both peptides in buffer alone (Figure 1c and d). In the presence of anionic POPC/POPG ((1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine)/(1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)]) lipid vesicles, PSM δ , but not PSM γ , became more α -helical (Figure 1d).

Next, a spectroscopic analysis of tryptophan in PSM γ was carried out to evaluate the capacity of the peptide to associate with lipid membranes, a characteristic consistent with the amphipathic structure predicted by the wheel plot. As tryptophan was present only in PSM γ , only this peptide was amenable to this form of spectroscopic analysis. In buffer alone, PSM γ 's tryptophan emitted maximally at 339 nm, whereas in the presence of POPC/POPG lipid vesicles the maximal emission shifted to 332 nm, indicating a more buried state (Figure 1e and g). The addition of urea successfully unfolded and dissociated peptide oligomers of PSM γ , as shown by a red shift of the maximal emission to 355 nm. However, in the presence of lipid vesicles, the maximal emission in urea remained low (335 nm), indicating a continued strong membrane association (Figure 1f and g). These data confirmed that PSM γ strongly associates with lipid membranes even under the strongly dissociating condition of 5.5 M urea. Thus, the combined observations of being

α -helical, a secondary structural change in the presence of membranes, and a strong interaction with lipid vesicles confirmed that PSMs have similarities to AMPs in terms of secondary structure and membrane affinity.

S. epidermidis PSMs form multimeric complexes in solution

Another common characteristic of AMPs is their ability to form complexes. To determine whether PSM γ forms multimeric complexes, we generated unfolding curves of PSM γ as a function of peptide concentration in urea. In this two-state model, we considered "folded" to be that of a multimeric state that allows for stabilization and embedding of tryptophan. In the "unfolded" state, peptides are monomeric in solution and the tryptophan residue will be solvent exposed. PSM γ is shown to be in a multimeric state at 0 M urea and a monomeric state at 4 M (Figure 2a). The unfolding curve of 5 μ M PSM γ had a midpoint (C_m) of 2.14 M urea, a slope of 0.67 kcal M⁻¹ mol⁻¹ at the midpoint, and a $\Delta G_{(H_2O)}^\circ$ of 1.43 kcal mol⁻¹ (Figure 2a and b). Increasing the concentration of PSM γ to 25 μ M caused a shift in the midpoint to 3.2 M urea, a slope of 0.49 kcal M⁻¹ mol⁻¹ at the midpoint, and a $\Delta G_{(H_2O)}^\circ$ to 1.57 kcal mol⁻¹. This result indicated that a greater concentration of urea was required to unfold PSM γ in the presence of more peptide, indicating the formation of stable multimeric complexes.

In addition to multimeric complexes, we probed PSM δ and PSM γ for their ability to form hetero-multimeric complexes using CD. CD spectra of PSM γ were measured in the presence of increasing concentrations of PSM δ . The PSM δ spectrum alone was subtracted from the combined spectrum; hence, the output shows only the changes in the secondary structure of PSM γ . On addition of PSM δ , PSM γ signal increased in intensity, suggesting a greater α -helical character (Figure 2c). Thus, the alteration of PSM γ 's spectrum by PSM δ suggests that the peptides are interacting.

PSMs disrupt artificial membrane vesicles and kill skin pathogens

Next, to determine whether the physical similarities of PSMs to AMPs extend to the functional capacity to perturb lipid membrane vesicles, we tested their ability to perforate POPC/POPG vesicles. The vesicles evaluated were generated in such a manner that they encapsulated the fluorophore ANTS (8-aminonaphthalene-1,3,6-trisulfonic acid, disodium salt) and a quencher DPX (*p*-xylene-bis-pyridinium bromide). As shown upon addition of Triton X, the release of ANTS from vesicle resulted in increased fluorescence because of dissociation from the quencher (Figure 3a and b). Incubation of these vesicles with increasing concentrations of PSM γ or PSM δ induced greater fluorescence, thereby demonstrating a leakage of ANTS from vesicles (Figure 3a and b). Thus, PSMs are directly membrane active and can perforate POPC/POPG vesicles, a functional characteristic similar to mammalian AMPs such as LL-37.

To directly determine whether the capacity of PSMs to disrupt membranes would extend to the ability of these peptides to kill or inhibit the growth of potential skin pathogens, GAS, *S. aureus*, and *S. epidermidis* were

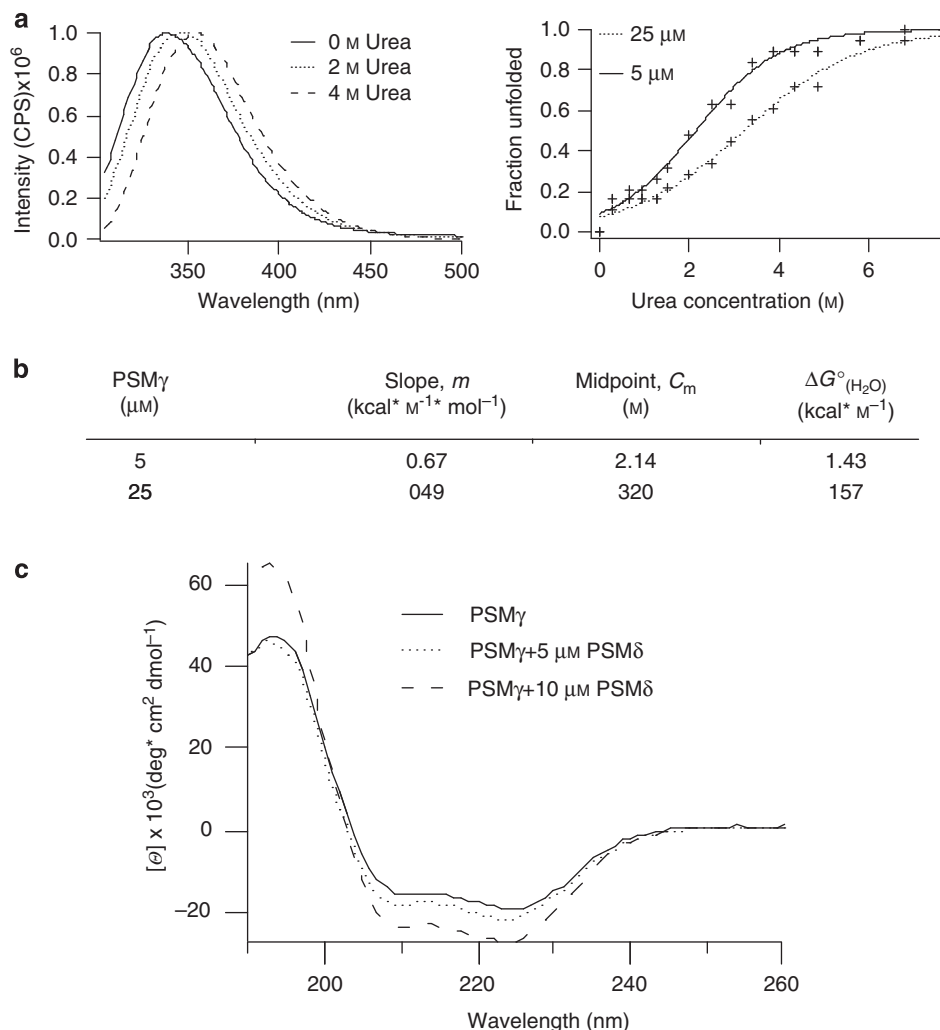


Figure 2. *S. epidermidis* PSMs form multimeric complexes in solution. (a), Tryptophan spectra of 5, 10, and 25 μM PSM γ in 20 mM potassium phosphate buffer, pH 7.3. Tryptophan shift in phenol-soluble modulins in the presence and absence of vesicles and urea. (b) Table of slope, midpoint, and $\Delta G^\circ_{(\text{H}_2\text{O})}$ from unfolding curves of PSM γ at 5 and 25 μM . Increased concentrations of PSM γ results in a shift of the $\Delta G^\circ_{(\text{H}_2\text{O})}$ from 1.43 to 1.57 $\text{kcal} \cdot \text{mol}^{-1}$, which indicates greater stability and complex formation. (c) Circular dichroism spectra of 20 μM PSM γ in the presence and absence of 5 or 10 μM PSM δ . PSM δ curves were subtracted from PSM γ curves. Curves show structural changes of PSM γ due to interaction with PSM δ .

evaluated. *S. epidermidis*, GAS, or *S. aureus* was incubated with PSM γ and cells fixed within 20 min for electron microscopy. This analysis showed that membrane blebbing of both GAS and *S. aureus* was induced by PSM γ (Figure 3g). This effect was not observed in *S. epidermidis* treated similarly. The degree of membrane blebbing observed in GAS and *S. aureus* was similar to that seen when bacteria were treated with mammalian AMP CRAMP (Figure 3g). PSM γ and PSM δ also induced dose-dependent membrane leakage in cultured mammalian cells as measured by cell propidium iodide (PI) uptake in cultured normal human keratinocytes (Figure 4). This capacity to disrupt mammalian cell membranes is a property previously reported for PSMs (Wang *et al.*, 2007) and similar to AMPs also found on the skin, such as LL-37. Cell permeability was measured by PI uptake in cells exposed to peptides in culture. The number of PI-positive fluorescent cells per field indicates relative

permeability effects on the cell population. Maximal PI uptake in the population occurred at 8 μM for both LL-37 and PSM γ . Thus, PSMs cause membrane leakage and membrane perturbation in bacteria and mammalian cells, and this function is similar to that of innate cutaneous AMPs.

Next, on the basis of the spectroscopic observations suggesting interactions of PSM γ and PSM δ , we determined whether PSM δ and PSM γ would show increased antimicrobial action when combined. GAS was incubated with increasing concentrations of both PSM δ and PSM γ . As shown previously, 8 μM of PSM γ alone completely inhibited GAS. The addition of 2 μM of PSM δ reduced the concentration of PSM γ needed to completely inhibit GAS growth to 4 μM (Figure 5a). Similarly, PSMs also cooperated with LL-37 to inhibit GAS survival (Figure 5b and c). These data show that PSMs function better together, and will further increase the potency of existing host AMPs on skin, such as LL-37.

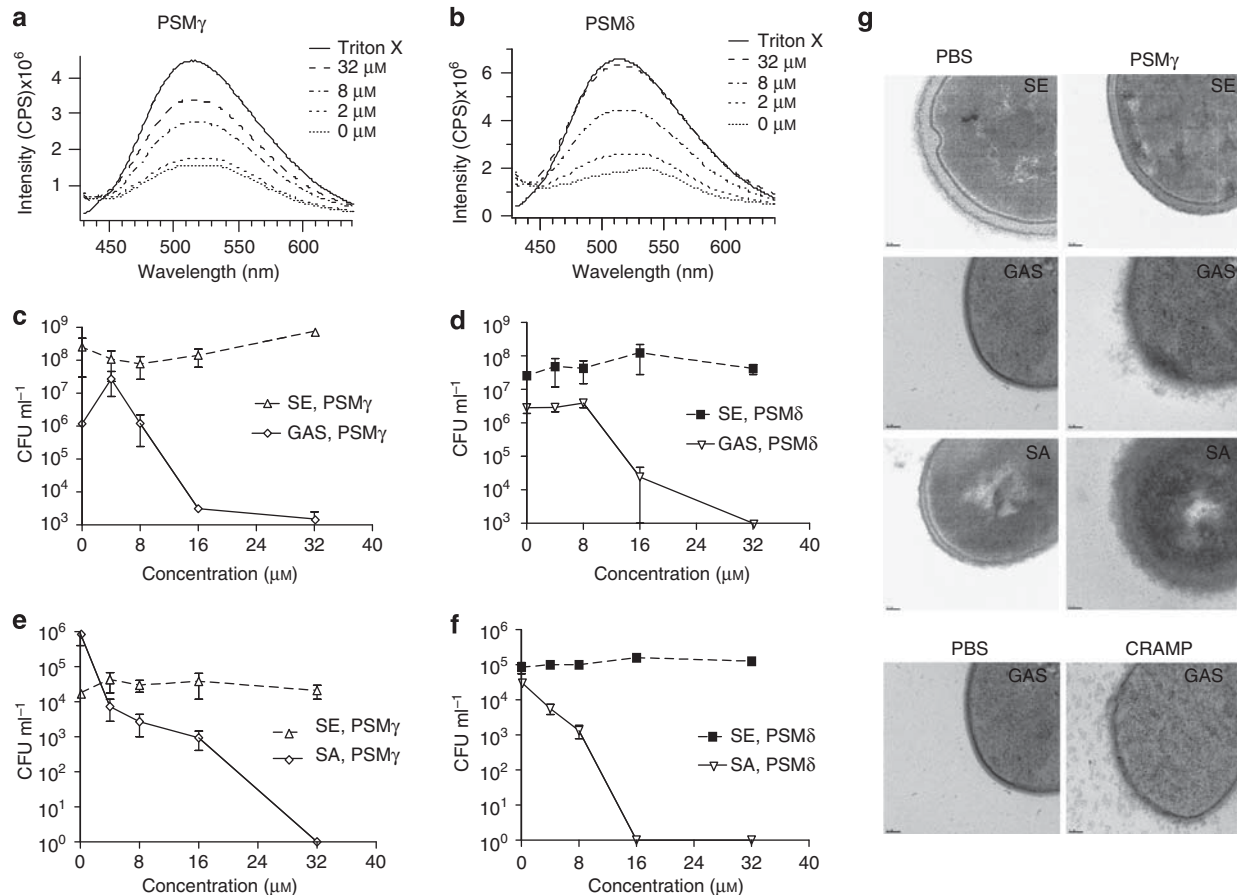


Figure 3. Phenol-soluble modulins disrupt artificial membrane vesicles and selectively kill skin pathogens. Synthetic vesicles encapsulating ANTS (fluorophore) and DPX (quencher) show dose-dependent fluorescence (leakage) in the presence of increasing concentrations of (a) PSM γ or (b) PSM δ . Both PSMs exhibit selective dose-dependent inhibition. GAS, but not *S. epidermidis* (SE), is susceptible to (c) PSM γ and (d) PSM δ . Similarly, (e) PSM γ and (f) PSM δ exert selective antimicrobial killing with *S. aureus* (SA) but not SE. (g) TEM analysis of SE, GAS, and SA membranes after incubation with PBS, PSM γ , or CRAMP (with GAS only). GAS and SA, but not SE, showed membrane blebbing after incubation with PSM γ . The effect of PSM γ on GAS membranes was similar to the blebbing that occurred after incubation with CRAMP. Images at original magnification $\times 30\,000$ and bars represent 50 nm.

PSMs are present on the surface of the human skin and act *ex vivo* to selectively kill GAS

As *S. epidermidis* is located on the epidermis, we sought to determine whether the presence of PSMs could prevent pathogen survival on the skin's surface. Sterilized skin explants from wild-type mice were treated with PSMs and then challenged with GAS or *S. epidermidis*. Similar to the *in vitro* minimal bactericidal concentration data that illustrate selective antimicrobial activity, PSM γ and PSM δ reduced GAS survival but not *S. epidermidis* on the skin's surface (Figure 6a and b).

DISCUSSION

In this study, we tested the hypothesis that *S. epidermidis* may have a beneficial or mutualistic relationship with human skin. Such findings have precedence with microbiota of the gut (Blum and Schiffrin, 2003; Backhed *et al.*, 2005; Gao *et al.*, 2007; Grice *et al.*, 2008), but has not been shown for cutaneous epithelia. In this study, our findings show that *S. epidermidis* produces two PSM peptides that have antimicrobial properties similar to those of host AMPs. Biophysical properties of PSMs supporting this conclusion

include observations that PSM γ forms multimeric complexes and exhibits a strong α -helical structure in solution that is modified by the presence of lipid membranes, thus disrupting the aggregates in solution. These findings suggest that PSM γ acts through the barrel-stave mechanism to disrupt microbial membranes and kill the organism, a mechanism of action similar to that of some other AMPs. Furthermore, the antimicrobial properties of these PSMs directly coincide with the predictions based on biophysical measurements. Importantly, PSMs seem to provide a selective advantage for *S. epidermidis* as they inhibit several common skin microbes but do not inhibit its own growth. Thus, the observation that *S. epidermidis* typically resides harmlessly on the human skin surface, combined with the potent antimicrobial properties of PSM γ and PSM δ , suggest that they could provide benefit to the host as additional epithelial AMPs.

There is clear evidence that the presence of AMPs at epithelial surfaces is beneficial. For example, we have previously shown that mice lacking cathelicidin (*Camp* $^{-/-}$) become much more susceptible to invasive GAS infections of the skin (Nizet *et al.*, 2001). Similarly, the transgenic expression of an additional AMP in the small intestine

Table 1. MBC activities of phenol-soluble modulins

Phenol-soluble modulin- γ	MBC
<i>Staphylococcus epidermidis</i> , 12228	> 64 μM
<i>Staphylococcus epidermidis</i> , 1457	> 64 μM
MRSA, Sanger 252	> 64 μM
MRSA, USA 300	> 64 μM
<i>Staphylococcus aureus</i> , 113	4–8 μM
<i>Streptococcus pyogenes</i> , NZ131	16 μM
<i>Escherichia coli</i>	8–16 μM
Phenol-soluble modulin-δ	
<i>Staphylococcus epidermidis</i> , 12228	> 64 μM
<i>Staphylococcus epidermidis</i> , 1457	> 64 μM
MRSA, Sanger 252	16–32 μM
MRSA, USA 300	32 μM
<i>Staphylococcus aureus</i> , 113	8 μM
<i>Streptococcus pyogenes</i> , NZ131	16 μM
<i>Escherichia coli</i>	8 μM

MBC, minimal bactericidal; MRSA, methicillin-resistant *Staphylococcus aureus*.

provides protection against fatal infections by salmonella (Salzman *et al.*, 2003), and expression in keratinocytes protects against skin infection (Braff *et al.*, 2005b). However, there is no evidence yet that cutaneous microbes offer a similar benefit to host immunity. Despite the many reports of microbial-produced antimicrobial molecules, such as bacteriocins (Hale and Hinsdill, 1975; Sahl, 1994; Ekkelenkamp *et al.*, 2005), the consequences of AMP production by resident microbes have not yet been investigated on skin. In contrast, microbes living on epithelial surfaces within the gut have been suggested to provide immune education and reciprocal benefit for the nutrient-rich niche (Backhed *et al.*, 2005). On the basis of mounting data on the benefits of resident microbes, combined with clear evidence of an important role for AMPs in skin innate immunity, it is reasonable to conclude that AMPs produced by microbes on the skin can be beneficial.

The properties described in this study for PSMs as AMPs are not inconsistent with previously described properties of these peptides as virulence factors (Wang *et al.*, 2007). In the setting of an immunocompromised host, and often in conjunction with a broken skin barrier with catheters, the production of PSMs by *S. epidermidis* can lead to tissue damage and cell lysis that contributes to virulence (Mehlin *et al.*, 1999; Hajjar *et al.*, 2001; Liles *et al.*, 2001; Vuong and Otto, 2002; Vuong *et al.*, 2004). Similarly, host AMPs such as LL-37 can also lead to disease when abnormally expressed (Howell *et al.*, 2004, 2006; Yamasaki *et al.*, 2007; Hollox *et al.*, 2008). Thus, membrane-active peptides often present a double-edged sword, providing defense but also potentially causing harm. In the situation with *S. epidermidis* PSMs, it is possible that they are beneficial when present on the surface

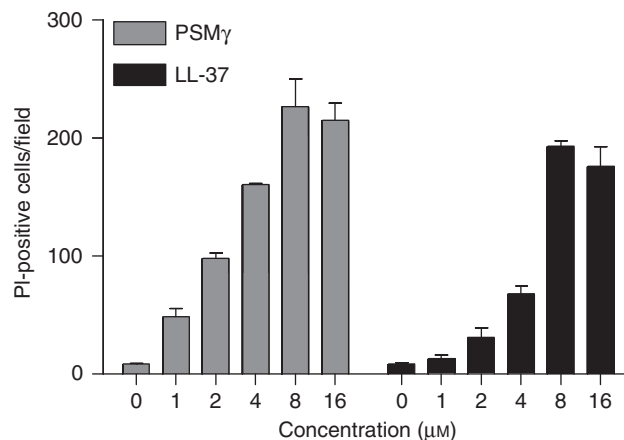


Figure 4. LL-37 and phenol-soluble modulin- γ induce similar dose-dependent changes in keratinocyte membrane permeability. Normal human epidermal keratinocytes, incubated with increasing concentrations of LL-37 or PSM γ for 18 h, were stained with propidium iodide (PI) to evaluate disruption in membrane permeability. PI uptake was measured by manually counting PI-positive fluorescent cells per microscopic field. Data represent mean \pm SEM of three random fields and are representative of two independent experiments.

of intact skin, but become potentially dangerous to the host when the barrier is deeply invaded by live *S. epidermidis*, and these bacteria then inhibit leukocytes attempting to repel their invasion. On the basis of the rare occurrence of this phenomenon in normal skin, it is unlikely that PSMs do more harm than good.

An important aspect of our data is that it shows that PSMs have a unique and highly desirable function for selective removal of pathogenic organisms on the skin, such as *S. aureus* and GAS. Importantly, PSMs do this while retaining normal flora, such as *S. epidermidis*, thus enabling normal flora to maintain their proposed beneficial effects while eliminating pathogens. The mechanism of action for selective killing by PSMs likely involves a cooperative interaction with other native AMPs released by the host, thus boosting innate immune defense in an immediate and selective manner. This finding presents the possibility for a topical antimicrobial strategy to kill common pathogens while the microbiome is preserved, an approach that would be likely to extend the duration of maximal immune defense and prevent repopulation by pathogens. This selective activity could become an important part of a normal microbial defense strategy against colonization and transmission of hospital-acquired bacterial pathogens, and could also be exploited for a role in future anti-infective therapeutics.

MATERIALS AND METHODS

Peptides

PSM δ and PSM γ were commercially synthesized and purified by HPLC (Quality Controlled Biochemicals, Hopkinton, MA). PSM γ purity was >95% and PSM δ purity was >70%. Mouse cathelicidin-related antimicrobial peptide and LL-37 were commercially synthesized and purified by HPLC to a purity >95% as previously described (Braff *et al.*, 2005a).

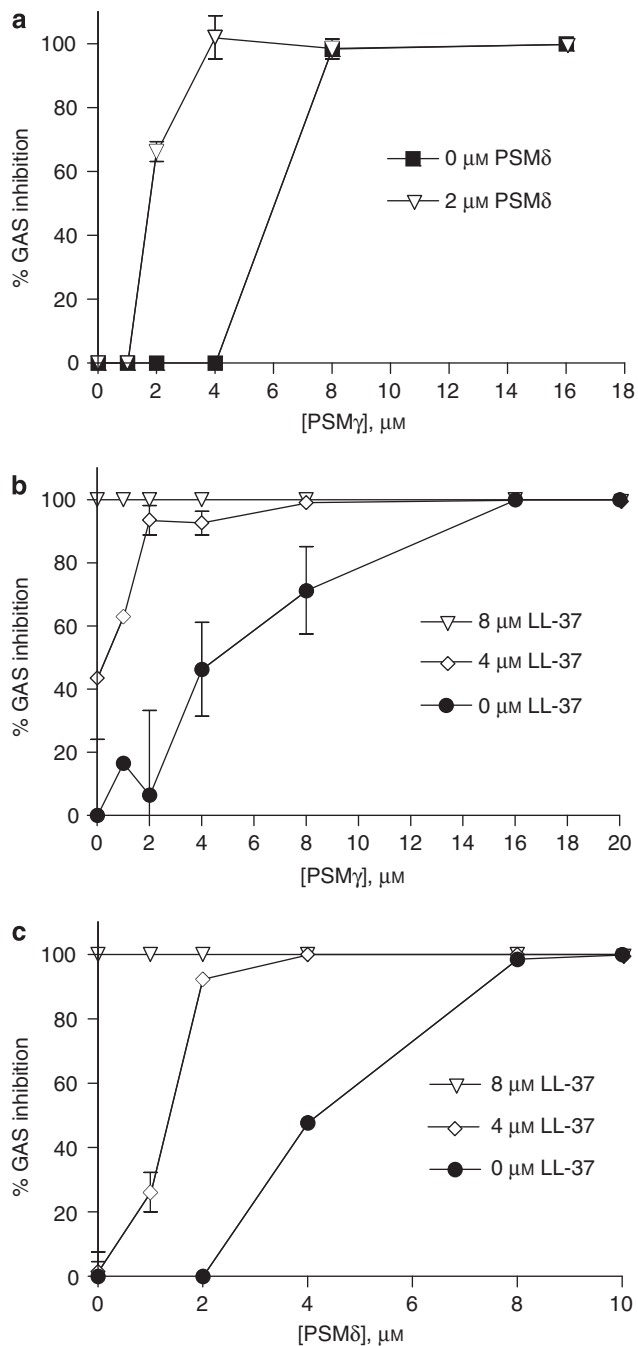


Figure 5. Phenol-soluble modulins cooperate with each other and host AMPs to kill GAS. (a) Co-incubation of GAS with PSM γ and PSM δ shows cooperative antimicrobial effect. Co-incubation of GAS with LL-37 and PSM γ (b) or PSM δ (c) shows cooperative antimicrobial effect. Data representative of two individual experiments performed in triplicate. Data are mean \pm SEM of a single experiment performed in duplicate and are representative of two independent experiments.

Cell culture

Normal human epidermal keratinocytes (Cascade Biologics, Portland, OR) were grown in EpiLife medium (Cascade Biologics) supplemented with 0.06 mM CaCl₂, 1% EpiLife-defined growth supplement, and penicillin/streptomycin (100 U ml⁻¹ and 50 μg ml⁻¹, respectively). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂.

Vesicle preparation and leakage assay

POPC and POPG (Avanti Polar Lipids) were combined at a 2:1 M ratio of POPC/POPG, dried under argon gas, resuspended by bath sonication in 20 mM potassium phosphate buffer (pH 7.3), or in 20 mM potassium phosphate buffer (pH 7.3), 50 mM DPX, and 50 mM ANTS. Vesicles were extruded through a 0.2- μm polycarbonate film and run over a size exclusion column to remove unencapsulated dye. Leakage assays were carried out by incubating vesicles (1:5 dilution) with the desired concentration of peptide for 1 h. Conditions for ANTS fluorescence were the following: excitation wavelength of 385 nm, emission wavelength of 400–700 nm, excitation and emission bandpass of 5 nm, 1 nm per step scan speed, and integration time of 0.2 s.

CD assay

The molar ellipticity ($[\theta] \times 10^3$, deg* cm²dmol⁻¹) of synthetic PSM γ and PSM δ was determined at 25 °C. For vesicle studies, 20 μM synthetic peptide in 20 mM potassium phosphate buffer, pH 7.3, was incubated with or without 1 mM of 2:1 POPC/POPG lipid vesicles for 1 h at 25 °C. For peptide interaction studies, 20 μM PSM γ was incubated with 5 or 10 μM of PSM δ for 1 h at 25 °C. Spectra were collected over 190–260 nm in an AVIV model Circular Dichroism Spectrometer (Aviv Biomedical, Lakewood, NJ) with a 0.1-cm path length, collecting data at 1 nm intervals. Five repeat scans were taken for each sample, and the averaged baseline spectrum was subtracted from the sample average. For vesicle studies, 20 mM potassium phosphate, pH 7.3, in the presence or absence of vesicles, was used as baseline. For peptide interaction studies, 5 or 10 μM of PSM δ was used as baseline.

Spectroscopic measurements

Synthetic peptide at concentrations of 5 and 25 μM were incubated in solutions containing 20 mM potassium phosphate, pH 7.3. Urea concentrations were determined by refractive index measurements taken on an Abbe 3L Bausch & Lomb Refractometer (Rochester, NY) (Shirley, 1995). Fluorescence measurements were taken on a Jobin Yvon SPEX FL3-11 spectrofluorometer (Edison, NJ) equipped with an R928 Photomultiplier Tube (Hamamatsu, Bridgewater, NJ). Conditions for tryptophan fluorescence are as follows: excitation wavelength of 290 nm, emission excitation and emission bandpass of 8 and 4 nm, respectively, 1 nm steps, and an integration time of 1 s. Unfolding curves assumed a two-state system (Pace, 1986) and reversible unfolding, and were performed in 20 mM potassium phosphate buffer, pH 7.3, in the absence of lipid vesicles. Emission maximum was determined for all protein samples and normalized to a scale of 0–1. Fraction unfolded, f , was plotted against urea concentration and was fit to the following equation (Pace, 1986):

$$f = \frac{\exp\left[-m\left(\frac{C_m - C}{RT}\right)\right]}{1 + \exp\left[-m\left(\frac{C_m - C}{RT}\right)\right]} \quad (1)$$

The fit-determined values for the slope, m , and midpoint urea concentration, C_m , were used to calculate the free energy of unfolding in the absence of denaturant, using the following equation (Pace, 1986; Sanchez *et al.*, 2008):

$$\Delta G_{(\text{H}_2\text{O})}^\circ = mC_m \quad (2)$$

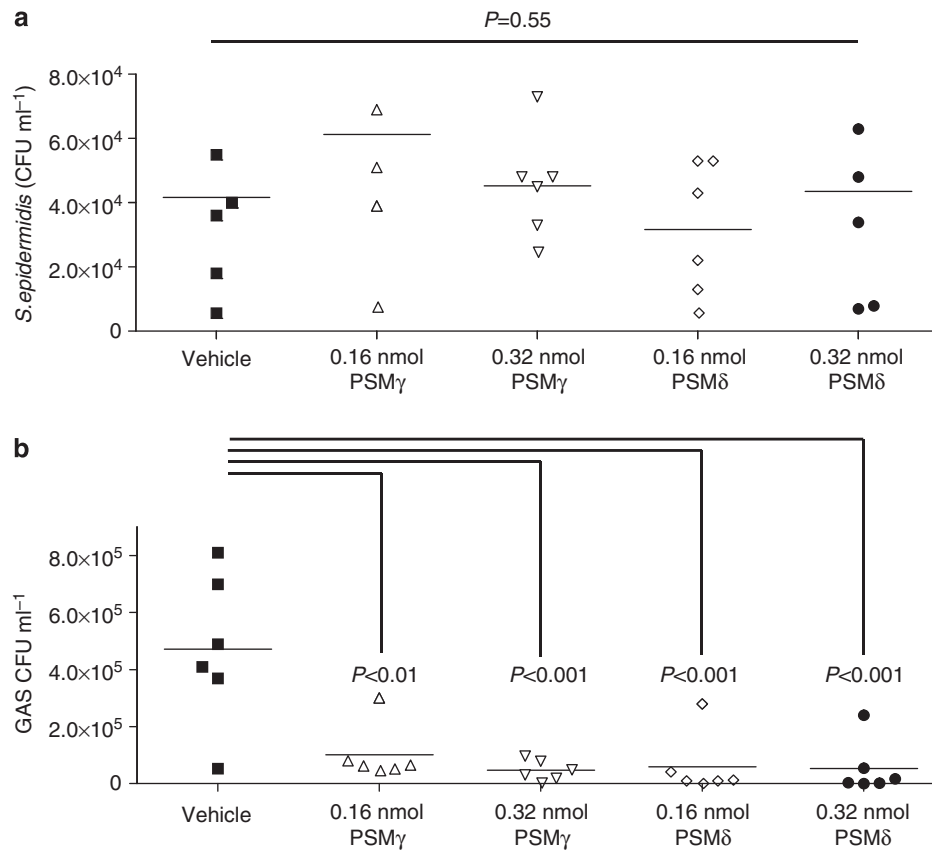


Figure 6. Phenol-soluble modulins selectively kill GAS but not *S. epidermidis* on the skin's surface. (a) Survival of *S. epidermidis* on mouse skin treated with PSMs at 0.16 or 0.32 nmol. Addition of PSMs on skin did not affect colonization of *S. epidermidis*. (b) GAS survival on skin was greatly reduced by the addition of PSMs at 0.16 or 0.32 nmol. Data are means of two independent experiments performed in triplicate. *P*-values were calculated using ANOVA with Bonferroni *post hoc* test when applicable.

In vitro antibacterial studies

Synthetic peptide (Quality Controlled Biochemicals) minimal bactericidal concentrations and bacterial killing curves were determined as before in the presence of carbonate, with the only modification being with GAS (in 25% Todd-Hewitt Broth, 75% of 1 × Dulbecco's (d) PBS (phosphate-buffered saline)), as GAS would not grow in media containing carbonate (Dorschner *et al.*, 2006). As a control for the survival curve, GAS and *S. epidermidis* were grown in the same medium. For electron microscopy analysis, GAS, *S. epidermidis*, and *S. aureus* were grown to midlog phase in Todd-Hewitt Broth. Cells were washed with 1 × dPBS and resuspended at 10⁸ CFU ml⁻¹ in 1 × dPBS. A final concentration of 16 μM peptide was added to the cells and incubated for 20 min on ice. The cells were submitted for EM analysis.

Keratinocyte toxicity study

Normal human epidermal keratinocytes (Cascade Biologics), grown to 75% confluence in EpiLife media containing 0.02 mM calcium and epidermal growth factor supplement (Cascade Biologics), were incubated with 0, 1, 2, 4, 8, and 16 μM of PSM γ , PSM δ , or CRAMP for 18 h. The cells were stained for permeability using 50 μg ml⁻¹ PI in 1 × dPBS for 10 min. The number of PI-positive fluorescent cells was counted per × 400 field. Three fields per well were enumerated and averaged. The experiment was performed twice with triplicate wells. In the representative experiment, error bars represent ± SEM.

Ex vivo mouse skin infections

The backs of 10- to 12-week-old wild-type C57BL/6 mice (Charles River, Wilmington, MA) were shaved. Nair (a depilating agent) was applied for 2 min and removed with a wet towel. At 18–24 h after hair removal, the mice were killed using CO₂. The skin was cleaned with 70% ethanol. Full-thickness punch biopsies of size 8 mm were floated on EpiLife media containing 0.06 mM calcium and epidermal growth factor supplement (Cascade Biologics). Synthetic PSM γ at 5 μl or PSM δ at 64 μM (0.32 nmol) or 32 μM (0.16 nmol) in 1 × dPBS was added to the punch biopsies for 5–10 min. A volume of 5 μl of GAS (*S. pyogenes* NZ131) or *S. epidermidis* (ATCC 12228, Manassas, VA) at 2 × 10⁵ CFU (colony-forming unit)/ml was then added to skin punches treated with the peptides. The samples were incubated at 37 °C for 4 h. CFUs were recovered by bead beating with 1 mm zirconia beads (which do not disrupt bacteria) in 1 ml 1 × dPBS for 1 min. The samples were placed on ice for 5 min and then bead beaten again for 1 min. Supernatant was serially diluted onto Todd-Hewitt agar for CFU enumeration. Experiment was performed twice in triplicate. In the combined experiment, error bars represent ± SEM. All experiments using mice were conducted according to institutional guidelines for animal experiments.

Statistical analysis

All statistical analyses were carried out using GraphPad Prism 4.0. One-way analysis of variance and Bonferroni's *post hoc* test were

used to determine significance for experiments with three more groups. An unpaired *t*-test was used to determine significance for experiments with only two groups. Values of $P < 0.05$ were considered significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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