Constitutive and Inflammation-Dependent Antimicrobial Peptides Produced by Epithelium Are Differentially Processed and Inactivated by the Commensal *Finegoldia magna* and the Pathogen *Streptococcus pyogenes*

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Constitutive and Inflammation-Dependent Antimicrobial Peptides Produced by Epithelium Are Differentially Processed and Inactivated by the Commensal Finegoldia magna and the Pathogen Streptococcus pyogenes

Inga-Maria Frick,* Sara L. Nordin,† Maria Baumgarten,* Matthias Mörgelin,* Ole E. Sørensen,* Anders I. Olin,*,† and Arne Egesten†

Epithelial linings serve as physical barriers and produce antimicrobial peptides (AMPs) to maintain host integrity. Examples are the bactericidal proteins midkine (MK) and BRAK/CXCL14 that are constitutively produced in the skin epidermal layer, where the anaerobic Gram-positive coccoïd commensal Finegoldia magna resides. Consequently, this bacterium is likely to encounter both MK and BRAK/CXCL14, making these molecules possible threats to its habitat. In this study, we show that MK expression is upregulated during inflammation, concomitant with a strong downregulation of BRAK/CXCL14, resulting in changed antibacterial conditions. MK, BRAK/CXCL14, and the inflammation-dependent antimicrobial β-defensins human β-defensin (hBD)-2 and hBD-3 all showed bactericidal activity against both F. magna and the virulent pathogen Streptococcus pyogenes at similar concentrations. SufA, a released protease of F. magna, degraded MK and BRAK/CXCL14 but not hBD-2 nor hBD-3. Cleavage was seen at lysine and arginine residues, amino acids characteristic of AMPs. Intermediate SufA-degraded fragments of MK and BRAK/CXCL14 showed stronger bactericidal activity against S. pyogenes than F. magna, thus promoting survival of the latter. In contrast, the cysteine-protease SpeB of S. pyogenes rapidly degraded all AMPs investigated. The proteins FAF and SIC, released by F. magna and S. pyogenes, respectively, neutralized the antibacterial activity of MK and BRAK/CXCL14, protein FAF being the most efficient. Quantitation and colocalization by immunoelectron microscopy demonstrated significant levels and interactions of the molecules in in vivo and ex vivo samples. The findings reflect strategies used by a permanently residing commensal and a virulent pathogen, the latter operating during the limited time course of invasive disease. The Journal of Immunology, 2011, 187: 000–000.

Epithelial linings constitute a physical barrier against environmental microbes that present potential threats to the host (for instance, pathogenic bacteria) but are also colonized by bacteria that are part of the normal microbiota. In addition to being a physical barrier, epithelial cells have a constitutive production of antimicrobial agents; for example, antimicrobial peptides (AMPs) such as the antibacterial chemokine BRAK/CXCL14 (1–3). Another example is midkine (MK), an antibacterial growth factor that is constitutively expressed in the basal parts of the epidermal layer (4, 5). It is likely that there is a delicate balance among constitutively produced AMPs, the ecological niche inhabited by bacteria of the normal microbiota, and the prevention of infection caused by pathogenic bacteria. In the case of injury and invasion of pathogenic bacteria, the expression of several AMPs of epithelial cells is induced, resulting in production of, for example, the β-defensins human β-defensin (hBD)-2 and hBD-3 (6, 7).

The normal human microbiota includes several bacterial species that inhabit the skin without causing any inflammation. An example of a skin commensal is the anaerobic Gram-positive coccus Finegoldia magna. This bacterium is also an opportunistic pathogen causing several clinical conditions, such as soft tissue infections, wound infections, and bone/joint infections in immunocompromised hosts (8). In addition, a number of recent studies reported the presence of F. magna in chronic wounds, including diabetic ulcers (9–11). F. magna resides in the lower parts of the epidermal layer, where it binds to BM-40, which is part of the basal membrane (BM), via the surface-associated protein FAF (12). It is therefore likely that F. magna encounter the constitutively expressed AMPs MK and BRAK/CXCL14 during normal, noninflamed conditions. Most strains of F. magna express a subtilisin-like enzyme, subtilase of F. magna (SufA), which is associated with the bacterial surface (13). Both FAF and SufA are produced at substantial amounts during early logarithmic phase, and, in addition to being surface associated, they are released to the environment (12, 13). Thus, it is likely that high local concentrations of these proteins can be reached in vivo. Studies on the proteolytic activity of SufA demonstrated that the enzyme cleaves
and inactivates antibacterial molecules such as LL-37 and MIG/CXCL9 (13). In addition, F. magna can neutralize the activity of AMPs by release of protein FAF from its surface (12, 14).

Streptococcus pyogenes is a highly virulent pathogen causing both superficial and deep severe infections, such as pharyngitis, erysipelas, necrotizing fasciitis, and septic shock (15). During infection and dissemination in tissues, S. pyogenes releases the cysteine-protease SpeB that can degrade several molecules of the host response (16). In addition, S. pyogenes releases protein SIC that inhibits complement activation and neutralizes AMPs (17–21). Both SpeB and SIC are produced in high amounts during early growth phase (20, 22), and both proteins are important for the virulence of S. pyogenes (22–27).

In this study, we show that the commensal F. magna can circumvent the bactericidal activity of MK, BRAK/CXCL14, and hBD-3 by releasing SufA and protein FAF. In contrast, the virulent pathogen S. pyogenes inactivates MK, BRAK/CXCL14, hBD-2, and hBD-3 by release of SpeB and protein SIC. The findings explain how F. magna can create a protected ecological niche during noninflamed conditions, whereas S. pyogenes possesses potent means to avoid a broad range of host defense molecules produced in response to inflammation during invasive infection. These properties are likely to reflect different strategies used by a permanently colonizing commensal and a highly virulent pathogen, the latter operating during the limited time course of invasive disease.

Materials and Methods

Proteins and peptides

Native SufA and SpeB were purified as previously described (13, 28). Recombiantly expressed protein FAF (aa 28–616) was obtained as a fusion protein with GST as described (12). Human recombinant MK and BRAK/CXCL14 were from PeproTech (Rocky Hill, NJ), hBD-2 and hBD-3 from Creative Peptides (Shirley, NY).

Human skin samples

Samples from human skin wounds were obtained under protocols approved by the Ethics Committee at Lund University (LU 509-01 and LU 708-01). Nonwounded human skin was obtained by taking punch biopsies from healthy donors, and skin wound samples were obtained by making new punch biopsies from the edges of the initial biopsies. Parts of the samples were fixed in formalin (10%) for immunohistochemistry. For cDNA microarray analysis, as much dermal tissue as possible was removed by dissection from the biopsies. The remaining tissue was washed thoroughly in sterile sodium chloride (0.9%) to remove infiltrating inflammatory cells. This procedure ensured that the samples mainly consisted of epidermis. RNA was isolated from these samples and used for cDNA microarray analysis.

To obtain ex vivo infestation, skin biopsies from healthy skin were incubated with F. magna bacteria (strain AL8B) as described (12). In brief, punch biopsies were incubated with F. magna (2 × 10^{9} bacteria) in PBS for 1 h, washed, and incubated under anaerobic conditions at 37°C for an additional 48 h. Following a washing step with PBS plus 0.05% Tween 20, samples were fixed in 2.5% glutaraldehyde in 0.15 M sodium cacodylate (pH 7.4), postfixed in 1% osmium tetroxide, and prepared for transmission electron microscopy (see below).

A skin biopsy from a patient presenting with erysipelas (S. pyogenes infected), a kind gift from Dr. Adam Linder, Lund University, Skåne University Hospital, was processed as described (29).

Immunohistochemistry for light microscopy

After fixation in formalin (10%), the specimens were dehydrated, embedded in paraffin, placed on Superfrost Plus glass slides (Menzel-Gläsur, Braunschweig, Germany), incubated for 2 h at 60°C, deparaffinized in Tissue Clear (Sakura Finetek, Zoeterwoude, The Netherlands), and rehydrated in graded alcohol and water. The slides were then treated with Dako Avidin Biotin Blocking Kit (DakoCytomation, Glostrup, Denmark) for 40 min at 97°C, followed by blocking in TBS supplemented with 0.05% Tween 20, 1% BSA, for 45 min at room temperature. After three 15-min washings, the specimens were incubated at room temperature for 1 h with polyclonal Abs diluted (5 μg/ml) in TBS supplemented with 0.05% Tween 20, 1% BSA. After three 20-min washings in TBS with 0.05% Tween 20, the slides were incubated with a MACH 3 Probe alkaline phosphatase Polymer kit (Biocare Medical, Concord, CA) for 10 plus 10 min, followed by three 15-min washes. Color was developed using Vulcan Fast Red chromogen (Biocare Medical, Concord, CA), and the slides were counterstained with Harris Hematoxylin (Histolab, Göteborg, Sweden).

Cell culture and stimulation of cells

Primary human keratinocytes were obtained from Cascade Biologics (Portland, OR) and cultured in serum-free medium (KGM Bullet kit) from Cambrex (Kiel, Germany). Cells were stimulated, beginning 24 h after complete confluence was reached with conditioned medium from PBMCs that had been stimulated with LPS (100 ng/ml, Escherichia coli O55:B5; Sigma-Aldrich, St. Louis, MO) for 4 d as described (27). After stimulation of the keratinocytes for 48 h, the cells were solubilized in TRizol (Invitrogen, Carlsbad, CA) and RNA purified according to the instructions from the manufacturer.

RNA isolation and microarray analysis

Total RNA was isolated with TRizol (Invitrogen, Carlsbad, CA) according to the recommendations of the supplier and resuspended in 0.1 M EDTA. The concentration was determined by spectrophotometric measurement. For gene expression analysis, total RNA was biotinylated and hybridized to Human Genome U133 Plus 2.0 GeneChips (Affymetrix, Santa Clara, CA) according to the instructions by the manufacturer. The microarray fluorescence signals were normalized using the GeneChip Operation Software (GCOS ver. 1.4; Affymetrix). The analysis was performed essentially as previously described (30). To calculate fold change in mRNA expression, the cDNA hybridization signals for MK and BRAK/CXCL14 obtained from wounds on day 4 were compared with the signals obtained from nonwounded skin. Microarray data are available through the Minimum Information About a Microarray Experiment database (http://www.ebi.ac.uk/arrayexpress; accession number E-MEXP-3305; username: Reviewer_E-MEXP-3305; password: 19dxiehx).

Quantitative real-time RT-PCR

cDNA was synthesized from 400 ng purified RNA from the keratinocyte culture using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) according to the instructions given by the manufacturer. The expression of MK and BRAK/CXCL14 together with G3PDH was analyzed using iQ SYBR Green Supermix (Bio-Rad). Primers for real-time PCR were: MK forward, 5′-GGT TGC CAG AA-3′; MK reverse, 5′-AGC TGT TGC GAC TAA-3′; BRAK/CXCL14 forward, 5′-CTC AAG TTI GGT TGC CAG AA-3′; and BRAK/CXCL14 reverse, 5′-GAA ATC CCT AGG TTG GGA AA-3′. Amplification was performed at 55°C for 40 cycles in the iCycler Thermal Cycler (Bio-Rad) and data analyzed using iCycler iQ Optical System Software (Bio-Rad). To calculate fold change in mRNA levels, the threshold cycles (Ct) of MK and BRAK/CXCL14, respectively, were subtracted from the Ct of the G3PDH housekeeping gene, thus obtaining normalized Ct. To calculate the difference in mRNA levels for MK and BRAK/CXCL14, values of resting keratinocytes were subtracted from those of stimulated keratinocytes and expressed as ddCT. Negative value of ddCT denotes downregulation, and positive ddCT denotes upregulation.

Bacterial strains and growth conditions

F. magna strains 505 and AL8B were isolated at Lund University Hospital (Lund, Sweden). S. pyogenes strain API (40/58) of serotype M1 was from the World Health Organization Collaborating Centre for Reference and Research on Streptococci, Prague, Czech Republic. API was grown in Todd–Hewitt broth (TH; BD/Difco, Franklin Lakes, NJ) at 37°C with 5% CO_{2}.

Bactericidal assay

F. magna bacteria (505 strain) were cultivated for 24 h (midexponential growth phase, OD_{560} ≈ 0.4), washed in incubation buffer (10 mM Tris/HCl, 5 mM glucose [pH 7.5]), and adjusted to a concentration of 2 × 10^{6} CFU/ml. Fifty microliters bacterial solution (∼10^{5} CFU) was incubated in buffer or with various concentrations of MK, BRAK/CXCL14, hBD-2, or hBD-3 for 1 h under anaerobic conditions. To quantify the bactericidal activity, serial dilutions of the mixtures were plated on TH-agar (supplemented with Tween-80) in duplicates and incubated at 37°C for 3 d under anaerobic conditions. The number of CFUs was then determined.
**FIGURE 1.** MK and BRAK/CXCL14 expression in skin. A, Bound specific Abs against MK and BRAK/CXCL14, respectively, were detected by secondary alkaline phosphatase-conjugated Abs, reacting with a substrate that is deposited as a red stain. MK and BRAK/CXCL14 are detected in epidermis of normal skin. MK shows a stronger staining in the basal part, whereas BRAK/CXCL14 is more evenly distributed (top panel). In skin from an acute wound (day 4; bottom panel), dispersed expression of MK is seen in the epidermal layer, whereas BRAK/CXCL14 expression is decreased. Replacement of the specific Abs with rabbit control IgG resulted in loss of staining. B, Electron micrograph showing overview with basal cell at the epidermal/dermal junction (stratum germinativum, asterisk; top left panel) and prickle cell in stratum spinosum in the epidermis of healthy skin. Scale bar, 5 μm.

**C**

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<th>Wound Day 4</th>
<th>Healthy skin</th>
<th>MK</th>
<th>BRAK/CXCL14</th>
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<td>Stained keratinocytes</td>
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**Transmission electron microscopy**

For negative staining, bacteria were incubated with intact AMPs or proteolytic fragments as described above, followed by processing for transmission electron microscopy as previously described (32). In short, samples (5 μl) were absorbed for 2 min onto the grids followed by two washes with distilled water. The samples were stained for 3 s with 0.75% uranyl formate droplets, followed by staining for an additional 30 s with 0.75% uranyl formate.

For immunolabeling, fixed and washed samples were subsequently dehydrated in ethanol and further processed for epon embedding. Sections were cut with an LKB ultratome (LKB, Bromma, Sweden) and mounted on gold grids. For immunostaining, the grids were floated on top of drops of 3% (w/v) glutaraldehyde and 2% (w/v) osmium tetroxide in 0.1 M sodium metaperiodate for 2 h, followed by staining for an additional 30 s with 0.75% uranyl formate.

**Mass spectrometry**

Obtained Coomassie-stained gel protein bands after SufA digestion were selected, cut, washed with 40% (v/v) acetonitrile, and evaporated to dryness. Reduction was performed with 10 mM DTT at 56°C for 30 min, alkylation with 50 mM iodoacetamide, and digestion with Asp-N endopeptidase (Roche Diagnostics, Mannheim, Germany; 25 μg/ml) in 25 mM ammonium bicarbonate buffer (pH 7.8) for 16 h at 37°C. After extraction of gel pieces with 75% acetonitrile in 5% trifluoroacetic acid, peptides were subjected to reversed-phase HPLC separation (CapLC; Waters, Manchester, U.K.) and subsequent sequencing on a Q-TOF Ultima API MS/MS (Waters, Manchester, U.K.). MK and BRAK/CXCL14 incubated with SufA were also taken directly to liquid chromatography tandem mass spectrometry (MS/MS) for analysis of obtained shorter peptide products. The MS/MS results were interpreted using the Peptide Mass Fingerprint tool of the Mascot search engine (http://www.matrixscience.com). Settings of fixed and variable modifications were used. Experimental data were compared with Asp-N cleavage of the MK and BRAK/CXCL14 sequences using the PeptideCutter tool (http://www.expasy.org).

**Cleavage of AMPs with SufA and SpeB**

Digestion of MK, BRAK/CXCL14, hBD-2, or hBD-3 with SufA and SpeB was performed at 37°C for 1 and 18 h, respectively. For cleavage with SufA, 5 μg peptides were incubated with 0.5 μg enzyme in PBS. The reactions were terminated by the addition of PMSF (Sigma-Aldrich) to a final concentration of 5 mM. In the case of SpeB, 5 μg peptides were incubated with 0.5 μg enzyme in PBS supplemented with DTT (final concentration 10 mM). The reactions were terminated by the addition of the cysteine-protease inhibitor E64 (final concentration 10 mM). The cleaved peptides were separated and analyzed by Tricine SDS-PAGE.

**SDS-PAGE**

SDS-PAGE was performed as described by Laemmli using a total polyacrylamide concentration of 10% and 3.3% cross-linking. Analysis of peptide degradation was performed with Tricine SDS-PAGE (31) using a polyacrylamide concentration of 16.5% and 3.3% cross-linking. Gels were stained with Coomassie R-250.

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polyclonal anti-MK, and anti-BRAK/CXCL14 at 4°C. Signal for binding was detected with EM goat anti-rabbit IgG 10 nm Gold (BBInternational, Cardiff, U.K.) and EM rabbit anti-goat IgG 20 nm Gold (BBInternational), respectively. Samples were finally stained with uranyl acetate and lead citrate before being observed and evaluated as above. They were examined with a Jeol JEM 1230 EX transmission electron microscope (Jeol) operated at 60 kV accelerating voltage. Images were recorded with a Gatan Multiscan 791 charge-coupled device camera (Gatan, Pleasanton, CA).

To evaluate the concentrations of the AMPs and the bacterial proteins in skin, the gold probe density on specific primary Abs and on normal rabbit Ig controls was quantified by taking 30 random electron micrographs on individual skin samples. Micrographs were masked with overlays representing 1 μm². The number of gold particles within different 1-μm² windows was determined manually by point counting in each micrograph. Molar Ag concentrations were then calculated on characteristics of Ag retrieval procedures (33–35) and predictions on immunolabeling efficiency, surface relief of thin sections, and surface accessibility of randomly dispersed molecules as described (36).

Surface plasmon resonance spectrometry

Protein FAF, protein SIC, or hBD-3 was immobilized via amine coupling in flow cells of a CM5 sensorchip (BIAcore). Different concentrations of proteins were injected over the coated surface at 10 μl/min and 25°C (in running buffer: 10 mM HEPES [pH 7.5], 150 mM NaCl, 0.005% surfactant P20, and 3.4 mM EDTA). The FAF, SIC, or hBD-3 surfaces were regenerated by injection of a 200-μl 2-min pulse of running buffer containing 2 M NaCl followed by an extensive wash procedure. After X and Y normalization of data, the blank curves from the control flow cell of each injected concentration were subtracted. The association (κa) and dissociation (κd) rate constants were determined simultaneously using the equation for 1:1 Langmuir binding in the BIA Evaluation 3.1 software (BIAcore). The binding curves were fitted locally, and the equilibrium dissociation constants (Kd) were calculated from mean values of the obtained rate constants.

Results

Expression of MK and BRAK/CXCL14 in the epidermis during healthy and inflamed conditions

Using immunohistochemistry, both MK and BRAK/CXCL14 could be detected in the epidermis of skin obtained from healthy donors (Fig. 1A). Labeling for MK was most intense in the basal parts of epidermis. However, in biopsies from wound edges 4 d postinjury, a more dispersed staining for MK was observed, whereas the staining for BRAK/CXCL14 was less intense, suggesting a reciprocal gene regulation for these genes during inflammation.

Subcellular localization of MK and BRAK/CXCL14 in keratinocytes was determined using immuno-electron microscopy. Both MK and BRAK/CXCL14 are seen in association with the BM (lamina densa; asterisks, Fig. 1B), at the nuclear membrane, and in association with the plasma membrane in keratinocytes of healthy skin, whereas only weak labeling for BRAK/CXCL14 was seen in keratinocytes of skin wounds after 4 d (Fig. 1B).

Using cDNA microarray, an ∼3-fold increase in MK expression was seen 4 d after injury, whereas BRAK/CXCL14 was downregulated (Fig. 1C). To investigate the influence of inflammatory mediators on MK and BRAK/CXCL14 expression in vitro, PBMC were stimulated with LPS (100 ng/ml) for 4 d. The conditioned medium was then used to stimulate primary human keratinocytes for 48 h. The conditioned medium doubled the MK expression, whereas BRAK/CXCL14 expression was downregulated by 12 threshold cycles (Fig. 1C). Downregulation of BRAK/CXCL14 expression in inflammatory skin disorders has been described previously (3).

Bactericidal activity of MK, BRAK/CXCL14, hBD-2, and hBD-3 against F. magna and S. pyogenes

Previous studies have demonstrated that MK and BRAK/CXCL14 both possess antibacterial activity (3, 5). Having seen the strong upregulation of MK and the downregulation of BRAK/CXCL14 during inflammation, we therefore investigated whether the activity of the proteins differ depending on the bacterial species being a commensal or a pathogen. In these experiments, we compared the sensitivity of the commensal F. magna (strain 505) and the pathogenic S. pyogenes (strain AP1) in a viable count assay. Interestingly, MK was more potent in killing S. pyogenes bacteria, whereas BRAK/CXCL14 killed F. magna bacteria more efficiently (Fig. 2A, 2B). Thus, at a MK concentration of 0.1 μM, ∼100% of S. pyogenes bacteria were killed, whereas 1 μM of the peptide was required to achieve 100% killing of F. magna bacteria. In contrast, 0.1 μM BRAK/CXCL14 killed ∼80% of F. magna bacteria, but only 20% of the S. pyogenes bacteria. During infection and inflammation, the β-defensins hBD-2 and hBD-3 are upregulated and secreted by keratinocytes, and the bactericidal activity of these peptides was also compared against F. magna (strain 505) and S. pyogenes (strain AP1). Of these peptides, hBD-3 was the most potent and efficiently killed both strains at 1 μM (Fig. 2D). At this concentration, hBD-2 only slightly affected F. magna bacteria (Fig. 2C) and, to achieve 100% killing of both F. magna and S. pyogenes bacteria with this peptide, concentrations of 10 μM were required.

Proteolytic activity from SufA of F. magna and SpeB of S. pyogenes against MK, BRAK/CXCL14, hBD-2, and hBD-3 and characterization of residual bactericidal activity

SufA of F. magna has proteolytic activity against LL-37 and the chemokine MIG/CXCL9 (13). Also, SpeB of S. pyogenes is known to cleave LL-37 (37) and several chemokines (38). Thus, we incubated MK, BRAK/CXCL14, hBD-2, and hBD-3 with SufA and SpeB, respectively, for 1 and 18 h, and the resulting fragments were analyzed by Tricine SDS-PAGE (Fig. 3). SpeB
rapidly cleaved all polypeptides, and after 1 h of incubation, they were almost completely degraded (Fig. 3A–D). In contrast, SufA cleavage of MK generated several smaller fragments, and two stable fragments were observed over time (Fig. 3A), whereas BRAK/CXCL14 was completely degraded after 18 h of incubation (Fig. 3B). To identify the cleavage sites in MK and BRAK/CXCL14, the protein bands, indicated in Fig. 3A and 3B, were excised and subjected to MS/MS analysis. In addition, cleaved peptides in solution were also analyzed by MS/MS. The predicted SufA cleavage sites are indicated in the amino acid sequences of MK and BRAK/CXCL14 (Fig. 3E,3F). Furthermore, in the models of these molecules, the amino acid residues prone to cleavage are indicated (Fig. 3E,3F). The bactericidal activity of MK has been located to a region in the N-terminal domain and to the C-terminal tail (5). As shown in Fig. 3E, several cleavage sites for SufA are identified in these regions. Cationic amino acids (i.e., arginine and lysine) are characteristic features of AMPs, and the majority of cleavage sites included either of these amino acids. SufA did not degrade the defensins hBD-2 and hBD-3, neither as seen after SDS-PAGE (Fig. 3C,3D) nor as determined by MS/MS.

Next, the bactericidal activity of the SufA-generated fragments of MK and BRAK/CXCL14 was tested to compare the activity against F. magna (505 strain) and S. pyogenes (API strain). The fragments generated after 1 h of digestion with SufA were still able to efficiently kill S. pyogenes, but killed F. magna to a significantly lesser degree. The bactericidal activity was lost following further digestion (Fig. 4A,4B). Electron microscopy revealed rupture of bacterial membranes and leakage of intracellular contents in the presence of the intact polypeptides, but to a lesser extent in the case of F. magna, when incubated with SufA-cleaved MK and BRAK/CXCL14, respectively (Fig. 4C). This suggests that the activity of SufA against these constitutively expressed AMPs will provide survival advantages for F. magna, at least during healthy conditions and during early stages of infection.

FIGURE 3. Proteolytic activity of SufA and SpeB, respectively, against MK (A), BRAK/CXCL14 (B), hBD-2 (C), and hBD-3 (D). The peptides (5 μg) were incubated with the respective enzymes (0.5 μg) for 1 and 18 h at 37˚C, and cleavage products were analyzed by Tricine SDS-PAGE. The fragments of MK and BRAK/CXCL14 generated by SufA after 1 h were characterized by MS/MS. The primary amino acid sequences and models of MK and BRAK/CXCL14 with predicted SufA cleavage sites indicated in red are shown in E and F, respectively. No cleavage of hBD-2 or hBD-3 with SufA occurred as determined by MS/MS.
Neutralizing capacity from protein FAF of F. magna and protein SIC of S. pyogenes against MK and BRAK/CXCL14

Most strains of F. magna express the surface protein FAF, which also is released into the surrounding environment, partly through action of SufA (12, 14). Thus, we asked whether FAF could bind and inhibit the activity of the AMPs described above. First, possible interference of FAF with the killing of F. magna bacteria by MK and BRAK/CXCL14 was investigated. The F. magna 505 strain was incubated with bactericidal concentrations of MK (1 μM), BRAK/CXCL14 (1 μM), and hBD-3 (1 μM), respectively, in presence of various ratios of FAF. As shown in Fig. 5A, FAF dose dependently blocked the activity of all three peptides. Notably, the defense hBD-3, which was resistant to cleavage by SufA, was the peptide most efficiently blocked by FAF. To investigate binding characteristics between FAF and the various peptides, surface plasmon resonance spectrometry was used. FAF was immobilized to the surface of a chip, and MK, BRAK/ CXCL14, or hBD-3 were injected and left to interact to saturation. Binding to FAF was obtained with MK (K_D = 13 nM) and BRAK/CXCL14 (K_D = 8.9 nM), but not hBD-3. However, when, instead, hBD-3 was immobilized and FAF injected over the surface, a stable interaction was observed (K_D = 0.7 nM) (Fig. 5A). Due to the relatively low and sometimes inconsistent bactericidal activity of hBD-2 in viable counts experiments, blocking experiments were not performed with this peptide. In addition, surface plasmon resonance experiments showed no or low binding of FAF to hBD-2 (data not shown).

Then we analyzed whether protein SIC, which is produced by some strains of S. pyogenes including the highly virulent M1 serotype (21), is also able to inhibit the bactericidal activity of MK and BRAK/CXCL14. SIC blocked the killing of S. pyogenes with these peptides, although not as efficiently as protein FAF (Fig. 5B). Furthermore, using surface plasmon resonance spectrometry, SIC was found to bind to both MK and BRAK/CXCL14, but with a more transient kinetic compared with the interaction of FAF (Fig. 5B). SIC has previously been shown to bind and interfere with the activity of human β-defensins and was therefore not analyzed (19, 39).

Colocalization of MK and BRAK/CXCL14 with SufA and FAF of F. magna and SpeB and SIC of S. pyogenes in infested skin

Healthy skin infested ex vivo with F. magna and biopsies from S. pyogenes-infected skin (erysipelas) were investigated using electron microscopy (Fig. 6A, upper panels). In both cases, bacteria were seen in the vicinity of keratinocytes, with characteristic morphological features (i.e., being coccoid and, in the case of S. pyogenes, an extracellular rim of M protein).

Using immunoelectron microscopy on healthy skin biopsies infested with F. magna ex vivo, MK and BRAK/CXCL14 were colocalized with SufA and FAF in close proximity of F. magna bacteria (Fig. 6A). In skin biopsies from erysipelas (caused by S. pyogenes), MK and BRAK/CXCL14 colocalized with SpeB and SIC, respectively, close to the bacterial surface (Fig. 6A). To determine the concentrations of the AMPs and the bacterial proteins in the skin samples, the gold probe density on the specific primary Abs and on normal rabbit Ig controls was quantified as described in Materials and Methods. The values obtained (Fig. 6B) confirmed increased production of MK and a decreased production of BRAK/CXCL14 during infection. Skin infested ex vivo with F. magna may resemble the situation during an opportunistic infection causing inflammation rather than colonization as a commensal. The findings are summarized in Fig. 7.

Discussion

In this study, we show that F. magna, a member of the normal microbiota, and the virulent pathogen S. pyogenes differently modulate the activity of constitutively and induced AMPs, respectively. Although F. magna possess the means (i.e., SufA and protein FAF) to circumvent the constant pressure of the constitutively expressed AMPs MK and BRAK/CXCL14, S. pyogenes possess a broader activity (via SpeB and protein SIC) including the important AMPs hBD-2 and hBD-3 that are expressed during inflammation (summarized in Figure 7).

During healthy, noninflamed conditions, F. magna colonize the basal parts of epidermis and are likely to encounter the
constitutively expressed MK and BRAK/CXCL14, as demonstrated in the current study. However, during inflammation, BRAK/CXCL14 production is downregulated, as seen in atopic dermatitis and psoriasis (3, 40). Epidermal growth factor is upregulated during inflammation and wound healing and has a strong inhibitory activity on BRAK/CXCL14 gene expression (41). MK is upregulated during inflammation in some tissues of the adult and can be detected in plasma of healthy individuals (42, 43). Binding of many proinflammatory cytokines to their corresponding receptors and activation of TLRs by pathogen-associated molecular patterns result in activation of NF-κB–dependent gene transcription (44). MK has an NF-κB–responsive element in its promoter region and is thus likely to be upregulated during inflammation (45). Another property of MK is that it recruits and activates neutrophils to sites of inflammation, suggesting several important roles for this growth factor (46).

In a previous study, it was shown that BRAK/CXCL14 has antibacterial activity against fungi and both Gram-positive and -negative aerobic bacteria (3). The finding in the present work that BRAK/CXCL14 had higher activity against F. magna than S. pyogenes might reflect this chemokine’s role in controlling the host burden of commensal bacteria. During inflammation caused by more virulent bacteria, additional AMPs such as hBD-2 and hBD-3 are upregulated to restrict the spread of the infection (47). Thus, in such an event, it is necessary for commensal bacteria to cope with another set of host defense molecules to survive. It is therefore not surprising that F. magna is less sensitive to MK. Interestingly, hBD-2 showed relatively weak antibacterial activity against both F. magna and S. pyogenes compared with the other AMPs investigated. During invasion, S. pyogenes, being a virulent pathogen, operates during a limited timeframe. This may be one explanation for SpeB being more efficient and less specific than...
SufA, enhancing successful invasion and dissemination, overwhelming the host. In contrast, it is not in the interest of \textit{F. magna}, being a colonizing commensal, to disturb homeostasis.

Common characteristics for AMPs are a high content of the positively charged amino acids arginine and lysine. Interestingly, SufA preferentially cleaves both MK and BRAK/CXCL14 at these positions and thereby reduces the antibacterial activity of these molecules. For a commensal bacterium, this is an important mechanism that will lead to improved survival, unarming constitutively produced AMPs, at the site of the colonization. The preference of SufA to cleave at positions of basic amino acids has been described for other subtilases of human and plant origin (48). In contrast, SufA cannot cope with the \(\beta\)-defensins, possibly because they are more compact molecules. However, \textit{F. magna} strains that express FAF have an advantage through the efficient neutralizing capacity of this molecule against hBD-3. The peptide hBD-2 has a relatively low bactericidal activity and may not present a similar threat to the bacterium.

In the case of a pathogen, such as \textit{S. pyogenes}, it is important for the bacterium to modulate a broad spectrum of AMPs to

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure6}
\caption{Colocalization of MK and BRAK/CXCL14 with SufA and FAF of \textit{F. magna} and SpeB and SIC of \textit{S. pyogenes} in infested skin. \textbf{A}, Healthy skin biopsies, ex vivo infested with \textit{F. magna} (left panels) and biopsies from \textit{S. pyogenes}-infected skin (erysipelas; right panels), were investigated using electron microscopy. Bacteria are seen in the vicinity of keratinocytes (top left panel, arrows). Scale bars, 2.5 \(\mu\)m. At higher magnification, individual bacteria are visualized. In the case of \textit{S. pyogenes}, a typical rim of M protein is seen (upper right panel). Scale bars, 0.5 \(\mu\)m. Immunelectron microscopy shows colocalization of MK (as detected by 10-nm colloidal gold particles conjugated with secondary Abs) and BRAK/CXCL14 (10-nm colloidal gold particles) with SufA (5-nm colloidal gold particles) and FAF (5-nm colloidal gold particles), respectively, in the close proximity of \textit{F. magna} bacteria (bottom left panels). Scale bar, 0.1 \(\mu\)m. In a skin biopsy from erysipelas (caused by \textit{S. pyogenes}), MK (as visualized by Abs labeled with 10-nm colloidal gold particles) and BRAK/CXCL14 (10-nm colloidal gold particles) colocalize with SpeB (5-nm colloidal gold particles) and SIC (5-nm colloidal gold particles), respectively, close to the bacterial surface (bottom right panels). Scale bar, 0.1 \(\mu\)m. \textbf{B}, Morphometric evaluation of AMPs and bacterial protein concentrations in skin. The probe density of gold-labeled Abs was quantified on electron micrographs of individual skin samples. Molar Ag concentrations were calculated based on the number of gold particles per \(\mu\)m\(^2\).}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure7}
\caption{Schematic illustration of bacterial survival strategies during noninflamed and inflamed conditions, respectively. \textit{F. magna} resides in the basal parts of epidermis, where it adheres to BM-40 of the BM. Keratinocytes constitutively produce MK and BRAK/CXCL14, for which bactericidal activity \textit{F. magna} can attain by the release of SufA and FAF. Upon invasion of the virulent pathogen \textit{S. pyogenes}, the host responds with inflammation, resulting in downregulation of BRAK/CXCL14 expression and a concomitant upregulation of MK, hBD-2, and hBD-3. \textit{S. pyogenes} can neutralize these AMPs by release of the cysteine protease SpeB and the AMP-binding protein SIC, whereas the countermeasures executed by \textit{F. magna} are less efficient in the inflamed context, because SufA has no effect on hBD-2 and hBD-3.}
\end{figure}
efficiently invade host tissues. In this study, we find that SpeB rapidly degrades all investigated AMPs (MK, BRAK/CXCL14, hBD-2, and hBD-3). This is in line with previous studies showing a broad capacity for SpeB to cleave AMPs (38).

In the case of *F. magna*, we are not aware of other enzymes or proteins interacting with AMPs, whereas *S. pyogenes* produces several additional enzymes counteracting innate and adaptive immune mechanisms (e.g., SpyCep [degrading IL-8/CXCL8], C5a-peptidase [degrading C5a], IdeS [degrading iG], and EndoS [glycanase activity against IgG]). However, the latter enzymes have a very selective mode of action (49).

The release of protein FAF and protein SIC by *F. magna* and *S. pyogenes*, respectively, equips the bacteria with means to inactivate AMPs at a distance. Interestingly, FAF was proven to bind and neutralize the antibacterial activity of both MK and BRAK/CXCL14 more efficiently than SIC. The survival of *F. magna* bacteria is critically dependent on balancing the pressure from constitutive host-defense activities, limiting its proliferative activity and virulence, whereas virulent pathogens such as *S. pyogenes* bacteria do not have a biology that needs such considerations. Together, the current study demonstrates how *F. magna* can counteract the activities of constitutively produced AMPs, thus creating a protected habitat within the epidermis.

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