Domain 5 of High Molecular Weight Kininogen Is Antibacterial*

Emma Andersson Nordahl‡, Victoria Rydengård‡, Matthias Mörgelin¶, and Artur Schmidtchen‡

From the Department of Clinical Sciences, Lund, ‡Section of Dermatology and Venereology and ¶Section of Clinical and Experimental Infectious Medicine, Lund University, Biomedical Center, Tornavägen 10, SE-221 84 Lund, Sweden.

Running Title: Antimicrobial activity of domain 5 of HMWK

To whom correspondence should be addressed: Emma Andersson Nordahl, Department of Clinical Sciences, Lund, Biomedical Center B14, Tornavägen 10, SE-221 84 Lund, Sweden, Tel: +46 46 120921, Fax: +46 46 157756, E-Mail: emma.nordahl@med.lu.se

Antimicrobial peptides are important effectors of the innate immune system. These peptides belong to a multifunctional group of molecules that apart from their antibacterial activities also interact with mammalian cells, glycosaminoglycans, and control chemotaxis, apoptosis, and angiogenesis. Here we demonstrate a novel antimicrobial activity of the heparin-binding and cell-binding domain 5 of high molecular weight kininogen. Antimicrobial epitopes of domain 5 were characterized by analysis of overlapping peptides. A peptide, HKH20 (H479-H498), efficiently killed the Gram-negative bacteria Escherichia coli, Pseudomonas aeruginosa, and the Gram-positive Enterococcus faecalis. Fluorescence microscopy and electron microscopy demonstrated that HKH20 binds to and induces breaks in bacterial membranes. Furthermore, no discernible hemolysis, or membrane permeabilizing effects on eukaryotic cells, were noted. Proteolytic degradation of high molecular weight kininogen by neutrophil-derived proteases as well as the metalloproteinase elastase from Pseudomonas aeruginosa yielded fragments comprising HKH20 epitopes, indicating that kininogen-derived antibacterial peptides are released during proteolysis.

The innate immune system, based on antimicrobial peptides (AMPs), provides a rapid and non-specific response against potentially invasive pathogenic microorganisms. AMPs, first isolated from human leukocyte extracts by Zeya and Spitznagel in 1963 (1), were subsequently discovered in invertebrates (2) and cold-blooded vertebrates (3). At present, over 880 different AMPs have been identified in eukaryotes (www.bbcm.univ.trieste.it/~tossi/pag5.htm). During recent years it has become increasingly evident that many antimicrobial peptides are multifunctional (4,5). They are found to mediate chemotaxis (LL-37, defensins)(6-8), apoptosis (lactoferricin, LL-37) (9-11), and angiogenesis (PR-39, LL-37) (12,13). Conversely, molecules previously not considered as AMPs, including proinflammatory and chemotactic chemokines (14), neuropeptides (15-19) and peptide hormones (16,20), have recently been found to exert antibacterial activities. The proinflammatory, chemotactic and anaphylatoxic peptide C3a, generated during activation of the complement system displays potent antibacterial effects (21). Many AMPs, by virtue of their cationicity and amphipathicity, also interact with heparin (22,23).

High molecular weight kininogen (HMWK) is a multifunctional 120 kDa glycoprotein found in plasma (~80 μg/ml) (24) and in α–granules of platelets (25). The protein is composed of six domains, each having different properties and specific ligands (24). Domains D1-D3 have a cystatin-like structure, and the two latter domains serve as specific inhibitors of cysteine proteinases such as cathepsins and calpains. The D4 domain contains the bradykinin sequence, which is released by plasma kallikreins during contact activation (24,26). Biologically active kinins can also be generated by the cooperative action of mast cell tryptase and neutrophil elastase (27). Thus, similar to complement degradation, limited proteolysis of HMWK generates highly vasocative and proinflammatory peptides, which are formed at sites of tissue...
injury and inflammation. The heparin-binding (28), cell-binding (29) and antiangiogenic (30) D5 from HMWK, contains regions dominated by histidine, glycine, and in certain parts, interspersed lysine residues (31). The starting point for this study was the observation that this domain of HMWK shares many structural (cationicity, spacing of basic residues) and functional features with AMPs. Here we show that recombinant domain 5 (rD5), related peptide epitopes, as well as HMWK cleavage products function as "classical" AMPs, thus disclosing a previously unknown antibacterial activity of domain 5 of HMWK.

EXPERIMENTAL PROCEDURE

Biological materials – Blood drawn from healthy volunteers (Vacutainer tubes, Becton, Dickinson & Co, containing 2 mg/ml K3EDTA) was used for preparation of erythrocytes and EDTA plasma. For preparation of citrate plasma vacutainer tubes containing 1:9 (v/v), 129 mM sodium citrate, were used. The plasma was separated from the blood by centrifugation at 2500 g in 15 min. The pellet was removed and plasma samples were stored at –20°C. HaCaT keratinocytes were kindly provided by Dr. Robert Fusenig (Heidelberg University, Germany) and were cultured in DMEM containing 5.5 mM glucose and 10% fetal calf serum (FCS). Polyclonal rabbit antibodies raised against HKH20 were purchased from Innovagen AB, Lund, Sweden. Neutrophils were prepared by routine procedures (polymorphprep™, AXIS-SHIELD PoC AS, Oslo, Norway) from blood of healthy human donors. The cells were disrupted by freeze thawing and addition of 0.3% Tween 20.

Peptides/Proteins and enzymes – HMWK was obtained from the Binding site inc. (San Diego, CA, USA). The synthetic peptides: KHN20, GHG20, GHG21, GGH20, HKH20, LDD40, Texas Red conjugated HKH20, GKH17 (Fig. 1B) and LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) were synthesized by Innovagen AB. The purity (>95%) and molecular weight of these peptides was confirmed by mass spectral analysis (MALDI-TOF Voyager, Applied Biosystems, Foster City, CA, USA). Neutrophil serine protease, human leukocyte elastase (HLE) (0.2 μg/μl, 5.8 μM/μl) and *Pseudomonas aeruginosa* elastase (62 μM/μl) was kindly provided by Dr. H. Maeda, Kumamoto University, Japan.

Purification of the recombinant domain 5 – The expression vector (pET25b) (Novagen, Inc., Madison, WI, USA) containing rD5 was expressed in *Escherichia coli* strain BL21(DE3) generously provided by Dr. U. Sjöbring (32). One millimolar isopropyl thio-β-D-galactoside was added to exponentially growing bacteria to induce protein production. After 3 h incubation at 30°C, bacteria were harvested by centrifugation (2800 g for 10 min) and the pellet was resuspended in 3 ml sonication buffer (s-buffer: 50 mM phosphate, 300 mM NaCl, pH 8.0). The bacteria were lysed by repeated cycles of freeze thawing. The lysate was centrifuged at 30000 g for 30 min and the supernatant was mixed with 2 ml Ni/nitrilotriacetic acid Sepharose (QIAGEN GmbH, Hilden, Germany) equilibrated with s-buffer and incubated on rotation for 1 h at room temperature. The Sepharose gel was loaded into a column and washed with 10 ml s-buffer containing 0.1% (v/v) Triton X-100, 10 ml s-buffer, 5 ml s-buffer with 1 M NaCl, 5 ml s-buffer, 10 ml 20% ethanol, 10 ml s-buffer containing 5 mM imidazole and finally 10 ml s-buffer with 30 mM imidazole. The rD5 protein was eluted with s-buffer containing 500 mM imidazole.

Molecular modeling – A model structure of rD5 (Fig. 1A) was created based on the homologous protein hisactophilin from *Dictyostelium discoideum* (Protein Data Bank code 1HCE (33)). The sequence of human rD5 (residues 414-525) was aligned against the sequence of hisactophilin using the alignment described in (34). The modeling was performed using the Prime module (35) from the Schrödinger computational chemistry suite of programs (Schrödinger, L.L.C., Portland, OR, USA). The sequence identity was 32% and rotamers from the conserved residues was retained. Terminal tails and residues not derived from the template were minimized during structure building. All loops were refined one at a time using default sampling in the loop refinement protocol built into Prime. Most of the backbone torsion angles for non-glycine residues lie within allowed regions of the Ramachandran plot. The few non-glycine residues outside these regions (Trp 18, Gln 38 and His 92) are located in loop regions.

Microorganisms – *Escherichia coli* strain BL21(DE3) was used for expression of the rD5
The zones of E. coli respectively, were compared (Fig. 6 and HKH20 were compared with the same different concentrations (10, 50, 100 μM) of rD5 punched and 6 solidification, 4 mm-diameter wells were poured into a Ø 85 mm Petri dish. After agarose Tween 20 (Sigma-Aldrich). The underlay was consisting of 0.03% (w/v) TSB, 1% (w/v) low electroendosmosis type (EEO) agarose (Sigma-Aldrich, St Louis MO, USA) and 0.02% (v/v) Tween 20 (Sigma-Aldrich). The underlay was poured into a Ø 85 mm Petri dish. After agarose solidification, 4 mm-diameter wells were punched and 6 μl of test sample was added to each well. Plates were incubated at 37°C for 3 h to allow diffusion of the peptides. The underlay gel was then covered with 5 ml of molten overlay (6% TSB and 1% Low-EEO agarose in dH2O). Antimicrobial activity of a peptide was visualized as a zone of clearance around each well after 18-24 h of incubation at 37°C. Screening for antimicrobial activity of peptides derived from domain 5 (KHN20, GHG20, GHG21, GGH20, HKH20, GKH17, all at 100 μM) was performed against E. coli. For comparison, we used LL-37 (100 μM) (Fig. 2B). The zones of E. coli clearance generated by different concentrations (10, 50, 100 μM) of rD5 and HKH20 were compared with the same concentrations of LL-37 (Fig. 2C). The dose-response characteristics of RDA were used to determine the minimal effective concentration (MEC) of HKH20 against P. aeruginosa. The log10 concentrations of HKH20 were plotted versus the respective diameter of the zone of clearance (not shown). Linear regression using least squares was used to estimate the MEC-value, which was determined by triplicate experiments using eight serial 2-fold dilutions (starting at 512 μM) of HKH20. HMWK degradation products were tested for inhibitory effects against E. coli (Fig. 6A). The proteases (incubated for 1 h, at 37°C), used at equivalent concentrations as in the HMWK-degradations, were added as controls. Using RDA (E. coli), zones of clearance generated by LDD40 and LL-37, respectively, were compared (Fig. 6F).

Radial diffusion assay – Radial diffusion assays (RDA) were performed essentially as described earlier (36). Briefly, bacteria were grown to mid-logarithmic phase in 10 ml of full-strength (3% w/v) trypticase soy broth (TSB) (Becton, Dickinson & Co). The microorganisms were then washed once with 10 mM Tris, pH 7.4. 4 x 10^6 bacterial colony forming units were added to 5 ml of the underlay agarose gel, consisting of 0.03% (w/v) TSB, 1% (w/v) low electroendosmosis type (EEO) agarose (Sigma-Aldrich, St Louis MO, USA) and 0.02% (v/v) Tween 20 (Sigma-Aldrich). The underlay was poured into a Ø 85 mm Petri dish. After agarose solidification, 4 mm-diameter wells were punched and 6 μl of test sample was added to each well. Plates were incubated at 37°C for 3 h to allow diffusion of the peptides. The underlay gel was then covered with 5 ml of molten overlay (6% TSB and 1% Low-EEO agarose in dH2O). Antimicrobial activity of a peptide was visualized as a zone of clearance around each well after 18-24 h of incubation at 37°C. Screening for antimicrobial activity of peptides derived from domain 5 (KHN20, GHG20, GHG21, GGH20, HKH20, GKH17, all at 100 μM) was performed against E. coli. For comparison, we used LL-37 (100 μM) (Fig. 2B). The zones of E. coli clearance generated by different concentrations (10, 50, 100 μM) of rD5 and HKH20 were compared with the same concentrations of LL-37 (Fig. 2C). The dose-response characteristics of RDA were used to determine the minimal effective concentration (MEC) of HKH20 against P. aeruginosa. The log10 concentrations of HKH20 were plotted versus the respective diameter of the zone of clearance (not shown). Linear regression using least squares was used to estimate the MEC-value, which was determined by triplicate experiments using eight serial 2-fold dilutions (starting at 512 μM) of HKH20. HMWK degradation products were tested for inhibitory effects against E. coli (Fig. 6A). The proteases (incubated for 1 h, at 37°C), used at equivalent concentrations as in the HMWK-degradations, were added as controls. Using RDA (E. coli), zones of clearance generated by LDD40 and LL-37, respectively, were compared (Fig. 6F).

Viable count analysis – E. coli, E. faecalis and P. aeruginosa bacteria were grown to mid-logarithmic phase in Todd-Hewitt (TH) medium. Bacteria were washed and diluted in 10 mM Tris, pH 7.4 containing 5 mM glucose. Bacteria (50 μl; 2 x 10^6 cfu/ml) were incubated, at 37°C for 2 hours, with rD5 protein (Fig. 2A), HKH20 (Fig. 2D) or LDD40 (Fig. 6E) peptide at concentrations indicated in the figures. To test the time-dependence of bacterial killing, 30 μM HKH20 was used against E. faecalis and 0.6 μM HKH20 against P. aeruginosa and incubations were performed for 5, 15, 30 and 120 min (Fig. 3C). Activity of 10 μM HKH20 was also tested against P. aeruginosa diluted in 10 mM Tris, 0.15 M NaCl +/- 20% human EDTA-plasma. Significance was determined by using the Holm-Sidak method; one way repeated measures analysis of variance (ANOVA) and the statistical software used was SigmaStat, (SPSS Inc., Chicago, IL, USA) (Fig. 4). To quantify the bactericidal activity, serial dilutions of the incubation mixtures were plated on TH agar, followed by incubation at 37°C overnight and the number of colony-forming units was determined. 100% survival was defined as total survival of bacteria in the same buffer and under the same conditions as in the absence of peptide.

Heparin-binding assay – The rD5 protein and the synthetic HKH20 peptide (1, 2, 5 μM) were applied onto nitrocellulose membranes (Hybond™-C, GE Healthcare BioSciences, Little Chalfont, United Kingdom). Membranes were blocked (phosphate buffered saline (PBS), pH 7.4, 3% bovine serum albumin) for one hour and incubated with radiolabeled heparin (125I) (~10 μg/ml)(22). Unlabeled heparin (6 mg/ml) was added for competition of binding. The membranes were washed (3 x 10 min in 10 mM Tris, pH 7.4). A Bas 2000 radioimaging system (Fuji) was used for visualization of radioactivity.

Electron microscopy – Suspensions of P. aeruginosa (16 x 10^6 per sample) were incubated for 2 h at 37°C with the HKH20 peptide at 0.03 μM and 60 μM. For control, we included untreated bacteria. Each sample was gently transferred onto poly-L-lysine-coated Nylaflo® (GelmanSciences, MI) nylon membranes. The membranes were fixed in 2.5% (v/v) gluteraldehyde in 0.1 M sodium cacodylate, pH 7.2, for 2 h at 4°C, and subsequently washed with 0.15 M cacodylate, pH 7.2. They were then postfixed with 1% osmium tetroxide (w/v) and
Hemolysis assay – EDTA-blood was numerical-aperture oil-condenser. Plan Apochromat (x100 objective), and a high-a Hamamatsu C4742-95 cooled CCD camera, an inverted fluorescence microscope equipped with visualized by using a Nikon Eclipse TE300 Dako (Carpinteria, CA, USA). The bacteria were mounted on a slide by Dako mounting media, was poured away and the cover glass was bacteria were let to attach for 30 min. The liquid onto Poly-L-lysine coated cover glass and paraformaldehyde. The suspension was applied room temperature for 45 min in 4% fixed by incubation on ice for 15 min and in the incubation with bacteria. The bacteria were incubated at 37°C. The MTT containing medium was then removed by aspiration. Each well was washed gently with 100 μl PBS and the blue formazan product generated was dissolved by the addition of 100 μl of 100% DMSO per well. The plates were gently swirled for 10 min at room temperature, to dissolve the precipitate. The absorbance was monitored at λ 550 nm.

Proteolytic generation of peptides from HMWK – HMWK (16 μg) was incubated at 37°C for 10 or 30 min with either P. aeruginosa elastase (0.1 μg, 261 u/mg) or neutrophil elastase (0.4 μg, 29 u/mg) or freeze/thaw disrupted polymorphonuclear neutrophils (PMN) (17.7 μl, 1x10^6 cells/ml) in a total volume of 30 μl. HMWK (16 μg) incubated at 37°C for 30 min was used for control. Fifteen microliters of the material were analyzed on 16.5% precast sodium dodecyl sulfate polyacrylamide (SDS-PAGE) Tris-Tricine gels (BioRad, Hercules, CA, USA) under reducing conditions (Fig. 6B). Proteins/peptides were also transferred to nitrocellulose membranes (Hybond™-C, GE Healthcare Biosciences). Membranes were blocked by 3% (w/v) skimmed milk, washed, and incubated for 1 h with rabbit polyclonal HKH20 antibodies (1:5000) (Innovagen AB), washed again, and subsequently incubated (1 h) with HRP-conjugated secondary swine anti rabbit antibodies, 1:1000 (Dako). HKH20 proteins/fragments containing whole or parts of the HKH20 sequence were visualized using the ECL developing system (GE Healthcare Biosciences) (Fig. 6C).

Definition of kininogen cleavage product – Peptides generated by degradation of HMWK by
P. aeruginosa elastase were transferred from a 16.5% Tris-Tricine gel onto PVDF membranes (Hybond™-P, GE Healthcare Bio-Sciences Biosciences). One major peptide (indicated by an arrow in Fig. 6B) was cut out and sent for N-terminal sequencing (Protein Analysis Center, Karolinska Institutet, Stockholm, Sweden). The sequence LDDDLLE was obtained. The major detected MALDI signal (Protein Analysis Center, Karolinska) corresponded to the mass 4428.19 Da, thus identifying the peptide as LDD40 (Fig. 6D).

RESULTS

Antimicrobial effects of recombinant domain 5 and D5 derived peptides from HMWK.

To elucidate whether domain 5 of HMWK possess antibacterial activity, we initially investigated the effects of purified recombinant domain 5 (rD5) on E. faecalis (Fig. 2A) and E. coli (Fig. 2C). The results showed that rD5 was found to be antibacterial against these bacteria. Notably, rD5 exerted similar inhibitory activity in RDA as the classical AMP LL-37 (Fig. 2C).

In order to characterize functional antimicrobial epitopes of this domain of HMWK, we synthesized overlapping peptides (Fig. 1B) and tested these for antibacterial activities. The results showed that peptides derived from the histidine and lysine-rich parts of D5 (H469-K502, represented by peptides GGH20, HKH20, and GKH17) exerted potent antibacterial activities in RDA against E. coli (Fig. 2B). The calculated pI-values for these active peptides ranged between 9.70 and 10.78 (www.expasy.org/tools/pi_tool.html), and the net positive charge was +3 to +7, values comparable to those reported for many cationic AMPs. RDA analyses using P. aeruginosa and E. faecalis, identified the HKH20 peptide as the most potent epitope of D5 (not shown) and furthermore, the HKH20 peptide exerted antimicrobial effects comparable to both intact rD5 as well as LL-37 (Fig. 2C).

Likewise, molecular modeling of the rD5 sequence using the homologous protein hisactophilin from Dictyostelium discoideum as a template, suggested that the sequence H479-H498 expose critical cationic lysine residues, which enable interaction of rD5 with negatively charged bacterial membranes. Thus, in the following experiments, we focused on this specific epitope of D5 (Fig. 1A, B). In viable count assays, HKH20 exerted antibacterial effects against E. coli, P. aeruginosa and E. faecalis (Fig. 2D). Using RDA, the MEC-value of HKH20 against P. aeruginosa was estimated to 0.4 μM, which was comparable to the previously reported activity of LL-37 (21). As recently shown by us, a cross-functionality exist between AMPs and heparin-binding peptides (22), and this applies to classical AMPs such as LL-37 and defensin, the anaphylatoxin C3a, as well as several other heparin-binding peptides. Analogous to the studies by Pixley et. al. on the heparin binding of domain 5 of HMWK (28), rD5 and the HKH20 peptide bound to radiolabeled heparin at physiological salt concentrations (Fig. 2E), thus providing and additional link between rD5, HKH20, and cationic AMPs.

HKH20 binds to bacterial surfaces. The interaction between the HKH20 peptide and bacterial plasma membranes was examined by fluorescence and electron microscopy. HKH20 was labeled with the fluorescence dye Texas Red and incubated with P. aeruginosa. As demonstrated by fluorescence microscopy, the peptide was bound to the bacterial surface, and the binding was completely blocked by heparin (Fig. 3A, panel 2). Next, P. aeruginosa was incubated with HKH20 at 0.03 μM and 60 μM and analyzed by electron microscopy. We noted clear differences in the morphology of HKH20-treated bacteria in comparison with the control (Fig. 3B). HKH20 caused local perturbations and breaks along P. aeruginosa bacterial plasma membranes, and intracellular material was found extracellularly. These findings were similar to those seen after treatment with the antimicrobial peptide LL-37 (22). Most AMPs act rapidly on bacterial membranes, and thus, we next investigated the time-dependence of bacterial killing. As demonstrated in Fig. 3C, more than 80% of the Gram-negative P. aeruginosa were killed by 0.6 μM HKH20 within 5 min. Longer incubation times were required for efficient killing of the Gram-positive E. faecalis (~50% of bacteria killed by 30 μM peptide after 15 min).

Antibacterial activities at physiological conditions. Presence of plasma proteins and the ionic environment govern the activity of AMPs. For example, the antimicrobial activities of defensins are inhibited at physiological salt conditions (37). Thus, we examined the influence of physiological salt (0.15 M NaCl) as well as plasma on the antimicrobial activity of the HKH20 peptide. At 10 μM HKH20 (a concentration yielding complete killing of P. aeruginosa in 10 mM Tris buffer), the peptide
Notably, immunoreactive fragments were generated. The HKH20 antibody demonstrated that several peptides after a 30 min incubation time. Western blot analysis using an antibody to HKH20 revealed the generation of a major peptide of an apparent molecular mass of approximately 10 kDa. Notably, the major HKH20-reactive D5-fragment was synthesized and found to be antibacterial against E. coli and P. aeruginosa (Fig. 6E) and E. coli (Fig. 6F).

**DISCUSSION**

The main findings in our study is the identification of a potent antibacterial activity of recombinant domain 5 and the corresponding characterization of antimicrobial D5-derived peptides from HMWK, in concert with a conceptual proof that similar antibacterial epitopes are generated during proteolysis of HMWK in vitro. The results have implications for our understanding of novel properties of HMWK, and will enable future development of D5-derived AMPs for therapeutic use.

The D5 domain is multifunctional, and binds cellular receptors (29,39-41) as well as anionic surfaces (42), and inhibits angiogenesis by inducing apoptosis of proliferating endothelial cells (30). From a structural perspective, several lines of evidence indicate that the epitope involving the sequence H479-H498 of D5 is responsible for its antimicrobial activity. Although tentative, molecular homology modeling studies suggest that HKH20 is found at the surface of the domain 5 (Fig. 1A, yellow in upper panel). Thus, positively charged lysine residues of HKH20 (Fig. 1A, red in upper panel), protruding outward from the molecule have the capacity to interact with negatively charged structures, such as bacterial plasma membranes as well as heparin (Fig. 3A, Fig. 2E).

Interestingly, HKH20 as well as GKH17 contains the consensus sequence for heparin binding, XBBXBX (in reverse; 490NKGKK495, where X represents hydrophobic or uncharged amino acids, and B represents basic amino acids) (43), peptide motifs which were recently found to exert potent antibacterial activities (22). It is also of note that domain 5 of HMWK contains two subdomains, one His-Gly-rich (K420-D474) and one His-Gly-Lys-rich (K420-G502), the latter including HKH20 (Fig. 1A, B). Whereas, the heparin-binding capacity of the His-Gly-rich domain is Zn$^{2+}$ dependent, the His-Gly-Lys-rich part is able to bind heparin regardless of Zn$^{2+}$. The main findings in our study is the identification of a potent antibacterial activity of recombinant domain 5 and the corresponding characterization of antimicrobial D5-derived peptides from HMWK, in concert with a conceptual proof that similar antibacterial epitopes are generated during proteolysis of HMWK in vitro. The results have implications for our understanding of novel properties of HMWK, and will enable future development of D5-derived AMPs for therapeutic use.

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(28). Results with rD5 as well as HKH20 yielded similar antimicrobial activities in absence or presence of 50 μM Zn^{2+} (not shown). Likewise, heparin completely abolished the antimicrobial activity of both molecules, suggesting that the lysine-rich, and Zn^{2+}-independent domain comprising HKH20 is sufficient for the antimicrobial activity of the D5 domain.

The observation that insects and mammals utilize AMPs to counter microbial infections has, in combination with the growing problem of resistance to conventional antibiotics, spawned considerable interest in the discovery and subsequent development of novel AMPs for therapeutic use. Various strategies, (for reviews see (44,45) such as use of combinational library approaches, stereoisomers composed of D-amino acids or cyclic D,L-α-peptides are employed in the development of therapeutically interesting AMPs. Due to potential lytic properties of AMPs against bacterial as well as mammalian membranes, one of the challenges in designing new peptides relies on developing AMPs with high specificity against bacterial or fungal cells, i.e., a high therapeutic index (minimal hemolytic concentration/minimal antimicrobial activity; MHC/MEC). The finding that D5, as well as HKH20 displayed no lytic activities against mammalian cells, suggest a high amount of dissociation between antimicrobial and antieukaryotic activities. Biomembranes are highly complex structures, and currently, efforts are undertaken to gain a deeper understanding of the complex structure-activity relationships governing AMP specificity and selectivity (44,45). However, factors such as high hydrophobicity, α-helicity, as well as tendency to self-associate are linked to high hemolytic activity (as exemplified by our results with LL-37). The structural prerequisites for the selectivity and exact antimicrobial action of HKH20 remain to be characterized, however, the peptide has some unique features, such as high net charge, absence of hydrophobic residues, and a high amount of lysine and histidine, separating it from the group of classical helical amphipathic AMPs. The peptide exerted similar membrane breaking effects as LL-37. Analogously, recent studies indicate that the peptide, displaying random conformation in aqueous solutions, has a high specificity against negatively charged artificial liposomes, and little effects on zwitterionic membranes, thus in agreement with the herein noted bacterial specificity. Interestingly, P. aeruginosa, E. faecalis, Proteus mirabilis, Streptococcus pyogenes (46) and Staphylococcus aureus (47) all secrete proteases that degrade the cathelicidin LL-37. Unlike LL-37, HKH20 is highly resistant to various bacterial proteases (not shown), likely due to absence of hydrophobic residues in HKH20. Notably, the P. aeruginosa elastase, E. faecalis gelatinase, or 50 kDa metalloproteinase of P. mirabilis belong to the M4 peptidase family (thermolysin family) and have similar specificities requiring hydrophobic amino acids (L, I, F) at the P1’ position (48,49). Taken together, from a therapeutical standpoint, our results suggest that strategies based on utilizing endogenous, and protease-resistant, AMPs with a high therapeutic index could be highly rewarding.

From a biological perspective, it is well established that HMWK is proteolytically processed by endogenous as well as bacterial proteases, leading to release of different immunomodulating and bioactive peptides (for reviews see (24,26,50). During contact activation plasma kallikrein cleaves out the proinflammatory nanopeptide bradykinin from HMWK. In addition, mast cell tryptase and neutrophil elastase are both able to release a vascular permeability enhancing peptide (E-kinin) containing the bradykinin peptide (51,52). Furthermore, kinins may be released by cysteine proteinases of Porphyromonas gingivalis and of Streptococcus pyogenes (50,53,54).

The data presented in this study provide a first proof of concept that D5-derived antibacterial fragments comprising the HKH20 epitope are generated after proteolysis of HMWK. This observation was further substantiated by the structural characterization of a proteolytically generated and antimicrobial peptide fragment, LDD40, generated by elastase from P. aeruginosa (Fig. 6B, D, E, F). The observation that an bacterial elastase as well as endogenous neutrophil-derived proteases yielded similar HKH-containing fragments of domain 5 not only reflect the protease-resistance of this domain (as discussed above), but also suggests that this generation may reflect a common mechanism for generation of antimicrobial peptides from this domain in vivo. Highly relevant to our results, findings by other investigators indicate that AMPs derived from other parts of HMWK indeed may be generated. For example, apart from D5, bradykinin of domain 4 was found to possess antimicrobial activities (15). Furthermore, results at our
laboratory indicate that vascular permeability enhancing peptide (E-kinin), SLMKRPPGFS-PFRSSRI, containing the bradykinin peptide (51,52) generated by the concerted actions of mast cell tryptase and neutrophil elastase is also antimicrobial against both \textit{P. aeruginosa} and \textit{S. aureus} (not shown). Taken together, these findings indicate that multiple AMPs may be proteolytically released from HMWK. As illustrated in Fig. 6C, a mixture of neutrophil proteases generated low-molecular weight HKH20-like peptides, whereas neutrophil elastase yielded larger D5-derived fragments. This illustrates that multiple processing steps induced by the concerted action of various proteases on HMWK are likely to occur \textit{in vivo}. Interestingly, an analogous processing has indeed been described for the cathelicidin LL-37 (55), yielding enhanced antibacterial activities of the resulting peptide fragments. Thus, current investigations aim at characterizing D5-derived AMPs that are generated during various inflammatory and infective processes \textit{in vivo}, their resulting antimicrobial activities, and roles in innate immunity.

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FOOTNOTES

* This work was supported by grants from the Swedish Research Council (projects 13471), the Royal Physiographic Society in Lund, the Welander-Finsen, Söderberg, Groschinsky, Crafoord, Alfred Österlund, Lundgrens, Lions and Kock Foundations, DermaGen AB, and The Swedish Government Funds for Clinical Research (ALF).

1The abbreviations used are: AMP, antimicrobial peptide; HMWK, high molecular weight kininogen; rD5, recombinant domain 5; TR, PAELA, Pseudomonas aeruginosa elastase; HLE, human leukocyte elastase; RDA, radial diffusion assays; TSB, trypticase soy broth; EEO, electroendosmosis type; TH, Todd-Hewitt; PBS, phosphate buffered saline; LDH, lactate dehydrogenase; PMN, polymorphonuclear neutrophils.

2Ringstad, L., Schmidtchen, A., and Malmsten, M. Effects of peptide length on the activity of a kininogen-derived antimicrobial peptide on lipid bilayers, bacteria and cells, manuscript in preparation

Acknowledgement:
We wish to thank Ms Maria Baumgarten and Ms Mina Davoudi for their expert technical assistance and Dr. Björn Walse for the molecular model of domain 5.

FIGURE LEGENDS

Fig. 1. Molecular model and sequence of recombinant domain 5 and corresponding overlapping peptides. A, upper part, Homology model of rD5 (residues 414-525) showing the HKH20 peptide in yellow, with some of its positively charged lysine residues in red protruding outward from the molecule. The model was based on hisactophilin from Dictyostelium discoideum (33). The modeling was performed using the alignment described by Colman (34) and the Prime module (35) from the Schrödinger computational chemistry suite of programs (Schrödinger, L.L.C., Portland, OR, USA). A, lower part, The amino acid sequence of rD5. B, The sequences of the overlapping peptides from D5 used in this study.

Fig. 2. Activities of rD5 and D5-derived peptides. A, In viable count assays antibacterial activities for rD5 were detected against E. faecalis (— ■ —). 2 x 10⁶ cfu/ml of bacteria were incubated in 50 μl with peptides at concentrations ranging 0.3 to 100 μM. B, C, Peptides and rD5 protein were tested in RDA in low-salt conditions. E. coli (4 x 10⁶ cfu) was inoculated in 0.1% TSB agarose gel. Each 4 mm-diameter well was loaded with 6 μl of peptide at 100 μM in B and at the indicated concentrations in C. The zones of clearance correspond to the inhibitory effect of each peptide after incubation at 37 °C for 18-24 h. Negative controls (labeled Ctr in B, and at the top left of the plate in C) containing buffer (10 mM Tris, pH 7.4) were included. These clear zones correspond to the 4 mm well. D, In viable count assays antibacterial activities for HKH20 were seen against E. faecalis (— ▼ —), P. aeruginosa (— ● —) and E. coli (— ○ —). 2 x 10⁶ cfu/ml of bacteria were incubated in 50 μl with peptides at concentrations ranging from 0.03 to 60 μM. E, rD5 and HKH20 were both able to bind heparin. 1, 2 and 5 μg HKH20 were applied to nitrocellulose membranes. These membranes were then incubated in PBS (containing 3% bovine serum albumin) with iodinated (¹²⁵I) heparin. Unlabeled heparin (6 mg/ml) (+) was added for competition of binding. The membranes were washed (3 x 10 min in 10 mM Tris, pH 7.4). A Bas 2000 radioimaging system (Fuji) was used for visualization of radioactivity.

Fig. 3. Interactions of HKH20 with bacterial plasma membranes and kinetics for bacterial killing. A, Binding of Texas Red-labeled HKH20 to P. aeruginosa and inhibition of binding by excess of heparin. Image 2 shows red fluorescence of TR-HKH20 peptide (10 μg/ml) bound to bacteria (1 x 10⁷/ml) and image 4 shows bacteria incubated with heparin and TR-HKH20. Images in 2 and 4
were recorded using identical instrument settings. The corresponding Nomarski images are shown in image 1 and 3. Scale bar represents 10 μm. B, *P. aeruginosa* (16 x 10⁶ per sample), (left panel) was incubated for 2 h at 37°C with 0.03 μM (middle panel), and 60 μM (right panel) HKH20 and analyzed with electron microscopy. Scale bar represents 0.5 μm. C, The time-dependence of bacterial killing by HKH20 was analyzed by viable count assays. Concentrations of HKH20 used were 0.6 μM and 30 μM against *P. aeruginosa* (—○—) and *E. faecalis* (—▼—), respectively.

**Fig. 4.** Antibacterial activities of HKH20 under physiological conditions. *P. aeruginosa* bacteria were subjected to 10 μM HKH20 in 10 mM Tris pH 7.4 containing 0.15 M NaCl in presence or absence of 20% human EDTA-plasma. Identical buffers without peptide were used as controls. Significance was determined by using the Holm-Sidak method; one way repeated measures analysis of variance (ANOVA) and the statistical software used was SigmaStat, (SPSS Inc., Chicago, IL) P<0.001 (**).  

**Fig. 5.** Effects of HKH20 against eukaryotic cells. The activities of HKH20 (—●—) in the three different assays were compared with the effects of LL-37 (—○—) used at the same concentrations. The plotted values are mean out of three measurements and the error bars represent the standard deviation. A, Hemolytic effects of HKH20 and LL-37 were investigated. The cells were incubated with different concentrations of HKH20 or LL-37. 2% Triton X-100 (Sigma-Aldrich) served as positive control. The absorbance of hemoglobin release was measured at λ 540 nm and is expressed as % of TritonX-100 induced hemolysis (note the scale of the y-axis). B, HaCaT keratinocytes were subjected to HKH20 and LL-37. Cell permeabilizing effects were measured by the LDH based TOX-7 kit (Sigma-Aldrich). LDH release from the cells was monitored at λ 490 nm and was plotted as % of total LDH release. C, The MTT-assay was used to measure proliferation of HaCaT keratinocytes in the presence of different concentrations of HKH20 and LL-37. In the assay, MTT is modified into a dye, blue formazan, by enzymes associated with metabolic activity. The absorbance of the dye was measured at λ 550 nm.

**Fig. 6.** Generation of antimicrobial peptides by degradation of HMWK. A, Inhibitory effects of HMWK cleavage products were visualized as zones of bacterial clearance in RDA. Cleavages of HMWK were performed for 10 and 30 min at 37°C (see experimental procedures). 1: Control, *P. aeruginosa* elastase (PAELA); 2: Control, Human leukocyte (neutrophil) elastase (HLE); 3: Control, polymorphonuclear neutrophils (PMN); 4: Control, HMWK; 5 and 6: HMWK incubated with PAELA for 10 and 30 min, respectively, 7 and 8: HMWK incubated with HLE for 10 and 30 min, respectively, 9 and 10, HMWK incubated with PMN for 10 and 30 min, respectively. RDA was performed in low-salt conditions. *E. coli* (4 x 10⁶ cfu) was used as test organism. Each 4 mm-diameter well was loaded with 6 μl. A negative control, containing buffer (10 mM Tris, pH 7.4) was included in the well at the top left of the plate. B, Intact HMWK (HMWK) and cleavage products from the different incubations (indicated above) were analyzed by SDS-PAGE (16.5% Tris-Tricine gel). The black arrow indicates the fragment analyzed by N-terminal sequencing and MALDI-TOF analysis. Molecular mass markers are indicated to the left. C, Western blot analysis identified cleavage products recognized by polyclonal antibodies against HKH20. The white arrow indicates the size of the HKH20 peptide. D, The indicated fragment (black arrow) in B was analysed by N-terminal sequencing and MALDI-TOF. E, Antibacterial activity of LDD40 in viable count assay. 2 x 10⁶ cfu/ml of *E. faecalis* bacteria were incubated in 50 μl with the peptide at concentrations ranging 0.03 to 60 μM. F, Inhibitory effects of LDD40, compared with effects of LL-37, used at indicated concentrations against *E. coli* (4 x 10⁶ cfu). RDA was performed in low-salt conditions. Each 4 mm-diameter well was loaded with 6 μl. A negative control, containing buffer (10 mM Tris, pH 7.4) was included in the well at the top left of the plate. A representative experiment out of six was shown.
A

\[ \text{rD9:} \ 3^83^T\text{V}S\text{P}PHTS\text{MA} \ PAQDEER\text{DS}G \ KEQG\text{H}T\text{R}R\text{H}D \]
\[ \text{W}G\text{HEKQ}R\text{K}H\text{N} \ L\text{G}G\text{H}G\text{K}H\text{E}R\text{D} \ Q\text{G}G\text{H}QRR\text{G}H\text{G} \]
\[ \text{L}\text{G}\text{H}\text{G}EQ\text{Q}H\text{G} \ L\text{G}G\text{H}K\text{F}\text{K}L\text{D} \ DDLEHQGGHV \]
\[ \text{L}D\text{G}G\text{H}K\text{H}K\text{H}G \ H\text{G}\text{G}K\text{H}K\text{N}KG \ K\text{K}G\text{K}K\text{HNGWK} \]
\[ \text{TEHLASSSED} \ S^{513} \]

B

\[ \text{KHN20:} \ 4^20\text{K}\text{H}N\text{L}G\text{H}G\text{H}K\text{H} \ E\text{R}DQG\text{G}H\text{Q}R^{439} \]
\[ \text{GHG20:} \ 4^40\text{G}G\text{H}L\text{G}G\text{H}G\text{E}Q \ QHGLG\text{H}G\text{H}K^{459} \]
\[ \text{GHG21:} \ 4^54\text{G}G\text{H}GH\text{K}F\text{K}L\text{D} \ D\text{L}E\text{H}Q\text{G}G\text{H}V\text{L}D^{474} \]
\[ \text{GGH20:} \ 4^69\text{G}G\text{H}V\text{L}D\text{H}\text{G}H\text{K} \ HK\text{G}G\text{H}G\text{H}K^{488} \]
\[ \text{HKH20:} \ 4^79\text{H}K\text{K}G\text{H}G\text{H}G\text{K} \ K\text{N}G\text{K}K\text{N}G\text{K}^{498} \]
\[ \text{GKH17:} \ 4^86\text{G}K\text{H}K\text{N}K\text{G}K\text{K} \ G\text{K}\text{H}G\text{W}K^{502} \]
Figure 2

A) % Survival vs. μM peptide

B) Petri dishes with various peptides and bacterial species

C) Petri dish with bacterial growth at different concentrations of peptides

D) % Survival vs. μM peptide

E) Western blot analysis of peptides with and without heparin inhibitor

rD5, E. faecalis, HKH20, LL-37, KHN20, GHG20, GHG21, GGH20, HKH20, GKH17, LL-37, Ctr
Figure 3

A

B

C

% Survival

P. aeruginosa

E. faecalis

0 5 10 15 30 60 120 min

0 20 40 60 80 100

0.03 μM

60 μM

HKH20

HKH20 + Heparin

Ctr

HKH20: 0.03 μM

HKH20: 60 μM

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Figure 5

A  % Hemolysis

B  % LDH release

C  Abs. λ = 550 nm

µM peptide
Figure 6

A. Image of a bacterial plate with labeled wells.

B. Western blot analysis showing bands at different kDa markers.

C. Western blot analysis showing bands at different kDa markers.

D. Peptide sequence of LDD40: LDDLEHOGG HVLDHGKHK

E. Graph showing survival rate of E. faecalis with different peptide concentrations.

F. Western blot analysis showing bands at different peptide concentrations.
Domain 5 of high molecular weight kininogen is antibacterial
Emma Andersson Nordahl, Victoria Rydengård, Matthias Mörgelin and Artur Schmidtchen

*J. Biol. Chem. published online August 9, 2005*

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