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Interleukin-8-Derived Peptide Has Antibacterial Activity

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Chemokines are inflammatory mediators with effects on diverse processes associated with the immune response. Some of the proteins belonging to the CXC chemokine subfamily, one of four groups in the family, possess inherent antibacterial activity against a wide range of bacteria. The CXC chemokine interleukin-8 (IL-8) has not been ascribed any direct antibacterial activity, but the fact that several of the amino acids in the carboxy-terminal part of the protein are identical or similar to those in a bactericidal cecropin-like peptide [Hp(2–20)] from Helicobacter pylori suggests that processing of the cytokine might generate peptide fragments with antibacterial properties. Synthetic peptides representing the carboxy-terminal part of IL-8 were investigated for antibacterial activities. These fragments possessed an antibacterial activity absent in the full-length IL-8. The antibacterial effects were reduced at increasing salt concentrations whereas the activity was increased when the pH was lowered. The IL-8-derived peptide shared structural similarity with and was also functionally additive to the Hp(2–20) peptide. The IL-8-derived peptide lacked the proinflammatory effects of the full-length protein. We also showed that acid hydrolysis of IL-8 generated a major peptide fragment corresponding to the antibacterial carboxyl terminus of the protein. The results presented are of special interest when put in the context of the suggested importance of antimicrobial peptides for microbial colonization of the gastric mucosa.

Chemokines are inflammatory regulators with effects on a number of different processes of prime importance for our innate as well as acquired immune responses, affecting stem-cell survival, development, and homeostasis, as well as chemotaxis and angiogenesis (30). The cytokines are divided into four subfamilies, and based on their structure and primary amino acid sequence, they belong to the CXC, CC, C, or CX3C group. The CXC subfamily of chemokines consists of a number of different monocyte and granulocyte-activating proteins, including CXCL8, also termed interleukin-8 (IL-8) (30). This interleukin is generated and secreted by several different types of inflammatory cells as well as by epithelial cells. IL-8 is not only a very potent endogenous monocyte/neutrophil chemoattractant but also a potent secretagogue and activator of the immune system against microorganisms from evolutionarily old and primitive organisms to highly evolved organisms such as mammals. Accordingly, many chemokines possess, in addition to their regulatory activities, direct antibacterial properties (11, 35). The formation of a large positively charged area on the surface of the molecule is a common feature of the antimicrobial chemokines and a characteristic possibly explaining the bacterial inhibitory activities (35).

The CXC chemokine IL-8 is a homologous dimeric molecule, each monomer consisting of three antiparallel β-strands connected with loops and one α-helix made up of the 19 carboxy-terminal residues (3). The protein has not been linked to any antibacterial activities, but the fact that the carboxy terminus of IL-8 contains an α-helical structure suggests that this part of the molecule might have antibacterial properties even though the full-length protein is devoid of such an activity. It should be noted that it is a common feature for antibacterial peptides to be activated upon proteolytic cleavage from larger precursor proteins.

The cecropin-like peptide Hp(2–20), as well as LL-37, which belongs to the cathelicidin family of antibacterial peptides, are prominent examples of bactericidal peptides derived through such a cleavage mechanism (15, 22, 34). In addition, analysis of the amino acid sequence of IL-8 revealed similarities with Hp(2–20) derived from the gastric pathogen Helicobacter pylori, the causative agent of gastritis and peptic ulcers. This group of cecropin-like antibacterial peptides, first identified as the principal component of the humoral immune system of insects and later found in a variety of organisms (5), have in common an amphipathic α-helix structure which is an important structural feature. This is illustrated by the fact that amino acid replacements that break or stabilize the helical structure will reduce or abolish or increase the antibacterial activity, respectively (5, 14, 29). The cecropins as well as cecropin-like peptides exercise their antibacterial effect through the formation of pore structures in phospholipid bilayers, leading to depolarization of the membrane of the target microbe (13), an activity for which the α-helical structure is decisive (8).

In order to determine the antibacterial potential of IL-8, peptides corresponding to the carboxy-terminal part of the protein were synthesized and tested for antibacterial activity. These α-helical peptides possessed antibacterial properties, and acid hydrolysis of the intact IL-8 protein generated a major peptide identical to the antibacterial fragment.

Materials and Methods

Reagents. Recombinant human IL-8 was purchased from R&D Systems Europe Ltd. (United Kingdom) in a solution stabilized with bovine serum albumin (0.5%). The peptide KENWVRVEKFLKRAENS (IL-829) and the peptide corresponding to the acid hydrolysis-generated fragment, PKENWVRV...
VEKFLKRAENS (IL-8-g(1–73)), were synthesized and high-pressure liquid chromatography purified by KJ ROSS-PETERSEN AS (Horsholm, Denmark). No functional difference between the two peptides was found, and both are referred to as the IL-8 peptide. The cecropin-like peptide Hpl(2–20), with a sequence corresponding to the amino-terminal part of ribosomal protein L1 in H. pylori (AKKYFKRLKELFKSKGD/), was synthesized and high-pressure liquid chromatography purified by Innogen (Lund, Sweden). The peptides were dissolved in water and stored at −70°C until used. Horseradish peroxidase was from Roche Applied Science (Bromma, Sweden). A-6013 agarose used for casting all inhibition zone plates was from Sigma Chemical Co. (St. Louis, Mo.) as were isoluminol, luminol, superoxide dismutase, and the cometachic peptide formyl-Met-Leu-Phe (fMLF). Dextran and Ficoll-paque were purchased from Amaresham Biosciences (Uppsala, Sweden).

**Inhibition zone assay for determination of antimicrobial activity.** *Escherichia coli* strain MG1655 was grown overnight in Luria Bertani (LB) broth (1) at 37°C on a rotary shaker. A modified inhibition zone assay was used for detection of antibacterial activity (17, 19, 20). In short, standard LB agar (broth supplemented with 1% [wt/vol] agarose), except when noted, containing bacteria (approximately 5 × 10⁵ CFU in logarithmic growth phase per milliliter agar) was poured into petri dishes, diameter 92 mm. Wells, diameter 3 mm and depth 1 mm, were punched in the agar and peptide preparations (3 μl) diluted in distilled water unless otherwise stated, were added to the wells, whereupon the plates were incubated at ambient temperature for 45 min and then at 37°C overnight.

Peptide concentrations ranging from 50 μM to 2.5 mM were used in the assays.

The inhibition zone was detected as the diameter of the clear zone surrounding the well free of visible bacteria. The antibacterial effect on fungus and other bacteria was determined by lack of growth of strains used (as shown at the www.sigmaaldrich.com site), *Staphylococcus aureus* (CCUG 49245), *Candida albicans* (CCUG 49242), *Proteus mirabilis* (CCUG 49244), *Salmonella enterica* serovar Typhimurium MS 395 and MR10 (a kind gift from Olle Stendahl, University of Linköping, Linköping, Sweden), *Klebsiella pneumoniae* (CCUG 49243), *Bacillus subtilis* (strain ATCC 6051, from the Culture Collection, University of Göteborg, Göteborg, Sweden) and *Staphylococcus epidermidis* (strain ATCC 14990, from the Culture Collection, University of Göteborg, Göteborg, Sweden) and *Streptococcus pyogenes* (strain ATCC 14990, from the Culture Collection, University of Göteborg, Göteborg, Sweden), were grown microaerophilically (in a jar filled with 25 ml Anaerocult C from Merck [Darmstadt, Germany]) in serum broth (3.7% brain heart infusion [Difco 0037] in distilled water), except when noted, containing bacteria (approximately 5 × 10⁵ CFU in logarithmic growth phase per milliliter agar) was grown microaerophilically (in a jar filled with Brucella broth in the microaerophilic gas) in Brucella broth (base purchased from Sigma Chemical Co. [St. Louis, Mo.]) and spread onto agar plates made from Sigma Chemical Co. (St. Louis, Mo.) as were isoluminol, luminol, superoxide dismutase, and the cometachic peptide formyl-Met-Leu-Phe (fMLF). Dextran and Ficoll-paque were purchased from Amaresham Biosciences (Uppsala, Sweden).

**Analysis of the acid hydrolysis products from IL-8.** Acid hydrolysis was performed as described by Lin et al. (25). In short, recombinant human IL-8 (12 μM) was diluted (1/20) in trifluoroacetic acid (0.6% [vol/vol], pH 1), and the mixture was incubated for 16 h at 65°C. The sample was purified on a ZipTip-C18 (Millipore) according to standard procedures available at www.millipore.com. The sample was analyzed using a MALDI-LR (Micromass, Manchester, United Kingdom) matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) in reflectron mode; 0.5 μl of the sample was mixed with 0.5 μl matrix solution (12 mg/ml α-cyano-4-H-O-cinnamic acid in acetonitrile/water 1:1, 0.1% trifluoroacetic acid) directly on the MALDI probe and allowed to dry at ambient conditions. ACTH (18–39) MH⁺ 2465.199 was used as the external and tryptic autodigest and MH⁺ 2211.05 as the internal lock mass. The sample was eluted in 3.5 μl of acetonitrile/water (1:1) containing 0.1% formic acid, and the peak of interest identified in MALDI-TOF was fully sequenced by electrospray ionization-dual mass spectrometry (ESI-MSMS), on a Q-TOF Ultima API (Micromass, Manchester, United Kingdom). Fragment ion data were acquired by nano-flow electrospray using argon collision gas. The analyses were performed by the SwGene Proteomics Centre at the University of Göteborg.

**RESULTS**

**Physicochemical properties of the IL-8 fragment and sequence alignment with the cecropin-like peptide Hpl(2–20).** The sequence of IL-8 was compared to that of the antibacterial cecropin-like peptide Hpl(2–20), derived from *Helicobacter pylori*. The carboxy terminus of IL-8 showed a high degree of similarity with Hpl(2–20), manifested both as identity at the amino acid level and similarity in physicochemical properties and exchangeability of the constituting amino acids (Fig. 1A). The sequence alignments were performed using the Clustal W (1.81) software provided by the open access Web-site http://bio.lundberg.gu.se. The computer-based estimated three-di-}

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**Antimicrobial activity of synthetic IL-8-derived peptides against E. coli.** An inhibition zone assay was employed to determine the antibacterial activity of the synthetic peptides. The synthetic IL-8 peptides had dose-dependent antibacterial properties similar to those of Hpl(2–20) (Fig. 2a), but no such activity was evident when the full-length IL-8 molecule was applied at similar concentrations (Fig. 2b). The medium control (water) did not give rise to any inhibition zone at all (data not shown).
The inhibitory activity of antibacterial peptides is often influenced by the basic experimental conditions. This was the case for the IL-8 peptide, as illustrated by the fact that the antibacterial activity increased when the sodium chloride concentration was reduced, while the activity decreased when the concentration was raised. At around 350 mM NaCl the antibacterial activity was lost. In contrast, the H. pylori peptide Hp(2–20) was equally potent when the growth medium was supplemented with sodium chloride up to concentrations of 100 mM and the activity was still pronounced at 350 mM (data not shown). The differences between the IL-8 peptide and Hp(2–20) were quantified and the data are shown in Table 1.

In addition, the pH was of importance for the antibacterial activity of the IL-8 peptide. When the pH was lowered, a significant enhancement of antibacterial activity was obtained, whereas no such effect was observed for Hp(2–20) (Table 1). We could not use pH levels below 5.0 owing to the fact that the ions Mg$^{2+}$ and Ca$^{2+}$. Concentrations of Mg$^{2+}$ or Ca$^{2+}$ exceeding 15 mM totally abolished the antibacterial activity of the peptides (data not shown).

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solidification of the agarose was lost at these levels. An increment of pH to 9.0 reduced the antibacterial activity of both Hp(2–20) and the IL-8 peptide (data not shown).

**Combined antibacterial effects of the IL-8 peptide and Hp(2–20).** Despite the fact that the inhibition zone assay does not allow for a direct addition of one zone diameter to another when the concentration of a drug is increased or when two antimicrobial agents are combined, the technique can be used to determine additivity/synergy. Combined action of the IL-8 peptide and Hp(2–20) increased the antibacterial activity, and we concluded that the effects of the two peptides were additive. This conclusion was based on the fact that the zone-diameter obtained by Hp(2–20) could be increased through an addition of the IL-8 peptide, even when the latter was present in concentrations that were subinhibitory when acting alone (Fig. 2C).

**Effect of the IL-8 peptide and Hp(2–20) on different bacteria.** The antibacterial potential and inhibition profile differed between the IL-8 peptide and Hp(2–20) (Table 2). The two peptides were equally potent in inhibiting the test strain of *E. coli*, but this was not the case when *E. coli* was replaced by other gram-negative bacteria (Table 2). There was also a difference between the peptides with respect to the capacity to inhibit *H. pylori*; while the bacteria were resistant to Hp(2–20), a low degree of antibacterial activity was obtained with the IL-8 peptide. The two peptides efficiently inhibited streptococci (*Streptococcus pyogenes*) whereas other gram-positive bacteria tested were largely unaffected by either peptide, as was the fungus tested (Table 2).

**Proinflammatory activities of the IL-8 peptide.** Binding of full-length IL-8 to its neutrophil cell surface receptor (CXCR1) gives rise to a proinflammatory signaling cascade that results in activation of the phagocyte NADPH-oxidase (21). This activation in turn results in production and release of reactive oxygen species (Fig. 3). When the IL-8 peptide replaced the full-length protein, no production of reactive oxygen species could be detected (Fig. 3). Higher concentrations of the IL-8 peptide did not result in any activity either (data not shown).

The full-length IL-8 molecule (1.2 × 10⁻⁹–1.2 × 10⁻¹⁰ M) was also a potent neutrophil chemoattractant, but no such activity was induced when the IL-8 peptide (1.2 × 10⁻⁸–1.2 × 10⁻¹⁰ M) was introduced in the chemotaxis assay system (Fig. 3, inset). Accordingly, the IL-8 peptide was (in contrast to full-length IL-8) unable to activate neutrophils in terms of oxidase activity and induction of chemotaxis and had no proinflammatory activities similar to those earlier described for Hp(2–20) (7).

**Identification of one major cleavage fragment generated after acid hydrolysis of IL-8.** A major cleavage peptide with a molecular weight of 2,459 Da was identified and this fragment was subsequently found to be identical to the carboxy-terminal part of the full-length IL-8 molecule, i.e., a 20-amino-acid-long peptide of amino acids 80 to 99 with the sequence PKENWVQRVKFLKRAENS (Fig. 4). In fact, the fragment generated through acid hydrolysis contained the complete α-helix (ranging from amino acid 81 through 97).

**DISCUSSION**

We show here that the α-helical carboxy-terminal end of the cytokine IL-8 has antibacterial capacity that is absent in the full-length IL-8 molecule. The antibacterial potency was affected by ion composition and pH, and the antibacterial effect against *E. coli* was additive to that of a cecropin-like peptide from *H. pylori*, Hp(2–20). The peptide derived from IL-8 was unable to induce the proinflammatory activities ascribed to Hp(2–20) (9) or full-length IL-8.

Some of the CXC cytokine family members, for example GRO-α/CXCL-1, are antibacterial, whereas others are devoid of such activities (11, 35). Cationicity seems to be an important feature shared by the antibacterial chemokines, as well as by many other antibacterial peptides/proteins. The cationic criterion is fulfilled by the full-length IL-8 molecule, and this feature is also conferred on the IL-8 peptide possessing antibacterial activities. These molecules have isoelectric points of 8.9 and 9.7, respectively, and a pI value above 8.0 is a prerequisite, but not sufficient, for antibacterial activity (35). The topological formation of a large positively charged area on the protein surface appears to be an important feature in antibacterial chemokines (35), and this property is also shared by the IL-8 peptide of amino acids 80 to 99 with the sequence PKENWVQRVKFLKRAENS (Fig. 4). In fact, the fragment generated through acid hydrolysis contained the complete α-helix (ranging from amino acid 81 through 97).
molecule. Many physicochemical characteristics thus suggest that the full-length IL-8 should also possess antibacterial activities. A possible explanation to why the full-length IL-8 cytokine lacks antibacterial effect might be that the amphipathic parts at the carboxy-terminal end are hidden or disturbed by other parts of the molecule.

As illustrated by the fact that all members of the cathelinidin family of antimicrobial peptides are derived from the common cathelin precursor, many antimicrobial peptides are cleaved off from larger precursor proteins (15). Matrix metalloproteinases such as gelatinase B and neutrophil collagenase have been shown to cleave members of the CXC chemokine family (33), suggesting that processing of the carboxy terminus constitutes an important regulatory mechanism. Proteinases with specificity for the amino-terminal side of aspartic/cysteic acid residues or the carboxy side of proline residues are abundant in human cells. During interaction with IL-8, these types of proteinases should, according to the amino acid sequence, generate fragments such as IL-879–99 and IL-881–99 that contain the α-helical part of the molecule, suggesting that antibacterial peptides can be generated from IL-8 by several different mechanisms. In light of the results that show acid hydrolysis of IL-8 generates an antibacterial peptide, studies of the peptide in relation to antibacterial/inflammatory activities in the acidic environment of the intestine would be of interest. This is further emphasized when considering the similarity and cooperative mode of action for the H. pylori derived peptide Hp(2–20) and the IL-8 peptide.

In addition to IL-8, there may be other CXC chemokines with antibacterial activity residing from the carboxy-terminal part of the protein. For example GRO-α, β, and γ all have α-helical parts similar to the one found in IL-8, suggesting that the antibacterial property of these three proteins might be derived from this part of the molecule. This remains to be shown, however.

It is well known that increased ionic strength results in reduction of the activity exerted by many antibacterial peptides. The antibacterial peptide LL-37, for example, loses activity as the sodium chloride concentration is raised (4). The IL-8 peptide was more sensitive to increased sodium chloride concentrations than Hp(2–20). A positive correlation between peptide folding and antibacterial activity has been observed, and important determinants for proper folding of certain antimicrobial peptides seem to be anions such as SO$_4^{2–}$, HCO$_3^–$, and CFCOO$^–$ (16). However, in this study the effect of anions did not seem to be crucial for the antibacterial properties of the IL-8 peptide. Very small effects on the antibacterial activity were obtained through varying the concentration of NaCl or Na$_2$SO$_4$ whereas increasing concentrations of MgCl$_2$, MgSO$_4$, CaCl$_2$ or CaSO$_4$ totally abolished the antibacterial effects of the IL-8 peptide, suggesting that divalent cations are of great importance. This phenomenon has been described earlier (24).

The negative effect on the IL-8 peptide activity upon addition of divalent cations might emanate from unfavorable conformation changes in the peptide or from the effect on cellular factors in the bacteria that makes it more resistant to the inhibitory action of the peptide. Yet another plausible explanation that previously has been proposed is the effect exerted by divalent cations on lipopolysaccharide molecules in gram-negative bacteria (24, 32). Cationic antibacterial peptides cross the outer membrane of gram-negative by the self-promoted pathway and the initial step in this process is assumed to be a high affinity binding of the peptide to lipopolysaccharides in the bacteria. This causes a displacement of divalent cations that stabilize adjacent lipopolysaccharide molecules. The displacement is hypothesized to destabilize the outer membrane, resulting in uptake of the peptide across the outer membrane followed by channel formation in the cytoplasmic membrane, the primary target in both gram-positive and gram-negative bacteria, and bacterial death. The actions of cationic antibacterial peptides are inhibited at this initial step by high concentration of divalent cations (24, 32).

The antibacterial effect of the IL-8 peptide was enhanced by lowering of the pH. This potentiating effect on antibacterial activity in mildly acidic environments was not applicable to Hp(2–20) but has earlier been shown also for the defensin group of antimicrobial peptides and platelet microbicidal proteins (31, 36). The induction of positive conformational changes and/or influence on net charge by protonation of specific amino acids in the peptide might be possible explanations.

The synergistic action of antibacterial peptides when combined is a well-known phenomenon in the protection of epithelial surfaces, and it has for example been described for neutrophil defensins and cathelicids (27), two representative families of neutrophil antibacterial peptides. The heterogenic group constituting the antibacterial peptides has diverse properties and the exact mechanisms of killing are unknown. One essential, contributing effect seems to be the membrane disintegration accomplished through peptide-peptide and peptide-membrane interaction, in which the amphipathicity is very important (37). One important step in this process is the threshold concentration that has to be reached before the membrane can be lysed. Two antibacterial peptides...
resembling each other should be able to interact cooperatively with the target membrane. Such an interaction would add antimicrobial effects of two peptides, as observed for the IL-8 peptide and Hp(2–20). The IL-8 peptide and Hp(2–20) seem to have slightly different mechanisms of action since they vary in their antibacterial properties against bacteria other than *E. coli*. One could hypothesize that they bind to/interact with different structures on the bacterial membrane, resulting in various antibacterial capacity depending on the particular bacterial strain investigated.

The IL-8-derived peptides were devoid of the proinflammatory activities ascribed to full-length IL-8. This is not so surprising since the proinflammatory effects exerted on neutrophils by IL-8 derives from the amino-terminal end of the protein. For example, the ELR motif, positions 31 to 33 in unprocessed IL-8 (1–99) and positions 4 to 6 in the most common, processed form of IL-8 (28–99), is essential for the chemotactic activity exerted on neutrophils by the protein (10).

It is also worth noticing that *H. pylori*, the bacterium associated with peptic ulcers, is unique in its ability to adapt to long-term survival in the acidic environment of the human stomach. Interaction between the bacteria and host mucosal cells results in an induction of transcription and local production of the precursor to the IL-8-derived antimicrobial peptides (18, 26, 28) and the persistence of the bacteria in the mucosa has been suggested to be facilitated by antibacterial products giving a competitive advantage over other microorganisms (29). Such antibacterial peptides are produced by the bacteria themselves but locally produced host-derived antibacterial peptides originating from inflammatory cytokines might also contribute to the antibacterial activity. Considering the results presented here, induction of IL-8 production and cleavage of the cytokine should aid the innate immune clearance of invading bacteria, but also the colonization and persistence in the stomach mucosa of pathogenic microbes such as *H. pylori*, since the antibacterial capacity of the IL-8 peptide is much more pronounced against other bacteria compared to against *H. pylori*.

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