Hepcidin, a Urinary Antimicrobial Peptide Synthesized in the Liver*

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Cysteine-rich antimicrobial peptides are abundant in animal and plant tissues involved in host defense. In insects, most are synthesized in the fat body, an organ analogous to the liver of vertebrates. From human urine, we characterized a cysteine-rich peptide with three forms differing by amino-terminal truncation, and we named it hepcidin (Hepc) because of its origin in the liver and its antimicrobial properties. Two predominant forms, Hepc20 and Hepc25, contained 20 and 25 amino acid residues with all 8 cysteines connected by intramolecular disulfide bonds. Reverse translation and search of the data bases found homologous liver cDNAs in species from fish to human and a corresponding human genomic sequence on human chromosome 19. The full cDNA by 5’ rapid amplification of cDNA ends was 0.4 kilobase pair, in agreement with hepcidin mRNA size on Northern blots. The liver was the predominant site of mRNA expression. The encoded prepeptide contains 84 amino acids, but only the 20–25-amino acid processed forms were found in urine. Hepcids exhibit antifungal activity against Candida albicans, Aspergillus fumigatus, and Aspergillus niger and antibacterial activity against Escherichia coli, Staphylococcus aureus, Staphylococcus epidermidis, and group B Streptococcus. Hepcidin may be a vertebrate counterpart of cysteine-rich antimicrobial peptides produced in the fat body of insects.

Innate immunity relies on a variety of effector mechanisms to defend against microbial invasion. Among them are the abundant and widely distributed disulfide-linked cationic antimicrobial peptides found in both the plant and animal kingdoms. Generally, these peptides exhibit a broad range of activity against bacteria, fungi, protozoa, and enveloped viruses. Plants produce many cysteine-rich antimicrobial peptides including thionins, plant defensins, and the cysteine rich Ib-AMP (9, 10). Cysteine-rich antimicrobial peptides originating in the liver have been described previously for the isolation of human and 25-amino acid processed forms, which are found in urine. Hepcidins exhibited antifungal activity against Candida albicans, Aspergillus fumigatus, and Aspergillus niger and antibacterial activity against Escherichia coli, Staphylococcus aureus, Staphylococcus epidermidis, and group B Streptococcus. Hepcidin may be a vertebrate counterpart of cysteine-rich antimicrobial peptides produced in the fat body of insects.

MATERIALS AND METHODS

Purification from Urine—Cationic peptides were extracted from pooled urine of one to five healthy donors using methods that we described previously for the isolation of human β-defensins-1 (11). Briefly, urine was filtered to remove cells and extracted with the weak cation exchange matrix CM Macrogrop (Bio-Rad, Richmond, CA). Cationic peptides were eluted with 5% acetic acid and further purified by RP-HPLC1 (Waters Model 626; Waters, Milford, MA) on a Vydac C18 column (218TP510) equilibrated in 0.1% trifluoroacetic acid (v/v). Peptides were eluted with an acetonitrile (ACN) gradient of 4% ACN increment/min for 5 min and washed with 20% ACN for 5 min, followed by a 0.5% ACN increment/min for the last 30 min (12). HPLC peak fractions were analyzed by acid urea polyacrylamide gel electrophoresis, and peptide masses were determined by MALDI-TOF-MS (UCLA Mass Spectrometry Facility (Los Angeles, CA) or Emory University Microchemical Facility (Atlanta, GA)).

Amino Acid Sequence—Purified hepcidin was modified by reduction and carboxymethylation before amino acid sequencing. Lyophilized peptides were resuspended in reduction and alkylation buffer (0.5 M Tris buffer, pH 8, 6 M guanidine hydrochloride, and 20 mM EDTA) to a concentration of 1 mg/ml. Reduction was accomplished by the addition of a 1000-fold molar excess of dithiothreitol (DTT), and the solution was overlaid with N2 gas and then incubated at 52 °C for 5 h. Fresh DTT (500-molar excess) was added and incubated for an additional hour at 52 °C. The solution was cooled to room temperature for 10 min, and iodoacetamide freshly dissolved in reduction and alkylation buffer was added to a final concentration of 3-fold molar excess over DTT and incubated for 10 min in the dark. The reaction was stopped by the addition of DTT (1500-fold molar excess of the peptide). The peptide was then purified on a C18 RP-HPLC column equilibrated in 0.1% hexafluoroisopropanol acid (v/v). Peptides were eluted using linear ACN gradients at a flow rate of 3 ml/min and monitored by absorbance at 230 and 280 nm as follows: 1% ACN increment/min for 20 min, followed by a 0.5% increment/min for the last 60 min. HPLC fractions were lyophilized and resuspended in 5% acetic acid, and the peak fraction (32–35%...
ACN) was submitted for amino acid sequencing by Edman degradation at the UCLA Peptide Sequencing Facility.

**Circular Dichroism Spectroscopy**—CD spectra were recorded using an AVIV 62DS spectropolarimeter (AVIV Associates, Lakewood, NJ). Peptide at a concentration of 1 mg/ml was dissolved either in 10 mM NaPO₄, pH 7.4, alone or in a 1:1 (v/v) solution of trifluoroethylene:polystyrene 96-well plates (Costar, Corning, NY), and 20 μl of peptides were added to 100 μl of bacterial suspensions at a concentration of 10⁶ CFU/ml. An absorbance of 0.2 corresponds to 10⁷ CFU/ml for the bacterial strains tested. The plate was incubated at 30 °C in a humidified chamber in the dark. Germination was microscopically monitored for the appearance of hyphae using a light microscope. After 48 h, the absorbance was measured at 620 nm using a SpectraMax 250 EIA plate reader (Molecular Devices, Sunnyvale, CA). To test for activity in high salt conditions, the assay medium was supplemented with 150 mM NaCl.

**Gungical Assay**—To determine whether the effect of hepcidin was fungicidal or fungistatic, spores were incubated in the presence of Hep20 and Hep25 at the highest concentrations necessary to inhibit germination as described above. After 48 h at 30 °C, the well contents were transferred to sterile microfuge tubes and centrifuged for 5 min at 2000 × g. The supernatant containing the peptides was removed, and the spore pellet was resuspended in 100 μl of fresh media and transferred into a new 96-well plate. The spores were incubated for an additional 48 h at 30 °C and then analyzed for germination as described above. If no hyphae were observed, then the effect of hepcidin was judged to be fungicidal.

**Pepptide Synthesis and Refolding**—Peptide synthesis reagents including Fmoc amino acids (AnaSpec Inc., San Jose, CA) and coupling solvents (PE Biosystems, Applied Biosystems, Foster City, CA) were used to synthesize Hep20 peptide on an ABI 431A peptide synthesizer (Applied Biosystems) using double coupling for all residues. The scale of synthesis was 0.25 mmol using a FastMoc™ strategy (16) with PS-PEG resin (PE Perceptive Biosystems, Connecticut Path, MA). After cleavage, the crude synthetic peptide (sHep) was resuspended in reducing buffer (6 μM guanidine-HCl, 0.02 μM EDTA, and 0.5 μM Tris-HCl, pH 8.07) to a final concentration of 0.5 mg/ml (w/v), homogenized with 20 strokes in a Dounce homogenizer, and incubated in a 50 °C sonicating water bath for 30 min. The resuspended peptide was reduced by adding DTT to a final concentration of 0.1 mM, overlaying with N₂ gas, and then incubating in a 50 °C water bath for 2 h. The reduced peptide was loaded onto a C₁₈ Sep-Pak cartridge (Waters) equilibrated with 0.5% trifluoroacetic acid, desalted with 15% ACN and 0.1% trifluoroacetic acid, and eluted with 40% ACN and 0.1% trifluoroacetic acid. The 40% ACN fraction was lyophilized and then further purified by RP-HPLC on a C₁₈ column using the same gradient as above in purification of the native peptide from urine. The major peak was lyophilized, reconstituted to 0.03 mg/ml with distilled H₂O, adjusted to pH 7.5 with ammonium hydroxide, and then air-oxidized in an open vessel at room temperature with stirring for 18 h. The refolded peptide was then purified on a C₁₈ Sep-Pak cartridge followed by RP-HPLC as described for the reduced peptide.

### RESULTS

**Purification and Amino Acid Analysis**—Cationic proteins were extracted from urine using a weak cation exchange resin and then further purified by RP-HPLC (Fig. 1). and hepcidin peaks were identified by characteristically migrating bands in Coomassie Blue-stained acid-urea PAGE. The fractions corresponding to the hepcidin peptides eluted between 24% and 30% acetonitrile. The identity of each peptide in peaks A and B of Fig. 1 was confirmed by MALDI-TOF-MS and amino acid sequencing. The peptides were 20 and 25 amino acids long (Hep20 and Hep25, respectively) and differed by a single-amino acid molar truncation but preserved the cysteine-rich domain (Fig. 2). Mass analysis identified the peptide in peak C of Fig. 1 as the 22-amino acid form (Hep22). The mass data (Table 1) indicate that all 8 cysteine residues are connected by disulfide bonds. In urine, Hep20 and Hep25 are the major forms,
hepcidin was purified by RP-HPLC on a Vydac C18 column using the following linear gradient in 0.1% trifluoroacetic acid: 4% ACN increment/min for 5 min, 20% ACN wash for 5 min, then 0.5% ACN increment/min for the last 30 min. Peak A, Hepc20; peak B, Hepc25; peak C, Hepc22. Absorbance was monitored at λ = 215 nm.

**Fig. 1.** RP-HPLC purification of hepcidin from human urine. Cationic peptides in urine were purified by RP-HPLC on a Vydac C18 column using the following linear gradient in 0.1% trifluoroacetic acid: 4% ACN increment/min for 5 min, 20% ACN wash for 5 min, then 0.5% ACN increment/min for the last 30 min. Peak A, Hepc20; peak B, Hepc25; peak C, Hepc22. Absorbance was monitored at λ = 215 nm.

**Amino acid sequence of hepcidin and proposed cysteine connectivity.** Purified hepcidin was carboxymethylated and then sequenced by Edman degradation. The three processed forms differ by amino-terminal truncation as denoted by asterisks (*). The proposed cysteine linkage pattern is 1-4, 2-8, 3-7, and 5-6, as shown. The cationic residues are indicated by arrows (>).

**Fig. 2.** Amino acid sequence of hepcidin and proposed cysteine connectivity. Purified hepcidin was carboxymethylated and then sequenced by Edman degradation. The three processed forms differ by amino-terminal truncation as denoted by arrows (>). The proposed cysteine linkage pattern is 1-4, 2-8, 3-7, and 5-6, as shown. The cationic residues are indicated by asterisks (*).

**Characteristics of hepcidin peaks A-C**

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<th>HPLC peak</th>
<th>Amino acid residues</th>
<th>MALDI-TOF mass</th>
<th>Calculated average mass</th>
<th>Approximate concentration in normal urine (μg/l)</th>
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<td>2436</td>
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<th>Characteristic of hepcidin peaks A-C</th>
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<tr>
<td>HPLC peak</td>
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whereas Hepc22 is a minor species. Collectively, the concentration of hepcidin ranges between 10 and 30 μg/liter (4-12 nm) in urine from normal donors. The remaining large peaks visible in Fig. 1 had previously been identified as variably amino-terminal-truncated forms of another antimicrobial peptide, human β-defensin-1 (11).

**CD Spectroscopy**—The CD spectra (Fig. 3) in phosphate-buffered saline solution (100 mM sodium chloride and 20 mM sodium phosphate, pH 7.4) and the structure-promoting solvent system trifluoroethanol-20 mM phosphate buffer, pH 7.4 (trifluoroethylene:buffer, 1:1, v:v), were consistent with a structure that has a series of β-turns, loops, and distorted β-sheets.

**Northern Blot Analysis**—Membranes preloaded with human organ tissue poly(A) selected mRNA were probed with a radiolabeled probe generated by PCR. A: lane 1, spleen; lane 2, thymus; lane 3, appendix; lane 4, peripheral blood leukocyte; lane 5, bone marrow; and lane 6, fetal liver (FL). B: lane 1, heart (H); lane 2, brain; lane 3, placenta; lane 4, lung; lane 5, adult liver (AL); lane 6, skeletal muscle; lane 7, kidney; and lane 8, pancreas. C: lane 1, stomach; lane 2, thyroid; lane 3, spinal cord (SC); lane 4, lymph node; lane 5, trachea; and lane 6, adrenal gland. An RNA marker ladder is shown. Hepc mRNA, 0.4–0.5 kilobase, was detected in fetal liver (FL) and adult liver (AL), with trace message detected in the heart (H) and spinal cord (SC).

**Antimicrobial Activity**—The colony-forming unit assay (19) was used to determine the antimicrobial activity of purified hepcidin forms against various microbial strains. In the initial screen, all the strains were tested against 30 μM Hepc20 and
Hepcidin 7809

**Fig. 5. Comparison of hepcidin sequences from various species.** A BLAST search of GenBank™ EST entries revealed cDNA homologues of human hepcidin (hHEPC) in pig (pHEPC), rat (rHEPC), mouse (mHEPC), flounder (fHEPC), and the long-jawed mudsucker Gillichthys mirabilis (gHEPC). Putative peptide sequences were translated from the cDNA sequences isolated from the liver of each species. The positions of cysteine residues (outlined in gray boxes) are conserved.

![Image of hepcidin sequences comparison]

**Fig. 6. Hepcidin gene and precursor peptide.** An intron-exon diagram and the peptide sequences encoded by the three exons A, B, and C are shown. The exons encode an 84-amino acid prepropeptide. Arrows denote three processed forms isolated from urine. An arrow indicates the putative signal sequence (SS) cleavage site.

Hepc25 (Hepc22 was not tested because it is present in small concentrations in the urine). Both peptides were antimicrobial against E. coli ML35p and, to a lesser extent, against S. epidermidis, S. aureus, C. albicans, and group B Streptococcus, but both were inactive against Pseudomonas aeruginosa (Fig. 7). A dose-response CFU assay performed against E. coli and S. epidermidis showed that both hepcidin peptides were microbicidal at the highest concentration of 30 μM against both strains (data not shown). In this assay and in the initial screen, Hepc20 was more active than Hepc25; however, both peptides were inhibited by the addition of 100 mM NaCl to the assay mixture.

**Inhibition of Germination—** Spores of two human pathogenic strains of A. fumigatus and A. niger were subjected to a germination assay (Fig. 8). Hepc20 was more potent than Hepc25 against both strains. Hepc20 was antifungal against A. niger, where no hyphae were detected after 48 h at 20 μM, but 40 μM was required against the more resistant strain, A. fumigatus. Hepc25 only retarded spore germination in A. niger at the highest concentration of 40 μM and was ineffective against A. fumigatus at the same concentration. Neither peptide was active in the presence of 150 mM NaCl.

**Fungicidal Activity—** We assessed fungicidal activity by following germination after removal of the antifungal peptides and additional incubation in fresh media. No germination was observed after 48 h. At the highest concentrations, Hepc20 was fungicidal against both strains, as indicated by the lack of spore germination in fresh medium. Hepc25 did not exhibit fungicidal activity at any concentration used in this assay.

**Cytotoxicity Assay—** To test for cytotoxic effects of hepcidin, K562 cells were assayed for viability after incubation with Hepc20 and Hepc25, using trypan blue as an indicator of membrane integrity. At the highest concentration of 30 μM, cells incubated for 15 h in the presence of Hepc20 or Hepc25 were 88% and 74% viable, respectively. When cells were treated with 30 μM human α-defensin HNP-1, only 9% viable cells remained at the end of the incubation period. Thus, hepcidin is not cytotoxic at a concentration that is ~3000-fold higher than that found in the urine.

**Chemical Synthesis of Hepcidin—** Crude synthetic material was completely reduced with DTT and purified by RP-HPLC. The major HPLC peaks were analyzed by AU-PAGE and MALDI-TOF-MS. As expected, the peptide migrated more slowly upon reduction. The gain of 8 Da corresponding to eight additional hydrogen atoms in MALDI-MS confirmed that the synthetic peptide was completely reduced. The reduced peptide was refolded by air oxidation and compared with the native peptide. The synthetic Hepc20 had identical AU-PAGE migration, eluted identically in C18 RP-HPLC, and had an identical electrospray mass and antimicrobial activity against E. coli.

**DISCUSSION**

We report here the first member of a new vertebrate family of small antimicrobial peptides that contain 8 cysteine residues and are active against both bacteria and fungi. Hepcidin mRNA encodes a larger precursor and is found primarily in the liver, but the peptides were first discovered in and isolated from urine. The native peptide was purified from human urine by cation exchange chromatography and RP-HPLC and characterized by amino acid sequencing, MALDI-TOF-MS, and CD spectroscopy.

The human hepcidin forms, 2–3 kDa in size, have an overall charge of +3 at neutral pH and are only 20–25 amino acids in
length (8 of these amino acids are cysteine residues (~30% cysteine)). Mass spectrometry data confirm all 8 cysteines to be engaged in four intramolecular disulfide bonds. The symmetric arrangement of pairs of cysteines around a cationic segment (HRSK) resembles that of antimicrobial protegrins and tachyplesins, peptides that have a two-strand β-sheet structure stabilized by interstrand disulfide bonds. Based on this similarity and on preliminary molecular models, we propose the following cysteine connectivity pattern: 1-4, 2-8, 3-7, and 5-6 (Fig. 2).

This arrangement of disulfide bonds exhibits three possible β-turns and provides a fold with the least sterical hindrance among the side chains. The proposed disulfide connectivity pattern makes the hepcidin structure similar to the “cystine knot” class of antimicrobial peptides (20, 21).

The cDNA structure suggests that the peptide is translated as an 84-amino acid prepropeptide that is amino-terminally processed to the 20- to 25-amino acid peptide (Fig. 6). A strong consensus sequence score (σ = 16.45 using the PC Gene program) for a signal sequence cleavage site is located between Gly24 and Ser25 that would produce a 60-residue propeptide. Another likely processing site is carboxyl-terminal to Arg59, a consensus site for the subtilisin/kexin family of mammalian propeptide processing enzymes (propeptide convertases) with a preference for cleavage after the paired basic residues Lys-Arg and Arg-Arg. Among other functions, the propeptide convertases cleave propeptides to generate one or more bioactive peptides from a single precursor (18). The abundance of propeptide convertases in the liver may explain our inability to isolate the larger propeptide from native sources (liver tissue, bile, and blood serum). The small size of hepcidin, its conservation between animal species, and its compact folding pattern may account for our difficulties in producing a useful antibody.

A search of the protein data base revealed no homology to hepcidin in vertebrates and invertebrates. However, searching the EST data base revealed liver cDNA homologues in pig, rat, mouse, flounder, and the long-jawed mudsucker (Fig. 5). All 8 cysteine residues are conserved with particularly strong sequence similarity in residues 60–84 that encompass the processed forms isolated from the urine. It is noteworthy that the putative propeptide convertase cleavage sites are also conserved. In humans, the paired basic residues are Arg-Arg, whereas the other homologues have the same recognition sequence or an alternative recognition sequence of Lys-Arg.

It is possible that if the proregion is cleaved from the precursor as a single peptide, it could also have a biological function. To test this possibility, the proregion (Ser25–Arg59) was synthesized, and an antibody reactive with the synthetic peptide was produced (Research Genetics). Preliminary data utilizing the synthetic propiece peptide have shown that it exhibits an antibacterial activity equally potent to that of Hepc20 and Hepc25 (data not shown). However, a Western blot analysis capable of detecting 500 ng of the synthetic peptide did not detect the propiece peptide in cationic extracts of urine, serum, liver extract, and bile (data not shown), making it unlikely that this form is stable and abundant. Acid liver extracts did contain a peptide with a mass identical to Hepc25 that eluted at the same acetoniitrile concentration as Hepc25 from urine. While this study was under review, we noted an interim report that an antimicrobial peptide identical to Hepc25, LEAP-1, was isolated from human plasma ultrafiltrate (22). The presence of Hepc25 in ultrafiltrate suggests that the peptide originating in the liver reaches the kidney in blood plasma.

Hepcidin may be the functional homologue of the insect defensin-like peptide drosomycin. Hepcidin is expressed in human liver, whereas drosomycin is found in the fat body (the liver equivalent) of Drosophila; both peptides have four disulfide bonds, and both are potent antifungal peptides. In parallel studies, Pigeon et al. identified a murine hepcidin as a hepatic mRNA inducible in vivo by iron overload and lipopolysaccharide. The ability of lipopolysaccharide to increase hepcidin mRNA in vivo and in isolated hepatocytes is consistent with the proposed role of hepcidin in inflammation and host defense. Like hepcidin, drosomycin is expressed constitutively but is also inducible upon microbial challenge. Like drosomycin, both Hepc20 and Hepc25 forms inhibit spore germination in the two pathogenic strains of Aspergillus tested. Future studies will be necessary to determine whether hepcidin, like the cysteine-rich antimicrobial defensins (23), interacts with specific cellular receptors and functions also as a signaling molecule.

Acknowledgments—We thank Drs. Christelle Pigeon and Olivier Lounyal for several corrections to the manuscript and for sharing their own work with us prior to publication and Dr. Edith Porter for critical reading of the manuscript.

REFERENCES
