THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 11, Issue of March 16, pp. 7806–7810, 2001

Printed in U.S.A.

Hepcidin, a Urinary Antimicrobial Peptide Synthesized in the Liver*

Received for publication, September 29, 2000, and in revised form, November 9, 2000
Published, JBC Papers in Press, December 11, 2000, DOI 10.1074/jbc.M008922200

Christina H. Park‡, Erika V. Valore‡, Alan J. Waring§, and Tomas Ganz‡¶

From the §Department of Pediatrics, Harbor-UCLA Medical Center, and ‡Departments of Medicine and Pathology, UCLA School of Medicine, Los Angeles, California 90059

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 prepropeptide contains 84 amino acids, but only the 20–25-amino acid processed forms were 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.

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 1–4 (1–3). In insects, cysteine-rich antimicrobial peptides are produced in the fat body (functional homologue of the mammalian liver) and transcriptionally induced and released into the hemolymph in response to infection or injury. These include insect defensins, heliomicin, drosomycin, and thanatin (4–7). Mollusks also produce cationic and cysteine-rich antimicrobial peptides such as mytilin, mytimicin, and myticin (8). In mammals, similar antimicrobial peptides include α- and β-defensins and protegrins (9, 10).

Like the insect fat body, the vertebrate liver is also centrally involved in innate immune response to infection. The "acute phase" response to infection or inflammation is a pattern of increased hepatic synthesis of many secreted proteins involved in host defense and the selective suppression of synthesis of other secreted proteins. In contrast to the abundant fat body-derived antimicrobial peptides of insects, no vertebrate antimicrobial peptides originating in the liver have been described to date. In this work, we report the discovery of a novel hepatic antimicrobial peptide, hepcidin, whose processed form is found in urine.

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-defensin-1 (11). Briefly, urine was filtered to remove cells and extracted with the weak cation exchange matrix CM Macroprep (Bio-Rad, Richmond, CA). Cationic peptides were eluted with 5% acetic acid and further purified by RP-HPLC[1] (Waters Model 626; Waters, Milford, MA) on a Vydac C_{18} 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 peptide was 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 N_2 gas and then incubated at 52 °C for 2.5 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 C_{18} RP-HPLC column equilibrated in 0.1% *h*-heptafluorobutyric 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%

* This work was supported in part by National Institutes of Health Grant HL 46809. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Dept. of Medicine, CHS 37-055, UCLA School of Medicine, Los Angeles, CA 90095-1690. Tel.: 310-825-6112; Fax: 310-206-8766; E-mail: tganz@mednet.ucla.edu.

[1] The abbreviations used are: RP-HPLC, reverse phase-high performance liquid chromatography; ACN, acetonitrile; CFU, colony-forming unit; DTT, dithiothreitol; Hepc, hepcidin; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; UCLA, University of California Los Angeles; EST, expressed sequence tag; poly(A), polyadenylated.

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 $NaPO_4$, pH 7.4, alone or in a 1:1 (v/v) solution of trifluoroethylene: phosphate buffer. Peptide solutions were scanned in 0.1 mm light path demountable cells over a 270 nm to 185 nm wavelength range at 10 nm/min and a sample interval of 0.2 nm.

cDNA and Genomic Cloning—An initial search of the protein data base showed no homology of hepcidin to any known protein. Therefore, the primary amino acid sequence was reverse translated and searched against the GenBank™ human EST data base using the BLAST (tblastn) nucleic acid search program (13). A matching cDNA clone (American Type Culture Collection yb44e08.r1, clone 74054; GenBank™ accession number T48277) was purchased from American Type Culture Collection (Manassas, VA). The plasmid clone was transformed and amplified in Bluescript *Escherichia coli* (Stratagene, La Jolla, CA) and purified using a Qiagen Plasmid Midi Prep kit according to the manufacturer's instructions (Valencia, CA).

5′ Rapid Amplification of cDNA Ends (RACE)—The sequence of the 5′ end of the cDNA was determined using Marathon-Ready cDNA from a liver library (CLONTECH). The gene-specific primers for hepcidin are antisense (for 5′-RACE) 5′-CCCAAGACCTATGTTCTGG-3′ and 5′-TCTGTCTGGCTGTCCCACTGCTGG-3′ and sense (for 3′ RACE) 5′-CATGTTCCAGAGGCGAAGG-3′. PCR was carried out according to the manufacturer's instructions. PCR products were purified by phenol/chloroform extraction and ethanol precipitation (14). The PCR product was sequenced using an ABI Prism DNA sequencer (Applied Biosystems) at the UCLA Sequencing Facility. The Omiga program (Oxford Molecular Co.) was used to analyze and search for potential consensus sites within the gene.

Northern Blot Analysis: Human Organ Screen—The hepcidin cDNA clone and the gene-specific primers above were used in the PCR reaction to generate a 173-base pair product from a segment of the coding region. The PCR product was used to probe CLONTECH organ blot membranes as described previously (11). The organ blots contained poly(A) selected human mRNA from spleen, thymus, appendix, peripheral blood leukocyte, bone marrow, fetal liver, heart, brain, placenta, lung, adult liver, skeletal muscle, kidney, pancreas, stomach, thyroid, spinal cord, lymph node, trachea, adrenal gland, prostate, testes, ovary, small intestine, and colon.

Human Liver—Frozen normal human liver blocks obtained from the UCLA Human Tissue Resource Center (Los Angeles, CA) were pulverized in liquid nitrogen using a mortar and pestle. Approximately 0.1 g of tissue was homogenized in 1 ml of Trizol reagent (Life Technologies, Inc.), and total RNA was extracted according to the manufacturer's instructions. Poly(A) RNA was separated from the total RNA preparation using a Qiagen Oligotex mRNA isolation kit. Purified poly(A) RNA was separated on a 1% agarose gel (Bio-Rad) containing 3.3% formalin in 10 mM $NaPO_4$, transferred onto GeneScreen Plus (PerkinElmer Life Sciences, Boston, MA), and hybridized with a radioactive probe as described above.

Antimicrobial Assays—The 20- and 25-amino acid forms of hepcidin (Hepc20 and Hepc25), each of which was homogeneous by electrophoretic and mass spectrum analysis, were quantified by UV absorbance at 215 and 220 nm using the formula concentration (mg/ml) = $(A_{215} - A_{225}) \times 0.144$, and they were tested for antimicrobial activity against bacterial strains (*E. coli* ML35p, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and group B *Streptococcus*) and yeast (*Candida albicans*) in a CFU assay as described previously (11). Culture densities were measured spectrophotometrically at 620 nm for bacterial strains and at 450 nm for yeast and then resuspended to a final concentration of 10^6 CFU/ml. An A_{620} reading of 0.2 corresponds to 10^7 CFU/ml for the staphylococcal and streptococcal strains and 5×10^7 CFU/ml for *E. coli*. An A_{450} reading of 1.0 is equivalent to 2.86×10^7 CFU/ml for *C. albicans*. The organisms were incubated with various concentrations of peptide at 37 °C with constant shaking for 1, 3, and 24 h. Surviving microbes were plated in triplicate on trypticase soy broth plates (Microdiagnostic Products Inc., Lombard, IL) using a spiral plating system (Spiral Biotech, Bethesda, MD). In some experiments, the assay medium was supplemented with 100 mM NaCl to mimic higher salt conditions characteristic of blood plasma.

Germination Assay—The two human clinical isolates of filamentous fungi *Aspergillus fumigatus* and *Aspergillus niger* used in this assay were a generous gift from Dr. Dexter Howard (UCLA Department of Microbiology, UCLA, Los Angeles, CA). Spores were harvested as described previously (15). The glycerol stocks of spores from each strain were utilized in a spectrophotometric germination assay as follows: spores were diluted to a concentration of 1.25×10^4 /ml in culture medium (half-strength potato dextrose broth (BBL), 10 μg/ml tetracycline (Sigma), and 100 μg/ml cefotaxime (Life Technologies, Inc.). Eighty μl of each spore suspension was placed in sterile flat-bottomed polystyrene 96-well plates (Costar, Corning, NY), and 20 μl of peptide or peptide diluent (water) was added to yield a final spore concentration of 10^4 /ml. 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 600 nm using a SpectraMAX250 EIA plate reader (Molecular Devices, Sunnyvale, CA). To test for activity in high salt conditions, the assay medium was supplemented with 150 mM NaCl.

Fungicidal Assay—To determine whether the effect of hepcidin was fungicidal or fungistatic, spores were incubated in the presence of Hepc20 and Hepc25 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 $2000 \times g$. 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.

Cytotoxicity Assay—K562 cells (American Type Culture Collection leukemic cell line CCL-243) were cultured in RPMI 1640 with L-glutamine (Life Technologies, Inc.) and 10% fetal calf serum (Hyclone, Logan, UT) for 48 h and then washed and resuspended to 10^6 cells/ml in serum-free RPMI 1640 media. A volume of 50 μl of cells was aliquoted into sterile flat-bottomed 96-well plates (Nalgene Nunc, Rochester, NY). Various concentrations of 10× peptide stocks were added to the cells and incubated at 37 °C/5% CO_2 for 12–16 h. Viability was determined by trypan blue dye exclusion.

Peptide 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 Hepc20 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 M guanidine-HCl, 0.02 M EDTA, and 0.5 M 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 0.01 M, overlaying with N_2 gas, and then incubating in a 50 °C water bath overnight. The reduced peptide was loaded onto a C_{18} Sep-Pak cartridge (Waters) equilibrated with 0.1% 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 Vydac C_{18} column using the same gradient program as described above in purification of the native peptide from urine. The major peak was lyophilized, resuspended to 0.03 mg/ml with distilled H_2O, 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 C_{18} 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 (Hepc20 and Hepc25, respectively) and differed by amino-terminal 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 (Hepc22). The mass data (Table I) indicate that all 8 cysteine residues are connected by disulfide bonds. In urine, Hepc20 and Hepc25 are the major forms,

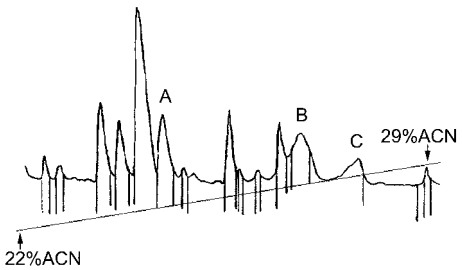

FIG. 1. **RP-HPLC purification of hepcidin from human urine.** Cationic peptides in urine were purified by RP-HPLC on a Vydac C_{18} column using the following linear gradient in 0.1% trifluoroacetic acid: 4% ACN increment/min for 5 min, 20% ACN wash for 5 min, then 0.5% ACN increment/min for the last 30 min. Peak *A*, Hepc20; peak *B*, Hepc25; peak *C*, Hepc22. Absorbance was monitored at λ = 215 nm.

FIG. 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* (*).

TABLE I
Characteristics of hepcidin peaks A-C

HPLC peak	Amino acid residues	MALDI-TOF mass	Calculated average mass	Approximate concentration in normal urine
		Da	*Da*	$\mu g/l$
A	20	2192	2191.77	10
B	25	2789	2789.40	10
C	22	2436	2436.06	4

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 173-base pair probe generated by PCR from the cDNA clone. mRNA was found to be highly expressed in fetal and adult liver, with a much lower signal detected in the heart and spinal cord (Fig. 4). A fourth membrane loaded with prostate, testes, ovary, small intestine, and colon was also probed, but no signal was detected (data not shown). A weak mRNA signal corresponding to 2.37 kilobases was seen in fetal and adult liver and spinal cord but was not further analyzed. Although the peptide was found in the urine, the mRNA was not detectable in the kidney or the bladder.

cDNA Cloning—A search of the protein data base revealed no sequence homology to any known peptides. Using the primary sequence of the peptide, the BLAST (tblastn) nucleic acid search program of the human EST data base identified a cDNA clone that spans the region of the peptide from Asp^{40} to Thr^{84} (I.M.A.G.E. consortium clone 74054; DNA GenBank™ accession number T48277; EST yb44e08) (17). An identical genomic sequence match was found on human chromosome 19 (GenBank™ accession number AD000684; locus CH19R30879). In addition, a search of EST data bases found matches to cDNA sequences from the liver of pig, rat, mouse, flounder, and the long-jawed mudsucker (Fig. 5). All of the matches are to peptides that have yet to be isolated and characterized. The similarity is strongest in the region that corresponds to the peptides isolated from urine, namely, residues Arg^{55} to Thr^{84}.

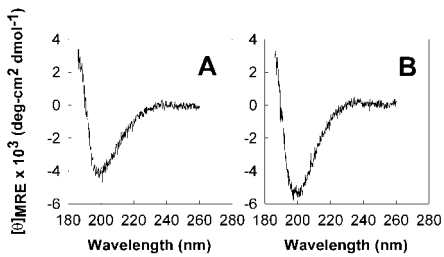

FIG. 3. **Circular dichroism spectra of Hepc20.** The CD spectra were recorded in (*A*) phosphate-buffered saline solution (100 mM sodium chloride and 20 mM sodium phosphate, pH 7.4) and (*B*) the structure-promoting solvent system trifluoroethanol-20 mM phosphate buffer, pH 7.4 (trifluoroethylene:buffer, 1:1, v:v).

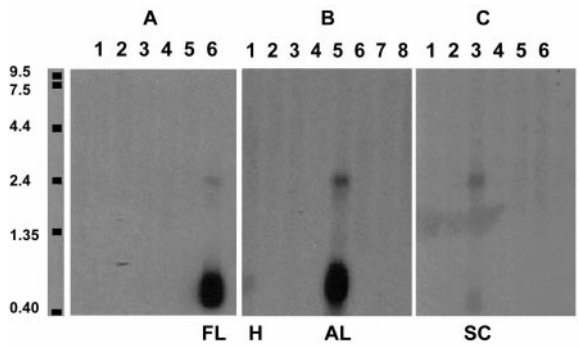

FIG. 4. **Northern blot analysis of human organ mRNA.** A membrane preloaded with human organ poly(A) selected mRNA was purchased from CLONTECH and probed with a radiolabeled probe generated by PCR. *A*: *lane 1*, spleen; *lane 2*, thymus; *lane 3*, appendix; *lane 4*, peripheral blood leukocyte; *lane 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*).

5′ RACE and Gene Analysis—The PCR products of the 5′ RACE reaction were sequenced and compared with the genomic sequence (Fig. 6). The mRNA, 0.4 kilobase in length (GenBank™ accession numbers AAG23966 for cDNA and P81172 for the corresponding hepcidin precursor), matched the estimated size on the Northern blot. The gene consists of three exons and two introns, and the third exon encodes the sequence of the peptides found in urine. The putative unprocessed peptide encoded by all three exons would be 84 amino acid residues long. A potential signal-peptide cleavage site between Gly^{24} and Ser^{25} would generate a 60-amino acid propeptide species. Consensus sites for myristylation sites and phosphorylation sites occur on Gly^{44} and Thr^{24}, respectively. In addition, a potential furin cleavage site is found at Arg^{59} (18). Since our initial studies, additional human EST sequences have appeared in the GenBank™ data bases (GenBank™ accession numbers AI937227, AI829866, and AI797446) with minor sequence variations in the noncoding regions.

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

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hHEPC ...RRRRRDTHFPICIFCCGCCHRSK-CGMCCKT
pHEPC ...RLRR-DTHFPICIFCCGCCRXAI-CGMCCKT
rHEPC ...KRRKRDTNFPICLFCCKCCKNSS-CGLCCIT
mHEPC ...KRRKRDTNFPICIFCCKCCNNSQ-CGICCKT
fHEPC ...RQKRHISHISLCRWCCNCCKANKGCGFCCKT
gHEPC ...SREKRGIK---CKFCCGCCTPGV-CGVCCR
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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.

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 assessed 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

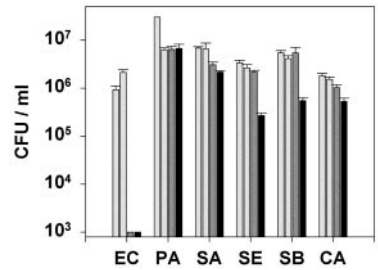

FIG. 7. **Antimicrobial assays.** Various microbial strains were subjected to a CFU assay using Hepc20 and Hepc25 at 30 μM concentration in 10 mM $NaPO_4$, pH 7.4, 0.01× trypticase soy broth after a 1-h incubation. The organisms tested were *E. coli* ML35p (*EC*), *P. aeruginosa* (*PA*), *S. aureus* (*SA*), *S. epidermidis* (*SE*), group B *Streptococcus* (*SB*), and *C. albicans* (*CA*). The amount of CFU/ml is indicated by the *bars*: *first gray bar*, microbial input (t = 0); *second gray bar*, medium only (t = 1 h); *dark gray bar*, Hepc25; *black bar*, Hepc20.

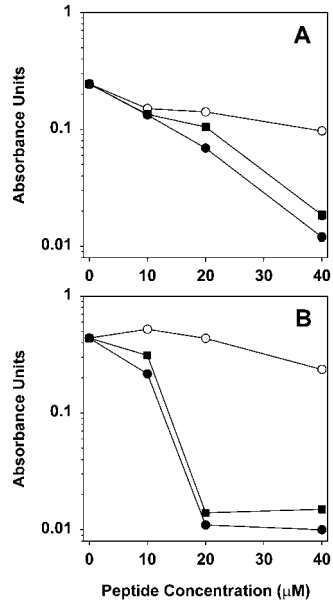

FIG. 8. **Fungal germination assay.** Graph of a germination assay is shown. Hepc20 (●), Hepc25 (○), and synthetic Hepc20 (■) were tested against the spores of two human pathogenic strains of *A. fumigatus* (*A*) and *A. niger* (*B*). In a 96-well plate, 10^4 spores were incubated with and without peptide at 30 °C in the dark. After 48 h, spore germination was monitored at λ = 600 nm.

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 C_{18} RP-HPLC, and had an identical electrospray mass and antimicrobial activity against *E. coli* in a CFU assay compared with native Hepc20.

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 steric 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 = 16.45 using the PC Gene program) for a signal sequence cleavage site is located between Gly^{24} and Ser^{25} that would produce a 60-residue propeptide. Another likely processing site is carboxyl-terminal to Arg^{59}, 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 (Ser^{25}-Arg^{59}) 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 acetonitrile 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.*[2] 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 Loreal 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.

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