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Characterization and bioactivity of hepcidin-2 in zebrafish: Dependence of antibacterial activity upon disulfide bridges

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ABSTRACT

Hepcidin is an antimicrobial peptide and iron-regulatory molecule with highly conserved disulfide bridges among vertebrates, but structural insights into the function in fish remains largely missing. We demonstrate here that recombinant hepcidin-2 from zebrafish is capable of inhibiting the growth of the Gram-negative bacteria *Escherichia coli* and *Vibrio anguillarum*, and the Gram-positive bacteria *Staphylococcus aureus* and *Bacillus subtilis* with minimum inhibitory concentrations (MICs) of 18, 15, 13 and 9 μM, respectively. We also show by TEM examination that recombinant hepcidin-2 is directly cidal to the cells of *E. coli* and *S. aureus*. Moreover, we find that hepcidin-2 displays affinity to LPS, LTA and PGN. All these data indicate that hepcidin-2 is both a pattern recognition molecule, capable of identifying LPS, LTA and PGN, and an antibacterial effector, capable of inhibiting the growth of bacteria. The data also show that the antibacterial activity of hepcidin-2 depends upon the disulfide bridges.

Introduction

Hepcidin is a cysteine-rich peptide which is structurally similar to the defensin family because of the four disulfide bridges in its tertiary structure. Hepcidin, also termed LEAP-1 for liver-expressed antimicrobial peptide, was discovered by two independent groups while looking for antimicrobial peptides in human body fluids in 2000 [9] and 2001 [14] and shown to possess antimicrobial activities. Subsequent studies have uncovered that hepcidin is a long sought iron regulatory hormone involved in hereditary hemochromatosis and anemia of chronic disease [4,13,21], which is part of the acute phase response to infection and inflammation [12,13]. Thus, hepcidin plays a dual role via acting as an iron homeostasis regulator as well as an antimicrobial agent.

Hepcidins have been identified in mammals, birds, frog and fish [1,3,5,10,11,17]. The sequences of hepcidins between mammalian species and those of amphibians and fishes are highly conserved. Hepcidin has been shown to be directly cidal against both Gram-positive bacteria (e.g. *Mycobacterium tuberculosis* and *Bacillus subtilis*) and Gram-negative bacteria (e.g. *Neisseria cinerea*) as well as yeasts (e.g. *Saccharomyces cerevisiae*) [9,18,20,24], thus comprising the first defense line of hosts against infections [19]. All hepcidins identified thus far contain basic amino acids that confer a positive total charge and a tendency to assume amphipathic secondary structures. This property has been proposed to enable hepcidins to permeate membranes of invading microbes. However, the mode of action of hepcidins against microbes remains largely unknown, and the structure–activity relationship is poorly defined.

The overall genomic organization of hepcidin shows remarkable conservation, in that hepcidin genes in all organisms studied so far consist of three exons interspaced by two introns. The exons encode an amino acid prepropeptide containing a typical leader peptide at the N-terminus, a proregion and a mature peptide containing the important cysteine residues at the C-terminus [3]. The number of hepcidin genomic copies varies with species, with one gene being identified in humans, two reported in mouse [15], three known in tilapia [8] and two present in zebrafish (GenBank accession number: AY363452 and AY363453; [17]). Zebrafish hepcidin genes code for a prepropeptide of 91 amino acids, and display an elevated expression in the abdominal organs, skin, and heart in fish that developed signs of infection following bacterial injection. However, antimicrobial activity of hepcidins in zebrafish remains to be tested.

The aims of this study were therefore to examine the antimicrobial activity of hepcidin-2 in zebrafish (*Danio rerio*) and its affinity to LPS, LTA and PGN. We also explored the relationship between disulfide bridges and antimicrobial activity.

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Materials and methods

Cloning and sequencing of hepcidin cDNA

Total RNA was extracted with RNAiso Plus (TaKaRa, Dalian, China) from adult zebrafish, and digested with RNase-free DNase (TaKaRa, Dalian, China) to eliminate the genomic contamination. Total cDNAs was synthesized with reverse transcription system (Promega) using oligo (dT) primer. The reaction was carried out at 42 °C for 50 min and inactivated at 75 °C for 15 min. Total cDNA was used as PCR template to amplify hepcidin gene. A pair of primers P1 and P2 (Table 1) specific of hepcidin-2 was designed according to the sequence of zebrafish hepcidin (GenBank accession number: NM_205583.2; http://www.ncbi.nlm.nih.gov/) using Primer Premier 5.0 program. The PCR amplification reaction was carried out at 94 °C for 5 min, followed by 32 cycles at 94 °C for 30 s, 53 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 7 min. The amplification products were gel-purified using DNA gel extraction kit (AXYGEN), cloned into the pGEM-T vector (Invitrogen), and transformed into Trans5α *Escherichia coli* (TransGen). The positive clones were selected and sequenced to verify for authenticity.

Sequence and phylogenetic analyses

The protein domain was analyzed using the SMART program (http://smart.embl-heidelberg.de/), and the signal peptide prediction was performed using SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/). The N-terminal cleavage site of the mature peptide was assigned based on the cleavage sites of the known mature hepcidins from human, white bass and large yellow croaker, and the proximity to the RX(K/R)R motif characteristic of processing sites for the propeptide convertases [16,22]. The theoretical molecular mass (MW) and isoelectronic point (pI) of the mature peptide were determined using ProtParam (http://www.expasy.ch/tools/protparam.html). Multiple alignments of the protein sequences and homologies among hepcidins were generated using the Clustal W program within the MegAlign of the DNASTAR software package (version 5.0). The Gap penalty and gap extension values were 10 and 0.2, respectively. Phylogenetic tree was constructed by MEGA (version 4.1) using p-distance based on the neighbor-joining method. The reliability of each node was estimated by bootstrapping with 1000 replications.

Construction of expression vector

The complete region encoding the mature peptide of hepcidin-2 was amplified by PCR using the upstream primer P3 and the downstream primer P4 (Table 1). The reaction was performed under the following conditions: an initial denaturation at 94 °C for 5 min, followed by 32 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s and a final extension at 72 °C for 7 min. The PCR product was digested with *EcoRI* and *XhoI* and sub-cloned into the plasmid expression vector pET-30a (Novagen) previously cut with the same restriction enzymes. The identity of insert was verified by sequencing, and the plasmid was designated *pET-30a/hepcidin-2*. Hence an expression vector including cDNA encoding the mature peptide of hepcidin-2 and a 5′ additional His-tag of pET30a was constructed.

Table 1
Sequences of the primers used in this study. *EcoRI* and *XhoI* sites are underlined. The mutations are shown in boldface and double underlined.

Primers	Sequence (5′–3′)
P1 (sense)	ATGAAGCTTTCTAACGTGTTTC
P2 (antisense)	TCAGAATTTGCAGCAGTATCCG
P3 (sense)	GGAATTCTGTGCAGATTCTGC
P4 (antisense)	CCGCTCGAGTCAGAATTTGCAG
P5 (sense)	TGCAAATGCTGTCGCAATAAA
P6 (antisense)	GCAGAATCTG**GC**AGGAATTC
P7 (sense)	CGCAATAAAGGC**GC**CGGATACTGC
P8 (antisense)	ACAGCATTTG**GC**G**GC**GAATCTGGC
P9 (sense)	GCCGGATAC**GC**C**GC**CTTTAAGACT
P10 (antisense)	GCCTTTATTGCG**GGC**G**GC**TTTGCA

Expression and purification of recombinant hepcidin-2

The recombinant peptide hepcidin-2 was induced by isopropyl-β-D-thiogalactoside and was purified by affinity chromatography on a nickel-nitrilotriacetic acid resin column. Cells of *E. coli* BL21 (DE3) were transformed with *pET-30a/hepcidin-2* and cultured overnight in Luria–Bertani (LB) broth containing kanamycin (100 μg/ml). The culture was diluted 1:100 with LB broth and subjected to further incubation at 37 °C for 3 h. The expression of hepcidin-2 was induced by adding isopropyl-β-D-thiogalactoside (IPTG) to the culture at a final concentration of 0.1 mM. After further incubation at 19 °C overnight, the bacterial cells were harvested by centrifugation at 5000 × *g* for 8 min, re-suspended in 20 mM PBS (pH 6.3) and sonicated on ice. The cell debris was removed by centrifugation at 12,000 × *g* for 30 min, and the supernatant was loaded onto a Ni-NTA resin column (Novagen). The column was successively washed with the PBS (pH 6.3) containing 10 mM, 20 mM, 30 mM and 40 mM imidazole, respectively, and then eluted with PBS containing 250 mM imidazole. The purity of the eluted samples was analyzed by 15% SDS-PAGE and staining with Coomassie brilliant blue R-250. The concentration of recombinant hepcidin-2, rHCD, was determined by the BCA method.

Disulfides are crucial for maintaining the spatial structure of antimicrobial peptides. To test the relationship between disulfide bridges and antimicrobial activity, site-directed mutation was performed using MutanBEST kit (TaKaRa) to generate mutant hepcidin. Alanine residues were used to replace cysteine residues in protein. As there was a difference of only a single sulfur molecule between alanine and cysteine, this would reduce the effect on protein molecular weight caused by mutation. Primers P5 and P6 were used to mutate the Cys^1 to Ala, and then Cys^2, Cys^3 and Cys^6 were mutated to Ala using the primers P7 and P8. Finally, the Cys^4, Cys^5, Cys^7 and Cys^8 were mutated to Ala using primers P9 and P10. The mutant was confirmed by DNA sequencing, and sub-cloned into the plasmid expression vector pET-30a (Novagen) previously cut with the same restriction enzymes, which was named as *pET30a/m-hepcidin-2*. Expression and purification of the mutated hepcidin-2, rmHCD, were carried out as above.

Western blotting

The extracts from *E. coli* Transetta (DE3) containing *pET-30a/hepcidin-2* and *pET30a/m-hepcidin-2* expression vectors both before and after the isopropyl-β-D-thiogalactoside induction and the purified recombinant hepcidin-2 were separated on a 15% SDS-PAGE gel, and electroblotted onto PVDF membrane (Amersham) by a semi-dry technique (Bio-RAD). The blotted membranes were blocked with 4% BSA in 10 mM PBS (pH 7.4) at room temperature for 2 h, and then incubated in the mouse anti-His-tag antibody (CWBIO) diluted 1:2000 with 10 mM PBS (pH 7.4) containing 1% BSA overnight at 4 °C. After washing in 10 mM PBS (pH 7.4), the membrane was incubated with horseradish peroxidase (HRP)-labeled goat anti-mouse IgG (CWBIO) diluted 1:5000 with 10 mM PBS (pH 7.4) containing 1% BSA at room temperature for 3 h. The bands were visualized using DAB kit (CWBIO) according to the manufacturer's instruction.