Molecular Cloning and Expression Analysis of Two Hepcidin Genes from Olive Flounder *Paralichthys olivaceus*

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Hepcidin is a cysteine-rich cationic antimicrobial peptide central to iron metabolism. We report a comparative analysis of the sequences, gene organization and expression of two hepcidin genes from olive flounder *Paralichthys olivaceus*. Both consist of two introns and three exons that encode a prepropeptide (81 amino acids for hepcidin I and 89 amino acids for hepcidin II). A TATA box and several consensus-binding motifs for transcription factors were found upstream of the transcriptional starting site. Hepcidin II was predominantly expressed in the liver and highly inducible under the effect of lipopolysaccharide (LPS), while a large amount of hepcidin I transcripts was detected in various tissues but did not appear to have a significant effect during LPS-stimulation.

Key words: hepcidin; antimicrobial peptide; *Paralichthys olivaceus*; gene expression

Antimicrobial peptides are a broadly distributed group of molecules that are important in host defense against microbial invasion. Most molecules are amphiphilic and contain both cationic and hydrophobic surfaces, enabling them to insert into biological membranes. Small cysteine-rich peptides exhibiting antimicrobial activity against various fungi and bacteria have recently been isolated from a blood ultrafiltrate, the human urinary tract, and the gill of bacterially challenged hybrid bass. These peptides, referred to as hepcidin, have been proposed to be the vertebrate counterpart of insect peptides induced in the fat body in response to infection. The hepcidin peptides share a distinctive cysteine bridge structure that is unique among other antimicrobial peptides. Besides its antimicrobial activity, recent reports argue in favor of the role of hepcidin in iron metabolism. The peptide sequences of additional hepcidins have been predicted from the expressed sequence tag databases of the liver of mouse, rat and various fish species including medaka, rainbow trout, Japanese flounder, winter flounder, and long-jawed mudsucker. In this study, we construct cDNA libraries from 8 tissues of olive flounder *Paralichthys olivaceus* distributed in Korea and analyzed 3,386 cDNA clones (data not shown). The expressed sequence tag analysis enabled us to find two distinct cDNA clones (HK319 and L275) whose amino acid sequences had significant similarity to hepcidin. The HK319 clone was derived from the head kidney cDNA library and named hepcidin I, and the L275 clone was from the liver cDNA library and named hepcidin II.

The 5′ region of the mRNA was determined by 5′ rapid amplification of the cDNA ends (RACE), and both cDNAs were completely sequenced. Hepcidin I cDNA (accession no. AY 533022) has 563 bases, excluding the polyA tail, and contains an open reading frame (ORF) of 267 bases with a coding capacity of 89 amino acids. Hepcidin II cDNA (accession no. AY533023) contains an ORF for the predicted polypeptide of 81 amino acids. The position of cleavage by the signal peptidase was predicted by PSORT, and the typical RX(K/R)R motif of propeptide convertases was identified. The signal peptide sequence was 24 residues in length, and the anionic prodomain was 38–39 amino acids, depending on the hepcidin variant. The amino acid sequences of the hepcidins were aligned with those present in dbEST, as well as with the recently reported hepcidin (Fig. 1). They were identical to those previously reported for Japanese flounder *P. olivaceus* in dbEST, with the exception of several amino acid changes in the pre-pro region. A stretch of eight cysteines, a significant feature of most mature hepcidins, was found with hepcidin I, whereas hepcidin II, Japanese flounder II and winter flounder II are missing two cysteine residues, indicating that a maximum of three disulfide bonds could be formed. The deduced amino acid sequences of the fish prepro-hepcidin-like peptides can be aligned with those from mammals throughout their length, but only showed...
Fig. 1. Amino Acid Sequence Alignment of Olive Flounder Hepcidin Peptides (Hepcins I and II) with Those of Japanese Flounder, Winter Flounder, Atlantic Salmon, Medaka, Bass, Human, Mouse, and Rat.

Conserved residues are highlighted, and the cysteine residues of the mature peptide are marked with asterisks. The predicted positions of signal peptidase and pre-protein cleavage are indicated by arrows. GenBank accession numbers are shown in parentheses. X is an uncompleted amino acid.

Fig. 2. Organization of Olive Flounder Hepcidin I (A) and Hepcidin II (B) Genes, mRNAs, and Upstream Regions.

Exons are depicted by boxes: unfilled boxes, untranslated regions; striped boxes, regions encoding a signal peptide; stippled boxes, regions encoding a prodomain; filled boxes, regions encoding a mature hepcidin peptide. GenBank accession numbers are shown in parentheses.
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High similarity in the portion corresponding to the processed peptides. However, within the fish, the signal peptide and prodomain were also very highly conserved.

To obtain a continuous genomic sequence, a long PCR product that included the hepcidin gene and promoter region was cloned and sequenced by the Universal Genome Walker kit (Clontech, Palo Alto, CA, U.S.A.) according to the manufacturer’s directions. The nucleotide sequences for the hepcidin I and II genes and their upstream regions were determined (accession nos. AY623817 and AY623818, respectively). The gene organization was similar in the two genes (Fig. 2). Both hepcidin genes consist of two introns and three exons. The first exon contains 5' UTR, the signal peptide, and part of the prodomain. The prodomain extends from exon 1 through to exon 3. Exon 3 also encodes the mature peptide and 3' UTR. Putative transcriptional binding sites were predicted by TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html). The 968-bp upstream sequence of the hepcidin I gene contains regulatory elements and putative binding motifs for several transcription factors. A sequence analysis revealed a TATA box 31 nucleotide upstream of the transcription starting site (nucleotide −31) and putative response elements for the three transcription factors, CAAT enhancer-binding protein C/EBPβ (nucleotides −750 and −571), hepatocyte nuclear factor HNF-3β (nucleotide −742) and NF-κB/c-Rel (nucleotide −384). The analysis of the 1532-bp upstream region of hepcidin II revealed one putative response element for the five transcription factors, C/EBPα and β (nucleotides −109 and −669), NF-κB/c-Rel (nucleotide −138) and HNF-4 and HNF-3β (nucleotides −197 and −249). However, we could not find the TATA box consensus sequence. The binding motifs for HNF, C/EBP, and NF-κB have also been described in the upstream region of the bass, human and mouse hepcidin genes. The transcription factors, C/EBPα and β, have been shown to activate both human and mouse hepcidin promoters. The NF-κB/Rel transcription factors are conserved from Drosophila to humans and play an important role in the Toll signaling pathway and host defense.

The tissue-specific expression of two hepcidin transcripts was assessed by RT-PCR. Products from the olive flounder were amplified by using gene-specific primers for types I and II hepcidin sequences (291 bp and 267 bp) and GAPDH (300 bp) (Fig. 3A). In the unstimulated olive flounder, the type I transcript was found at relatively high levels in the liver, spleen, pyloric caecae, head kidney, trunk kidney and gill, at low levels in the heart, brain and intestine, and at barely detectable levels in the stomach and rectum. The type II transcript was predominantly expressed in the liver and not detectable in the other tissues tested. The levels of hepcidin gene expression were assessed by semi-quantitative RT-PCR in three lipopolysaccharide (LPS)-stimulated and three phosphate buffered saline (PBS)-stimulated samples. Flounder mock-stimulated with PBS was used as the control ( ). The gel intensities were calculated relative to expression of GAPDH in the same tissues. The mean expression level ± standard deviation was calculated from three PBS-stimulated and three LPS-stimulated fish for each of the four tissues tested. A one-way ANOVA test was used for statistical analysis of the data generated. A P value below 0.05 was considered statistically significant.

Fig. 3. Determination of Olive Flounder Hepcidin Gene Expression by a Reverse Transcription-PCR Analysis.

(A) Hepcidins I and II and GAPDH gene expression were analyzed in different tissues of olive flounder by RT-PCR. The tissues assayed were heart (H), liver (L), stomach (ST), spleen (S), pyloric caecae (PC), intestine (I), rectum (R), head kidney (HK), trunk kidney (TK), gill (G) and brain (B). Primers were designed based on the two-hepcidin cDNA sequences. The forward and reverse primers for hepcidins I and II and the GAPDH gene were 5'-ATGAGGACTCAGCATTGCA-3' and 5'-TGTAGGTGTT-TGGGGAATCC-3', 5'-ATGAGAGTACAGTGTTGCA-3' and 5'-TTGGGATATACTGATATCCTC-3', and 5'-CCATGTTGAGTGTCATGACGG-3' and 5'-GAGCTCAGGGATGACCTTGCC-3' respectively. First strand cDNA was synthesized from 2 μg of total RNA by using the Omniscript RT kit (Qiagen, Hilden, Germany), and aliquots of the reaction products were subjected to PCR by using PCR Master (Roche Applied Science, Mannheim, Germany). The PCR conditions were an initial denaturation step of 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s, and a final extension step of 7 min at 72 °C. Amplified cDNA was resolved by electrophoresis on 2% agarose gel. (B) Olive flounder hepcidin gene expression following LPS-stimulation was determined by semi-quantitative RT-PCR. Three olive flounders (200 g) were anaesthetised by immersing in 50 mg l−1 TMS, injected intraperitoneally with 100 μg of Escherichia coli 0127:B8 LPS (Sigma), and allowed to recover in sea water. One day ( ) and three days ( ) post-injection, the expression of hepcidin-I and II genes in PBS- and LPS-stimulated flounders was determined by RT-PCR as just described. Flounder mock-stimulated with PBS was used as the control ( ). The gel intensities were calculated relative to expression of GAPDH in the same tissues. The mean expression level ± standard deviation was calculated from three PBS-stimulated and three LPS-stimulated fish for each of the four tissues tested. A one-way ANOVA test was used for statistical analysis of the data generated. A P value below 0.05 was considered statistically significant.
(PBS)-stimulated olive flounder samples (Fig. 3B). The mock-stimulated flounder with PBS was used as a control. Type I was constitutively expressed in the liver, head kidney, spleen and gill, and in vivo stimulation of the olive flounder with LPS did not appear to have any significant effect on the expression of hepcidin I in any of the tissues examined. On the other hand, one day post injection, the type II transcripts were noticeably up-regulated in the liver, head kidney, spleen and gill in the LPS-stimulated group (P < 0.05) compared to the control group, while the expression of the type II gene was less in each of the tissues after 3 days compared to the control. Type I was constitutively expressed in the liver, head kidney, spleen and gill in the control group, while the expression of the type II gene was less in each of the tissues after 3 days than after one day. Increased hepcidin gene expression has also been noted following LPS-challenge in mice, and bacterial challenge in white bass and Atlantic salmon.

Interestingly, hepcidins I and II were differently expressed in response to LPS-stimulation in various tissues of the olive flounder: hepcidin I was ubiquitously expressed, whereas hepcidin II was enhanced by one day post-injection of LPS. Overall, our data suggest that both hepcidins I and II genes are important factors in the host response to pathogens, but could exhibit different activities and/or play distinct roles. Moreover hepcidin, an important component of innate immunity, appears to be regulated through complex systems in response to diverse environmental stimuli.

References