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Characterizations of two grass carp *Ctenopharyngodon idella* HMGB2 genes and potential roles in innate immunity

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ABSTRACT

High-mobility group box 2 (HMGB2) protein is a chromatin-associated nonhistone protein, involved in transcriptional regulation and nucleic-acid-mediated innate immune responses in mammalian. However, the function of piscine HMGB2 in innate immune responses is still unknown. In the present study, two HMGB2 homologue genes (CiHMGB2a, CiHMGB2b) were identified and characterized in grass carp (*Ctenopharyngodon idella*). Both CiHMGB2a and CiHMGB2b genes encode proteins with 213 amino acids, sharing 71.4% identities and containing two basic HMG boxes and an acidic tail. The deduced protein sequences showed the most identities to HMGB2a (93%) and HMGB2b (86.4%) of zebrafish (*Danio rerio*), respectively. Quantitative real-time RT-PCR (qRT-PCR) analysis showed that CiHMGB2a and CiHMGB2b were constitutively expressed in all the 15 tested tissues. Post grass carp reovirus (GCRV) infection, mRNA levels of CiHMGB2a and CiHMGB2b were strongly up-regulated in spleen and head kidney and mildly modulated in *C. idella* kidney (CIK) cells. Meanwhile, mRNA expressions of CiHMGB2a and CiHMGB2b were significantly regulated by viral pathogen associated molecular patterns (PAMPs) polyinosinic–polycytidylic potassium salt (poly(I:C)) and bacterial PAMPs lipopolysaccharide (LPS), peptidoglycan (PGN) challenge in CIK cells. In CiHMGB2a and CiHMGB2b over-expression cells, expressions of CiHMGB2a and CiHMGB2b facilitated each other; transcription levels of CiTRIF, CiMyD88, CiIPS-1 and CiMx1 were remarkably enhanced, whereas CiIFN-I was inhibited, compared with those in cells transfected with pCMV (control plasmid); after GCRV challenge, all those tested genes were up-regulated with divergent expression profiles. Antiviral activities of CiHMGB2a and CiHMGB2b were manifested by the delayed appearance of cytopathic effect (CPE) and inhibition of GCRV yield. All those results demonstrate that CiHMGB2a and CiHMGB2b not only mediate antiviral immune responses but also involve in responding to viral/bacterial PAMPs challenge, which provides novel insights into the essential role of HMGB2 in innate immunity.

1. Introduction

High-mobility group (HMG) proteins are abundant nonhistone chromosomal proteins which participate in the processes of modifying and organizing nucleosomes into high-order structures along with core histones (Zhang and Wang, 2010). Three distinct families of HMG proteins are defined and named after the structure of their DNA binding domains as well as their substrate binding specificity, including HMG-AT-hook families (HMGA), HMG-box families (HMGB) and HMG-nucleosome binding families (HMGN) (Bustin, 2001; Gerlitz et al., 2009). Canonical HMGB proteins are only present in multicellular animals, from sponges onwards (Sessa and Bianchi, 2007). The organizations of HMGB gene are very conserved during metazoan evolution, and divergent in *Caenorhabditis* and *Drosophila* (Sessa and Bianchi, 2007). Three HMGB family members (HMGB1, 2, 3) are present in cartilaginous fish, bony fish and tetrapods, and two co-orthologs of each HMGB gene are present in some teleosts (Moleri et al., 2011). Mammalian HMGB proteins contain four subtypes: the ubiquitous HMGB1, the tissue-specific HMGB2, the embryo-specific HMGB3 and a novel member HMGB4. Among them, HMGB1, HMGB2, HMGB3 are characterized by a tripartite domain organization, consisting of two HMG boxes and a C-terminal acidic tail, but HMGB4 lacks the acidic tail (Thomas and Travers, 2001; Muller et al., 2004; Catena et al., 2009). In all animal HMGB genes, HMG box A clearly belongs to one specific subclass of HMG box gene segment (containing 1 intron), while the HMG box B belongs to a different subclass (containing 2 introns). Thus, the ancestral HMGB gene most likely arose in the first multicellular animal by the juxtaposition of two genes, each encoding a single box (Sessa and Bianchi, 2007).

HMGBs function in a number of foundational cellular processes such as transcriptional regulation, DNA repair, recombination, differentiation and extracellular signaling as well as nucleosome

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remodeling (Stros, 2010; Ueda and Yoshida, 2010). Recent reports show that HMGB proteins (HMGB1, 2, 3) function as a ligand that can evoke inflammatory response, and as a sensor for nucleic-acid-mediated immune responses (Yanai et al., 2012). Particularly, HMGBs represent universal sentinels of immunogenic nucleic acids which are first recognized promiscuously and then subsequently delivered to more discriminative pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and other cytosolic receptors (Yanai et al., 2009; Yanai et al., 2011a; Bianchi and Celona, 2010). Signaling through TLRs can be divided into two pathways: namely the myeloid differentiation factor 88 (MyD88)-dependent pathway and the TIR-domain-containing adaptor inducing interferon-β (IFN-β) (TRIF)-dependent pathway (Kumar et al., 2009; Kim et al., 2013). While IFN-β promoter stimulator 1 (IPS-1, also known as MAVS/VISA) can pass the immune signals from RLRs to trigger a signal for IFN-I production (Taniguchi et al., 2011; Rudd et al., 2012). TLRs and RLRs discriminatively interact with TRIF, MyD88 and IPS-1 to phosphorylate IFN regulatory factor 3/7 (IRF3/7) or nuclear factor-κB (NF-κB), leading to synthesis of IFN-I and cytokines to respond to viral RNA pathogen associated molecular patterns (PAMPs) (Zou et al., 2010). Myxovirus-resistant (Mx) protein is a key component of the antiviral state induced by IFN-I in the response of viral infections. The Mx genes have shown antiviral activity in numerous fish species, such as gilthead seabream (*Sparus aurata*) (Fernandez-Trujillo et al., 2011), grass carp (*Ctenopharyngodon idella*) (Peng et al., 2012), and rare minnow (*Gobiocypris rarus*) (Su et al., 2009a). However, HMGBs can also strongly suppress the activation of innate immune responses through binding with nonimmunogenic nucleotides (Yanai et al., 2011b). In any case, promiscuous sensing of nucleic acids by HMGBs appears to be a more primitive feature of sensing 'non-self', which is crucial for subsequent pattern recognition by PRRs (Yanai et al., 2011a).

The innate immune system is a fundamental defense mechanism of fish and plays an instructive role in the acquired immune response and homeostasis (Su et al., 2012). In teleosts, essential roles of PRRs and their downstream genes are largely investigated in antiviral immune responses (Blasius and Beutler, 2010; Zou et al., 2010; Zhang and Gui, 2012). However, whether fish HMGBs possess similar functions with those of mammals in innate immune responses is still limited. So far, *Sciaenops ocellatus* HMGB1 can function as secreted cytokine in the event of bacterial infection and promote innate defense through the activation of macrophages (Zhao et al., 2011). In grass carp, two co-orthologs of HMGB1 have been identified and involved in mediating immune responses to viral/bacterial PAMPs and grass carp reovirus (GCRV) challenge (Yang et al., 2013). But no evidence confirms the functional properties of piscine HMGB2 in innate immune responses. So it is worth noting the potential role of fish HMGB2 in immune defense to invading pathogens.

In the present study, two HMGB2 homologue genes (CiHMGB2a, CiHMGB2b) were identified and characterized from grass carp which is an important economic aquaculture species in China, but GCRV, a double strand RNA (dsRNA) virus, causes severe hemorrhagic disease in juvenile grass carp (Su et al., 2009b). Meanwhile, the expression profiles of CiHMGB2a and CiHMGB2b were examined *in vivo* and *in vitro*. Two over-expression vectors (pHMGB2a, pHMGB2b) were constructed and transfected into *C. idella* kidney (CIK) cells. Expression profiles of CiHMGB2a and CiHMGB2b and the representative genes of TLRs and RLRs pathways: *C. idella* TRIF (CiTRIF), *C. idella* IPS-1 (CiIPS-1), *C. idella* MyD88 (CiMyD88), *C. idella* IFN-I (CiIFN-I) and *C. idella* Mx1 (CiMx1) in CiHMGB2a and CiHMGB2b over-expression cells were examined post GCRV infection. Besides, antiviral activity assay and virus detection were also conducted in CiHMGB2a and CiHMGB2b over-expression cells. Better understanding of the antiviral immune defense mechanisms of grass carp may contribute to the development of management strategies for disease control and long term sustainability of grass carp farming. This was the first explored the potential roles of HMGB2 in innate immune responses in teleost.

2. Materials and methods

2.1. Construction of cDNA library and EST analysis

A cDNA library was constructed with CIK cells post GCRV infected, using the Creator SMART cDNA Library Construction Kit (Clontech, USA). Random sequencing of the library using T7 primer yielded 10,228 successful sequencing reactions. BLAST analysis of all the expressed sequence tags (ESTs) revealed that one EST contig of 558 bp was homologous to the HMGB2a in zebrafish (*Danio rerio*) (GenBank accession No. NM_001037424) and another EST contig of 497 bp was highly similar to the HMGB2b in zebrafish (GenBank accession No. NM_001004674). According to the homology, they were designed as CiHMGB2a and CiHMGB2b, respectively. These EST sequences were then picked up for further cloning of the full-length cDNAs from grass carp.

2.2. Cloning of CiHMGB2a and CiHMGB2b genes

After analyzing the two EST contigs, 5′ ends of CiHMGB2a and CiHMGB2b were integral. The 3′ ends were obtained by 3′ RACE method, which was performed using the BD SMART™ RACE cDNA amplification kit (Clontech). RACE was performed on spleen-derived RNA. To get the 3′ ends of CiHMGB2a, primer pairs, HF629/adaptor primer UPM and HF630/adaptor primer NUP (Table 1), were employed for the primary PCR and the nested PCR respectively. For 3′ end of CiHMGB2b, the gene specific primers HF631 and HF632 (Table 1) were used for the primary and nested PCR respectively. The full length cDNA sequences were verified by sequencing the PCR products amplified by primers H2AF696 and H2AR697 for CiHMGB2a and primers H2BF698 and H2BR699 (Table 1) for CiHMGB2b, within the predicted 5′ and 3′ untranslated region (UTRs) respectively. The PCR products were purified and ligated into pMD18-T vector (TaKaRa, Japan), then transformed into competent *Escherichia coli* TOP10 cells, and plated on the LB-agar petri-dish. Positive clones containing expected size inserts were screened by colony PCR. Three of them were picked up and sent to Genscript Biotechnology Limited Corporation (Nanjing, China) for sequencing.

2.3. Sequence analysis

The searches for nucleotide and protein sequence similarities were performed with BLAST program (http://www.ncbi.nlm.nih.gov/blast) and Matrix Global Alignment Tool (MatGAT) (http://bitincka.com/ledion/matgat/). The deduced amino acid sequence was analyzed with Expert Protein Analysis System (http://www.expasy.org). The translation was showed by Sequence Manipulation Suite (http://www.bioinformatics.org/sms/). Protein domains were predicted using Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de) and the ScanProsite tool (http://us.expasy.org/tools/scanprosite). Multiple sequence alignments were created using the ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html) program and Multiple Alignment display program (http://www.bioinformatics.org/sms/).

2.4. Animal, viral challenge and sample collection

Grass carp (average weight 15–20 g) were collected from a fish farm in Shaanxi Province, China and acclimatized to laboratory