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Comparative study of two immunity-related GTPase genes in Chinese soft-shell turtle reveals their molecular characteristics and functional activity in immune defense

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ABSTRACT

The immunity-related GTPases (IRGs) are a family of proteins that play critical roles in innate resistance to intracellular pathogens. The number and diversity of IRG genes differ greatly in different species. Although IRG proteins have been well studies in mammals, they remain poorly characterized in lower vertebrates. In this study, we cloned two IRG genes, *PsIRG5* and *PsIRG8*, from the Chinese soft-shelled turtle and compared their characterization and functional activity with mammalian IRGs. The *PsIRG5* is a gene of 1896 bp that encodes a protein of 413 amino acid and *PsIRG8* is 1543 bp in length encoding another 413 aa protein. Sequence alignment between all turtle IRG-like genes and mammalian IRGs showed that both *PsIRG5* and *PsIRG8* were conserved with mammalian GKS IRGs, while *PsIRG5* appeared a closer evolutionary relationship with mammalian GMS IRGs. The expression and subcellular characterization revealed that *PsIRG5* was dramatically upregulated under *Aeromonas hydrophila* challenge and exhibited co-localization with lysosomes in cells; whereas *PsIRG8* was downregulated and has no distinct localization. Functional activity assay demonstrated that *Ps*IRG5 plays a role in autophagy induction and IFN-γ contributes to enhance the induction, since it has IFN-inducible elements in its promoter region. These data above unravel the molecular characterization and functional activity of IRGs in lower vertebrate for the first time and will provide insights into the comparative immunity and evolutionary relationships of IRGs between mammals and reptiles.

1. Introduction

Interferons (IFNs) are the most important components in innate immunity since they trigger the protective defenses against pathogens by inducing the expression of various antimicrobial effector proteins and then further activating pathways of cell autonomous immunity of host cells (Taylor, 2007). The IFN-inducible proteins enhance pathogen detection and work collaboratively to circumvent intracellular replication and survival of various microbial pathogens (Schneider et al., 2014). The IFN-inducible GTPases are key members among these effector proteins. Based on paralogy and molecular mass, four subfamilies are classified within the IFN-inducible GTPases family: the 21–47kDa immunity-related GTPases (IRGs), the 65kDa guanylate binding proteins (GBPs), the 72–82kDa Myxoma Proteins (Mx) and the 200–285kDa very large inducible GTPases (VLIGs/GVINs) (Kim et al., 2012; Meunier and Broz, 2016). Although several biochemical properties are conserved among the four subfamilies IFN-inducible GTPases, their defined roles in mediating innate resistance are most often distinct.

IRGs were first reported in 1990s as genes dramatically induced by infection via interferon-gamma (IFN-γ) in mice (Hunn et al., 2011). It is now well-established that IRGs play key roles in resistance against a wide variety of intracellular pathogens, such as *Toxoplasma gondii*, *Chlamydia trachomatis*, *Salmonella typhimurium*, *Listeria monocytogenes* (Coers et al., 2008; Collazo et al., 2001; Henry et al., 2007, 2009; Hunn et al., 2011). IRGs promote phagosome maturation and autophagy to destruct pathogen-containing vacuoles (PCVs) or induct host cell necrosis in immunity against intracellular pathogens (Gutierrez et al., 2004; MacMicking et al.,

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2003; Martens et al., 2005; Zhao et al., 2009). IRGs are subdivided into GKS IRGs and GMS IRGs according to the sequence of first nucleotide binding motif (G1) in the N-terminal GTPase domain (Hunn et al., 2011; Meunier and Broz, 2016). Although IRG proteins have been well studies on structure and function in mammalian species, they remain poorly characterized in other vertebrates. Previously, comparative evolutionary analysis revealed that IRG genes was presented in fish and some invertebrates, suggesting they likely play conserved immune functions (Bekpen et al., 2005; Li et al., 2009). In the recently released vertebrate genomes including the Chinese soft-shell turtle (*Pelodiscus sinensis*) genome, we also found many IRG homologous sequences by genome mining.

The Chinese soft-shelled turtle, regarded as health-promoting food with high nutritional and medicinal values, is a commercially important cultured species in Asian countries such as China, Japan and Korea. However, the species is vulnerable to suffer from a variety of pathogens infection including *Aeromonas* spp., *Paecilomyces lilacinus*, *Bacillus thuringiensis* and iridovirus in China turtle aquaculture (Chen et al., 1999; Zhou et al., 1999; Li et al., 2008; Chen et al., 2013, 2014). Up to date, many efforts have been devoted to identify immune relevant genes in Chinese soft-shelled turtle, such as immunoglobulin (Xu et al., 2009), Interleukin-8 (Zhou et al., 2009), IFN-γ (Fu et al., 2014), Interleukin-1β (Liang et al., 2016) and Toll-like receptor 4 (Zhou et al., 2016), however, relatively less is known for the immunity of turtles or immune responses upon infection. In addition, the Chinese soft-shelled turtle is an ectothermic amniotic reptile and represents the evolutionary lineage of vertebrate (Zimmerman et al., 2010). From evolutionary perspective, a greater understanding of Chinese soft-shell turtle immunity will provide important insights into the evolutionary history of vertebrate immunity. To these ends, the spleen transcriptome of Chinese soft-shell turtle after challenged by *Aeromonas hydrophila* was characterized in our lab, and plenty of immune relevant genes involved in the anti-bacteria response were found including some IRG-like homologs.

In this study, we described the molecular characterization and expression patterns of two IRG genes, *PsIRG5* and *PsIRG8*, from Chinese soft-shell turtle. We also characterized their subcellular localization in fish cells by *in vitro* expression. Importantly, we demonstrated functional activity of PsIRG5 in autophagy induction. Our data give the functional activity of IRGs in lower vertebrate for the first time and will provide insights into the comparative immunity and evolutionary relationships of IRGs between mammals and reptiles.

2. Materials and methods

2.1. Turtle management and bacterial challenge

The Chinese soft-shell turtles with an average weight of 150 ± 20 g were obtained from a turtle farm in Guangdong Province of China. The turtles were maintained at 28 °C in the laboratory for two weeks before experiments. Then turtles were randomly divided into two groups: one group (the experimental group) was intraperitoneally injected with 500μl *A. hydrophila* (3×10^8CFU/ml) and the other group (the control group) injected with 500μl phosphate buffered saline (PBS). At 24, 48 and 72 h post-injection, the tissues were collected from the two groups ($n > 3$). The collected samples were flash-frozen in liquid nitrogen and then stored at −80 °C until RNA extraction.

2.2. RNA extraction, reverse transcription and cloning of turtle IRGs sequence

Total RNA was extracted from the different tissues with the SV Total RNA isolation System Kit (Promega) according to the manufacturer's instruction. The first-strand cDNA was synthesized from the total RNA using random primers and the M-MLV reverse transcriptase Kit (Promega). To clone the full length of turtle IRGs cDNA sequence, primers were designed based on the obtained EST sequences of IRGs (Table 1), and 5′ and 3′-RACE PCR were performed using the Chinese soft-shell turtle cDNA library. All amplified DNA fragments were ligated into pMD18-T (Takara) for cloning and sequencing.

2.3. Bioinformatics analysis of turtle IRGs

All the other IRG-like genes were obtained from the genome database of Chinese soft-shell turtle at the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/). For multiple sequence alignments, amino acid sequences of all turtle IRGs and human IRGs were aligned using ClustalX 1.83 program (Thompson et al., 1997) and refined with the GeneDoc software. Based on the DNA sequences of turtle IRGs, several transcription factor binding sites including IFN-γ activated site GAS ($TTCN_{2\text{-}4}GAA$), IFN-stimulated response elements ISRE (NNTTTCNNTTTC) and NF-κB binding sites (NGGNNNTTCC) were screened in putative promoter regions (2 kb upstream of putative transcription start point) (Olszewski et al., 2006; Li et al., 2009). The

Table 1
Primers used in this study.

Primers	Sequence (5′–3′)	Usages
PsIRG5-F1	GCTCTCTTGCAGGTGCCATGG	Sequence cloning
PsIRG5-R1	GACCATCTCCCGACGGAACG	
PsIRG5-F2	TCGGGAGGATGGGCAAGAGG	
PsIRG5-R2	CACGAACACGTCTGCTGATCACTG	
PsIRG5-F3	CCAGTGCTCCCTGTCGCTTG	
PsIRG5-R3	TTGTGATGCCCGCAGTATTTG	
PsIRG8-F1	TTCAGACTCTGCCAGAACTCAG	
PsIRG8-R1	GATTTCAGGGCTTGTTTCCA	
PsIRG8-F2	TGCCCAGTACCTGTGAACC	
PsIRG8-R2	TGGAGCCTGAGCAAGCAAC	
5RACE-F	AAGCAGTGGTATCAACGCAGAGT	
3RACE-R	AGAGGCCGAGGCGGCCGACATG	
PsIRG5-N3F	ATACTCGAGATGGATCTGGGGTCCATC	Plasmid construction
PsIRG5-N3R	GCTGAATTCTGAAGCTTTTTCCTGCC	
PsIRG5-cDF	CGCGAATTCATGGATCTGGGGTCCATC	
PsIRG5-cDR	TGCCTCGAGCTGAAGCTTTTTCCTGCC	
PsIRG8-N3F	CTCGAGGGATGGCGGAAAATACAAA	
PsIRG8-N3R	GGATCCTTGGTTGTTCTCTGCTCCTTC	
PsIRG8-cDF	GGATCCGGATGGCGGAAAATACAAA	
PsIRG8-cDR	CTCGAGTTGGTTGTTCTCTGCTCCTTC	
GAPDH-F	GGATACACGGAGGACCAG	
GAPDH-R	ATACCAGGAGACCAGTTTGA	
PsIRG5-RTF	TGGCTTCTATCACACAGG	Real-time PCR
PsIRG5-RTR	CTGCTTTGTCGTTTCTGTC	
PsIRG8-RTF	TTCCCTCACTTCACTTGCT	
PsIRG8-RTR	GCTACCATCCCTTCTACCC	
LOC6549-F	GAAGTCCTCCCTCATCAAC	
LOC6549-R	GATAAGCCATTGTCTCCGT	
LOC6797-F	CAGCTACGACTTCTTCATCAT	
LOC6797-R	GGAGCGCACATAATAAAACT	
LOC7218-F	TCTGTCATCCAAAAGTCTCC	
LOC7218-R	CCATCAGGCTTCCAACTAT	
LOC8763-F	CATTGCTGCTGTCCCTAT	
LOC8763-R	AAAGTCCTGGCGGTAGTT	
LOC9006-F	TTGTGGCAGAATGTATGAGA	
LOC9006-R	TATGTTATTGTTGAGGCGAGT	
LOC1137-F	AACTGGCCTTTCCCTCTT	
LOC1137-R	ATGTCCCCACTGCTCTGT	
LOC1863-F	GACACCTACCTGGAGCAG	
LOC1863-R	GGAGCGGACAAAGTAGAA	
LOC6314-F	CTGTAATTTGGGGTGTGTTT	
LOC6314-R	TTTCCTCAGTCTCCTTCTCA	