Molecular cloning and functional characterization of peptidoglycan recognition protein 6 in grass carp *Ctenopharyngodon idella*

Jun Hua Li a,b, Zhang Long Yu a,b, Na Na Xue a,b, Peng Fei Zou a,b, Jing Yu Hu a, P. Nie a, Ming Xian Chang a,*

a State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, Hubei Province 430072, China

b Graduate University of Chinese Academy of Sciences, Beijing 100039, China

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A B S T R A C T

Peptidoglycan recognition proteins (PGRPs) are pattern recognition molecules of innate immunity. In this study, a long-form PGRP, designated as gcPGRP6, was identified from grass carp *Ctenopharyngodon idella*. The deduced amino acid sequence of gcPGRP6 is composed of 464 residues with a conserved PGRP domain at the C-terminus. The gcPGRP6 gene consists of four exons and three introns, spanning approximately 2.7 kb of genomic sequence. Phylogenetic analysis demonstrated that gcPGRP6 is clustered closely with zebrafish PGLYRP6, and formed a long-type PGRP subfamily together with PGLYRP2 members identified in teleosts and mammals. Real-time PCR and Western blotting analyses revealed that gcPGRP6 is constitutively expressed in organs/tissues examined, and its expression was significantly induced in liver and intestine of grass carp in response to PGN stimulation and in CIK cells treated with lipoteichoic acid (LTA), polyinosinic polycytidylic acid (Poly I:C) and peptidoglycan (PGN). Immunofluorescence microscopy and copy and Western blotting analyses revealed that gcPGRP6 is effectively secreted to the exterior of CIK cells. The over-expression of gcPGRP6 in CIK cells leads to the activation of NF-κB and the inhibition of intracellular bacterial growth. Moreover, cell lysates from CIK cells transfected with pTurbo-gcPGRP6-GFP plasmid display the binding activity towards Lys-type PGN from *Staphylococcus aureus* and DAP-type PGN from *Bacillus subtilis*. Furthermore, proinflammatory cytokine IL-2 and intracellular PGN receptor NOD2 had a significantly increased expression in CIK cells overexpressed with gcPGRP6. It is demonstrated that the PGRP6 in grass carp has a role in binding PGN, in inhibiting the growth of intracellular bacteria, and in activating NF-κB, as well as in regulating innate immune genes.

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1. Introduction

The immune system has traditionally been divided into innate and adaptive components. Innate immunity is the first line of defense in vertebrates and the only defense in invertebrates and plants (Medzhitov and Janeway, 1997; Janeway and Medzhitov, 2002). The components of innate immunity system that discriminate between microorganisms and self are able to recognize highly conserved structures found only in microorganisms but not in higher eukaryotes. These structures are referred to as pathogen-associated molecular patterns (PAMPs), and the receptors recognizing these structures in innate immune system are called pattern recognition receptors (PRRs) (Medzhitov and Janeway, 1997; Janeway and Medzhitov, 2002). Peptidoglycan recognition proteins (PGRPs) belong to a family of PRRs that were first identified in insects and then in mammals such as *Homo sapiens* and *Mus musculus* (Yoshida et al., 1996; Kang et al., 1998; Liu et al., 2001; Dziarski and Gupta 2006). Despite the structural similarities, PGRPs in invertebrate and vertebrate differ in their distributions in tissues/ organs, and in functions. Insect PGRPs are expressed mainly in immune competent sites such as fat body, gut and haemocytes (Werner et al., 2000). The activation of insect PGRPs by Gram-positive or Gram-negative bacteria may trigger Toll (Gobert et al., 2003) and immune deficiency (Imd) signal transduction pathways (Takehana et al., 2002) and the prophenol-oxidase cascade (Takehana et al., 2002), which results in the production of antimicrobial effectors. In addition, some insect PGRPs such as *Drosophila* PGRP-SC1 and PGRP-LB are N-acetylmuramoyl-L-alanine amidases (Kim et al., 2003; Mellroth et al., 2003), which can hydrolyze proinflammatory peptidoglycans. One insect PGRP, *Drosophila* PGRP-SA, which is not an amidase, has an L,D-carboxypeptidase activity for dianomopelic acid-type tetrapeptide peptidoglycan fragments (Chang et al., 2004). Mammals have four PGRPs, namely PGLYRP1, 2, 3, 4. PGLYRP1 is found mainly in granules of polymorphonuclear leukocytes; PGLYRP2 is expressed mainly in liver, and PGLYRP3 and PGLYRP4 are selectively expressed in skin, eyes, salivary glands, tongue, throat, esophagus, stomach and intestine (Kashyap et al., 2011). Initially, mammalian PGRPs were reported to have two
in multiple intracellular signaling pathways, such as TLR signaling, development and apoptosis signalings have also a role in mediating multiple intracellular signaling pathways. Different from insect and mammalian homologues, zebrafish PGRPs have both amidase activity and broad-spectrum bactericidal activity (Li et al., 2007). In addition, zebrafish PGRPs have also a role in mediating multiple intracellular signaling pathways, such as TLR signaling, development and apoptosis signalings (Chang and Nie, 2008; Chang et al., 2009; Park et al., 2011; Zenhom et al., 2011).

Grass carp, *Ctenopharyngodon idella*, one of the most popular and economically important species in aquaculture industry of China, has received increasing attention in relation to its immune system (Chen et al., 2012, 2010a, b, c; Xu et al., 2010; Xiao et al., 2010), large yellow croaker (Mao et al., 2010) and red drum (Li et al., 2007), pufferfish (Chang et al., 2007), rockfish (Kim et al., 2003; Dziarski and Gupta, 2006). Recently, it has been suggested that mammalian PGRPs have a role in modulating inflammation and immune responses (Saha et al., 2009, 2010; Park et al., 2011).

Grass carp kidney cell line (CIK cells) was maintained in M199 medium (Gibco) with the supplementation of 10% fetal bovine serum (FBS) (Sigma), 100 U/ml penicillin (P) and 100 μg/ml streptomycin (S) at 28 °C. *E. tarda*, an intracellular bacterial pathogen in aquaculture, was grown in tryptic soy broth (TSB, BD Biosciences) at 28 °C. After the spectrophotometrical determination of their number at absorbance of 540 nm, the bacteria were washed twice with TSB before being used for infection at a multiplicity of infection (MOI) as indicated in related figure legends. PAMPs used in the present study were peptidoglycan (PGN, catalog No. 53243) from *Micrococcus luteus*, lipoteichoic acid (LTA, No. L2515) from *S. aureus*, and polyinosinic polycytidylic acid (Poly I:C, No. P9528), which were all purchased from Sigma–Aldrich.

### 2. Materials and methods

#### 2.1. Cell line, bacterium and PAMPs

Grass carp (*C. idella*) kidney cell line (CIK cells) was maintained in M199 medium (Gibco) with the supplementation of 10% fetal bovine serum (FBS) (Sigma), 100 U/ml penicillin (P) and 100 μg/ml streptomycin (S) at 28 °C. *E. tarda*, an intracellular bacterial pathogen in aquaculture, was grown in tryptic soy broth (TSB, BD Biosciences) at 28 °C. After the spectrophotometrical determination of their number at absorbance of 540 nm, the bacteria were washed twice with TSB before being used for infection at a multiplicity of infection (MOI) as indicated in related figure legends. PAMPs used in the present study were peptidoglycan (PGN, catalog No. 53243) from *Micrococcus luteus*, lipoteichoic acid (LTA, No. L2515) from *S. aureus*, and polyinosinic polycytidylic acid (Poly I:C, No. P9528), which were all purchased from Sigma–Aldrich.

#### 2.2. Cloning of cDNA and genomic sequences of gcPGRP6

Grass carp fingerlings with the body weight among 50–80 g were collected from the Guanqiao Experimental Base of the Institute of Hydrobiology, Chinese Academy of Sciences in Wuhan, China. Following the manufacturer's instruction, the total RNA was extracted from spleen and intestine from a healthy grass carp using TriZol reagent (Invitrogen, USA), and cDNA was synthesized and amplified using a RevertAid™ first strand cDNA synthesis kit (MBI Fermentas, Germany). The primers PGRP6F and PGRP6R, designed on the basis of conserved sequences of PGRP genes from other vertebrates, were used to clone the internal region of gcPGRP6 gene, and 3′ and 5′ RACE were performed using gcPGRP6 gene, and 3′ and 5′ RACE were performed using

### Table 1

<table>
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<th>Primer</th>
<th>Sequence (5′ to 3′)</th>
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gene-specific primers and adaptor primers (UPM) for cloning the full cDNA sequence (Table 1). The specific primers for 3’ RACE were PGRP6Fout and PGRP6Fin, whilst for 5’ RACE PGRP6Rout and PGRP6Rin were used.

Genomic DNA was obtained from the tail fin of a healthy fish using salting-out method, as reported by Aljanabi and Martinez (1997). According to the full cDNA sequence, primers gPGRP6F/ gPGRP6R were designed and full genomic sequence was cloned. All primers used in the present study are listed in Table 1.

2.3. Sequence and phylogenetic analyses

Homologous sequences in the GenBank database were identified using BLAST program, with alignments performed using the ClustalW program (version 1.83). Prediction of theoretical signal peptide was conducted by using SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/), and identification of possible N-glycosylation sites was achieved by using NetNGlyc 1.0 program (http://www.cbs.dtu.dk/services/NetNGlyc/). Pfam protein family and physico-chemical parameters were analyzed through the Pfam HMM search program (http://pfam.janelia.org/) and the ProtParam program (http://www.expasy.ch/tools/protparam.html), respectively. Transmembrane domains were predicted with TMpred program. Construction of phylogenetic tree was performed using the neighbor-joining (N) algorithm within MEGA Version 4.0 on the basis of deduced amino acid sequences, with the reliability assessed by 1000 bootstrap repetitions.

2.4. RNA extraction, cDNA synthesis and quantitative RT-PCR

Constitutive expression of gPGRP6 was examined in head kidney, skin, gill, eye, liver, spleen, trunk kidney, intestine from 3 individual fish. In order to examine the inductive expression of gPGRP6 in tissues following the stimulation of PGN, two groups of fish were injected individually with 200 μl, 1 mg/ml PGN or 200 μl PBS, respectively, and intestine and liver were collected separately from three fish at each sampling occasion, i.e., 0, 3, 9, 12, 15, 18 and 24 h post injection (hpi). The expression of gPGRP6 was further examined in CIK cells following the stimulation of different ligands, and 1 x 10⁶ CIK cells per well were seeded into six-well plates, and stimulated for 6 h with 5 μg/ml LTA, 50 μg/ml Poly l:C and 50 μg/ml PGN.

The extraction of total RNA was achieved by using TRIzol reagent (Invitrogen) according to the manufacturer’s description. Samples were then treated with RNase-free DNase, and 2 μg RNA from different organs/tissues was reverse-transcribed with a RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) at 42 °C with oligo (dT)₁₈ primer. Forward and reverse primers for gPGRP6 and β-actin were designed by using Primer premier 5.0. The amplification efficiency of all primers was determined by standard curve, and the primers with amplification efficiency ranging from 0.95 and 1.0 were used in the present study (Table 1) on a CFX96 thermal cycler (Bio-Rad, USA). In a 96-well plate, each sample was run in triplicate along with the internal control gene. The relative expression level of gPGRP6 was normalized to the expression of β-actin, and calculated by using the 2⁻ΔΔCT method (Livak and Schmittgen, 2001). Data were presented as gene expression level relative to β-actin or fold change relative to the corresponding control group. Statistical analysis of Student’s t-test was carried out to examine the significant difference at P < 0.05.

2.5. Construction of expressing vectors for the production of gPGRP6 fusion and non-fusion proteins

Primers GFPgPGRP6F and GFPgPGRP6R (Table 1) were used to amplify the gPGRP6 ORF for constructing of the plasmid expressing of gPGRP6-GFP fusion protein. The resultant PCR fragment was digested with the restriction enzymes (Sac I and Bam HI) and inserted into the pTurboGFP-N vector (Evrogen, catalog No. #PF512). In addition, the gPGRP6 ORF was amplified using primers gPPgPGRP6/p1gPGRP6R (Table 1) by PCR and inserted into the Sac I and Bam HI sites of pGFP1 vector which contained two identical CMV promoters and SV40 3'UTRs to direct the expression of target gene and GFP gene independently in transfected cells (Chang et al., 2011).

2.6. Production of anti-serum against gPGRP6 and Western blotting analysis

pQE30 was used to express recombinant gPGRP6. The coding region of gPGRP6 gene was amplified by PCR using primer pair pQEPGRP6F/pQEPGRP6R (Table 1) for being inserted into the Bam HI and Hind III sites of pQE30 expression vector and expressed in Escherichia coli M15. The expression of recombinant gPGRP6 was induced in culture with IPTG, and the protein was purified by nickel resin chromatography for immunizing rabbit under denaturing conditions. The anti-serum against gPGRP6 was produced as previously described (Li et al., 2013). The specificity of anti-gPCRP6 antibody was evaluated by Western blotting using overexpressed gPGRP6-GFP fusion protein.

For identifying the protein expression, head kidney, skin, gills, eye, liver, spleen, trunk kidney and intestine of grass carp were washed with PBS, and then homogenized with RIPA Lysis and Extraction Buffer (Thermo Scientific, USA) by standard procedures. Western blotting was performed using SuperSignal West Pico Trial kit (Thermo, USA) and ECL Western blot system (LAS-4000mini, Fuji, Japan) according to the manufacturer’s instructions.

2.7. Cellular localization of gPGRP6

Transient transfection was achieved using LipofectAMINE 2000 transfection reagent (Invitrogen) according to the manufacturer’s protocol. The transfection efficiencies for pTurboGFP or pGFP1 vector were about 8–12% monitored using flow cytometer. For exogenous localization, 2 x 10⁶ CIK cells were transfected with 4 μg plasmid DNA including pTurboGFP and pTurbo-gPCRP6-GFP. 36 h later, cells were stained for 5 min with Hoechst 33342 at a concentration of 4 μg/ml, before being observed under a fluorescent microscope (Zeiss) with an ultraviolet (UV) excitation filter. For endogenous localization of gPGRP6, 2 x 10⁶ CIK cells were seeded per well in a six-well plate for one night, and immunofluorescence staining was proceeded using rabbit anti-gPCRP6 antibody (1:500, v/v) according to the methods previously reported by Li et al. (2013).

In order to further confirm the distribution of gPGRP6, CIK cells transfected with pTurbo-gPCRP6-GFP were used to extract protein by using RIPA lysis and extraction buffer plus protease inhibitor cocktail (Thermo Scientific, 89900). As no fluorescence was observed in CIK cells transfected with pTurbo-gPCRP6-GFP, the medium from CIK cells transfected with pTurbo-gPCRP6-GFP were concentrated by ultrafiltration and used for Western blotting analysis. The primary anti-pTurboGFP antibody (Evrogen, catalog No. #AB513) was diluted at 1:5000. The bands were detected using a SuperSignal West Pico Trial kit and ECL Western blot system, and quantified with the Quantity One software.
2.8. Transient transfection and luciferase reporter assays

Activation of the NF-κB pathway was measured using the luciferase reporter assay, as described previously (Chang et al., 2009). CIK cells were transfected with 100 ng NF-κB luciferase plasmid, 10 ng pRL-TK vector as well as 100 ng non-fusion gcPGRP6 plasmids. The pTGFp1 vector was used as control. 24 h later, the cells were lysed and the luciferase activity was measured. The means and standard deviations (SD) were calculated from triplicate samples. Statistical analysis was conducted using Student’s t-test with the significance of \( P < 0.05 \).

2.9. In vitro invasion and proliferation assay in CIK cells infected with E. tarda

Invasion and proliferation assay was performed as described in Kibardin et al. (2006) with minor modification. \( 1 \times 10^6 \) CIK cells were electroporated with 2 μg pTGFp1 or pTGFp1-gcPGRP6 using an AMAXA Nucleofector II transfection system under programme T20. The cells were infected with E. tarda at the multiplicity of infection (MOI) of 10 for 90 min. After the cells were washed for 3 times, fresh M199 medium containing 100 μg/ml gentamicin was added to each well to kill extracellular bacteria. The number of bacteria that successfully entered the cells was assessed after a 1 h incubation with gentamicin by plating the serial dilutions of cell lysates obtained after 20 min incubation with 500 μl PBS containing 1% (v/v) Triton X-100. The invasion coefficient was calculated as the ratio of the number of entered bacteria vs the number of bacteria applied. To assess intracellular proliferation, the incubation with gentamicin was prolonged to 6 h, with the proliferation index calculated as the ratio of the number of CFU at 6 h incubation relative to the number of entered bacteria. The experiments were performed in triplicate. Data were presented as mean ± SD, and statistical difference was determined using Student’s t-test with \( P < 0.05 \).

2.10. PGN binding assay

Insoluble L-type PGN from S. aureus (No. 77140) and DAP-type PGN from B. subtilis (No. 69554) were obtained from Sigma–Aldrich. The PGN binding assay was performed according to the procedure of Sang et al. (2005) with minor modification. The over-expressed pTurbo-gcPGRP6-GFP or pTurboGFP (50 μg) were incubated with 20 μg insoluble L-type PGN or DAP-type PGN. After 3 h rocking incubation at 4 °C, bound and unbound proteins were separated by centrifugation. After washing 4 times, bound proteins were recovered from PGN by boiling in 2 x SDS–polyacrylamide gel electrophoresis (SDS–PAGE) loading buffer, prior to 12% SDS–PAGE. Proteins were examined by Western blotting analysis using anti-pTurboGFP antibody (Evrogen).

2.11. Induction of gcPGRP6 on the expression of innate immune genes in CIK cells

Quantitative RT-PCR (QRT-PCR) was carried out to examine the expression of innate immune genes in CIK cells transfected with pTGFp1-gcPGRP6 or empty pTGFp1 vector. With the availability of gene information for grass carp and also their possible relevance to PGRPs, the genes examined were NOD1 (FJ937972) and NOD2 (FJ937973), which have been suggested to bind PGNs in teleost fish to PGRPs, the genes examined were NOD1 (FJ937972) and NOD2 (FJ937973), which have been suggested to bind PGNs in teleost fish. Real-time PCR analyses. As shown in Fig. 4C, a specific band of about 49 kDa, which corresponds to the estimated size of gcPGRP6, is detected in head kidney, skin, gills, eye, trunk kidney, liver and spleen, with strong detection in intestine. The data are consistent with Realtime PCR results, which showed that gcPGRP6 transcripts were detected in all examined organs/tissues, including head kidney, skin, gills, eye, liver, spleen, trunk kidney and intestine, with the highest expression in intestine, and then in spleen and liver (Fig. 4D).
To reveal the immune response of gcPGRP6 under bacterial infection, the pattern of gcPGRP6 mRNA expression in liver and intestine were examined following injection with PGN, and the expression of gcPGRP6 increased significantly from 15 h until 24 h (P < 0.05), with the highest expression at 18 h for liver (2.7-fold) (Fig. 5A) and at 24 h for intestine (3-fold) (Fig. 5B).

To reveal the response of gcPGRP6 in the CIK cells upon stimulation with different ligands, its expression was examined in CIK cells at 6 h following the stimulation with LTA, Poly I:C and PGN, with a significant increase (P < 0.05) at 10.2, 8.1 and 4.7 folds, respectively (Fig. 5C).

3.3. Subcellular localization of gcPGRP6 in CIK cells

To determine the subcellular localization of gcPGRP6, the open reading frame of gcPGRP6 was inserted into the pTurboGFP expression vector. Fluorescent microscopy demonstrated that no fluorescence was observed in cells transfected with gcPGRP6, while pTurboGFP vector was distributed diffusely throughout the entire cell (Supplementary data A). To determine the endogenous localization of gcPGRP6, rabbit anti-gcPGRP6 antibody was used, and no fluorescence was observed in cells (Supplementary data B). Further, Western blotting analysis showed that gcPGRP6 is secreted.

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**Fig. 1.** Nucleotide and deduced amino acid sequences of grass carp peptidoglycan recognition protein 6, gcPGRP6. The translation start codon ATG and termination codon TGA are shown in box; the mRNA unstable motif ATTTA are underlined. The cleavage site of signal peptide was indicated with arrow, and N-glycosylation site with ellipse.
and is also present in the cell lysate (Fig. 6). The amount in the lysate was 9.2-fold higher than in the medium, when measured using the semi-quantitative image analysis.

3.4. Effect of gcPGRP6 in NF-κB pathway

To determine whether gcPGRP6 is involved in NF-κB activation, CIK cells were transfected with NF-κB-luc reporter plasmid, with or without gcPGRP6 plasmid. As seen in Fig. 7, gcPGRP6 potently induced NF-κB activation.

3.5. Effect of gcPGRP6 on intracellular bacterial invasion and proliferation

To explore the ability of gcPGRP6 in inhibiting the invasion and growth of intracellular bacteria, grass carp CIK cells were infected with *E. tarda* after transfection with ptGFP1-gcPGRP6. The number of entered *E. tarda* was counted using the plate count method.

### Table 2

<table>
<thead>
<tr>
<th>Species</th>
<th>Amino acid identity/similarity (%)</th>
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<tr>
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<tr>
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</table>

Fig. 2. Genomic organization of gcPGRP6 (A) and multiple alignment of amino acid sequences of PGRP domain from gcPGRP6 and other vertebrate PGRPs (B). Exons are indicated as square boxes including white and black boxes, and introns as straight lines. PGRP domain was indicated in black box. Conserved cysteines forming possible internal disulfides and dimers are shaded in grey. PGRP sub-domains I, II and III are indicated by a double arrow. Zn²⁺-binding amino acids required for amidase activity are indicated in asterisks. The Gly (G), Trp (W) and Arg (R) which are believed to determine preferential DAP specificity are indicated in arrows. GenBank accession numbers for the sequences used in the analysis are: gcPGRP6, ADL41186; DrPGLYRP6, ABE01406; SaPGRP-L1, ADC593707; DrPGLYRP2, NP_001038631; PpGCLYRP2, ACJ10072; HsPGLYRP2, AAD41689; DrPGLYRP5, ABE01405. The Gly (G), Trp (W) and Arg (R) which are believed to determine preferential DAP specificity are indicated in arrows. GenBank accession numbers for the sequences used in the analysis are: gcPGRP6, ADL41186; DrPGLYRP6, ABE01406; SaPGRP-L1, ADC593707; DrPGLYRP2, NP_001038631; PpGCLYRP2, ACJ10072; HsPGLYRP2, AAD41689; DrPGLYRP5, ABE01405.
Little or no difference was observed in the number of entered bacteria between mock-transfected cells and cells transfected with ptGFP1-gcPGRP6 (Fig. 8A). However, *E. tarda* count was significantly lower in cells over-expressing the gcPGRP6 compared to control cells transfected with the ptGFP1 vector after incubation with gentamicin for 6 h. The number of intracellular bacteria increased 29.6-fold in control CIK cells, whereas 11.3-fold in cells transfected with ptGFP1-gcPGRP6 (Fig. 8B).

3.6. Binding of gcPGRP6 with peptidoglycan

To demonstrate whether gcPGRP6 binds PCN, pTurbo-gcPGRP6-GFP fusion expression plasmid was used. CIK cells were transiently transfected with pTurboGFP or pTurbo-gcPGRP6-GFP plasmid, respectively. Recombinant gcPGRP6 displayed the correct molecular weight as revealed with anti-pTurboGFP antibody (Fig. 9). As shown in Fig. 9, gcPGRP6 was able to bind both Lys-type PGN and DAP-type PCN, and such binding was not observed for the control plasmid.

3.7. Induction of innate immune genes by gcPGRP6

To examine the effect of gcPGRP6 on innate immune genes, CIK cells were transfected with ptFP1 or ptFP1-gcPGRP6 plasmid, and incubated for 24 h. The transient transfection of ptFP1-gcPGRP6 significantly induced the expression of itself (data not shown). As shown in Fig. 10, the expression of IL-2 and NOD2 was induced significantly at mRNA level by gcPGRP6 (4.8 and 3.8 fold).

**Fig. 3.** Phylogenetic relationship of gcPGRP6 with other vertebrate PGRPs. Full-length amino acid sequences were aligned using the clustal X program and the phylogenetic tree was constructed using the Neighbor-Joining algorithm within MEGA version 4.0. The tree was boosted for 1000 times and percentage of the bootstrap value is shown.
folds, respectively), whereas the expression of other innate immune genes such as TNF-α, NOD1, hepcidin, G-type lysozyme and perforin changed non-significantly.

4. Discussion

In mammals, human PGRPs including PGLYRP1, PGLYRP2, PGLYRP3 and PGLYRP4 are selectively expressed in different organs. PGLYRP1 was highly expressed in bone marrow, PGLYRP2 in liver, PGLYRP3 and PGLYRP4 in esophagus (Liu et al., 2001; Dziarski and Gupta, 2010). In teleost fish, the expression pattern of gcPGRP6 is similar to zebrafish PGRP6 (Chang et al., 2007), which was detected in all organs/tissues examined, with the highest level in intestine. Furthermore, gcPGRP6 is inducible in liver, intestine and CIK cells in response to PGN stimulation, which reflects the importance of gcPGRP6 in antibacterial immune response. Interestingly, the expression of gcPGRP6 in CIK cells can also be induced by other PAMPs such as LTA and Poly I:C, which is consistent with the report that mammalian PGRPs recognize diverse microbial ligands by conserved or similar oligosaccharide moieties (Tydell et al., 2006).

Mammalian PGLYRPs are secreted proteins expressed in polymorphonuclear leukocytes (such as PGLYRP1), liver (such as PGLYRP2), or on body surfaces, and could be found in mucous membranes, and in secretions (saliva, sweat) (PGLYRP3 and PGLYRP4) (Lu et al., 2006; Dziarski and Gupta, 2010). In zebrafish, immunohistochemistry data demonstrated that all three PGRP proteins were present in the lumen of blood vessels, which indicates that they are secreted (Li et al., 2007). In the present study, no fluorescence was observed within cells transfected with gcPGRP6, whilst gcPGRP6 was detectable in the medium of transfected cells, suggesting that gcPGRP6, containing a predicted signal peptide, was effectively secreted to the exterior. In mouse, Kibardin et al. (2003) showed that the tag7/PGRP gene family, such as TagL-α, was detected both in the cell lysate and supernatant of Cos1 cells transfected with TagL-α expression construct. Furthermore, both secreted and intracellular TagL-α were able to bind to Gram-positive and Gram-negative bacteria membranes. Another secreted protein, bovine peptidoglycan recognition protein-S (PGRP-S), is also found to localize to the dense/large granules of naive neutrophils using Immunogold electron microscopy (Tydell et al., 2006). Similar to mouse TagL-α and bovine PGRP-S, gcPGRP6 was detected by Western blotting in the cell lysate as well as in the med-
ium although no fluorescence was observed for exogenous or endogenous gcPGRP6. It may be possible that the concentration of gcPGRP6 protein in single CIK cells was so low that fluorescence could not be detected microscopically, however, for Western blotting, the lysate was concentrated that gcPGRP6 could be observed at significant levels. In addition, in transfected cells, the proportion of gcPGRP6-GFP retained in the cell lysate exceeded that observed in the medium. Whether this is the case for endogenous gcPGRP6 may need further investigation.

In fact, PGRP6 in zebrafish has been revealed as a secreted bactericidal protein (Li et al., 2007). However, the presence of transmembrane regions in gcPGRP may imply that PGRP6 in teleost fish may also function potentially as a cellular pattern recognition receptor. The existence of gcPGRP6 in cell lysate of transfected cells may at least to some extent indicate the potential recognition role of this PGRP in grass carp. The binding of gcPGRP6 towards Lys-type and Dap-type PGN, and the inhibited growth of E. tarda in gcPGRP6-transfected cells may provide evidence for its PAMP recognition role as well as its bactericidal role in fish.

In invertebrates and vertebrates, NF-κB was described as a transcriptional regulator in innate and adaptive immune systems (Hayden et al., 2006). Insect PGRPs have been shown to play an important role in detecting pathogens and activating NF-κB through the Toll and Imd pathways (Takehana et al., 2002; Gobert et al., 2003; Silverman et al., 2009). In vertebrates, PRRs such as TLRs and NODs were shown to participate in the signaling pathway leading to NF-κB activation (Shi et al., 2008; Hasegawa et al., 2008), whereas the link between PGRPs and NF-κB is still ambiguous. In a mouse model of cerebral ischemia, peptidoglycan recognition protein-S (PGRP-S) is upregulated by NF-κB (Lang et al., 2008), whereas zebrafish PGRP-SC and PGRP6 have a distinct regulation on the expression of NF-κB (Chang and Nie, 2008; Chang et al.,

**Fig. 5.** Inductive expression of gcPGRP6 in liver, intestine and CIK cells. RNA was extracted from liver (A) and intestine (B) of fish stimulated with PGN at 3, 9, 12, 15, 18 and 24 hpi. (C) gcPGRP6 in CIK cells following the stimulation with lipoteichoic acid (LTA), polyinosinic polycytidylic acid (Poly I:C) and peptidoglycan (PGN). 1 × 10⁶ CIK cells were seeded per well into six-well plates, before being stimulated in triplicate for 6 h with 50 μg/ml LTA, 50 μg/ml Poly I:C and 50 μg/ml PGN. Each column and bar represents the mean ± SD from three individuals. Asterisks (*) indicate significant (P < 0.05) difference between induced and corresponding control groups.

**Fig. 6.** Subcellular localization of gcPGRP6 in grass carp CIK cells. CIK cells transfected with pTurbo-gcPGRP6-GFP were used to extract cytoplasmic and medium proteins after 48 h transfection. Protein extracts were loaded onto the 12% SDS–PAGE gel, and detected by using anti-pTurboGFP antibody. Scanned TIFF images were processed with bands quantified using the Quantity One software.

**Fig. 7.** Activation of NF-κB by gcPGRP6. CIK cells were transfected with an NF-κB-luc reporter plasmid, pRL-TK vector as well as non-fusion gcPGRP6 expression plasmids. The data are presented as the ratio of Firefly to Renilla. Asterisks (*) indicate the significant (P < 0.05) difference between CIK cells transfected with ptGFP1 empty plasmid and non-fusion plasmid of ptGFP1-gcPGRP6.
The present study showed that gcPGRP6 can induce the activation of NF-κB, which suggests that gcPGRP6 may participate in immune responses through NF-κB activation.

In human corneal epithelial cells (HCECs) and oral epithelial HSC-2 cells, it is demonstrated that bacterial PAMPs could significantly induce the expression of PGLYRPs through the TLR- or NLR-mediated signal pathways (Ma et al., 2010; Uehara et al., 2005). In zebrafish, inhibition of zfPGRP6 expression in developing embryos using small interfering RNA significantly down-regulated the expression of TLR2 and TLR5 (Chang and Nie, 2008). In addition, in a peptidoglycan- or MDP-induced arthritis model, PGLYRP2 and NOD2 are both required for local chemokine and cytokine production, which accounts for the inflammatory arthritis (Saha et al., 2009). In this study, it was observed that the overexpression of gcPGRP6 in CIK cells could significantly up-regulate the expression of NOD2. It seems that, in addition to the role of vertebrate PGRPs as direct effector molecule (Dziarski and Gupta, 2006), PGRPs may play synergistic and complementary roles with TLRs and NLRs families in antibacterial immune response.

Insect PGRP proteins are important for the antimicrobial innate immunity by activation of Toll or Imd signal transduction pathways that result in the activation of transcription factors similar to NF-κB.
to mammalian NF-κB and induction of antimicrobial peptides (Tanji et al., 2007). In mammals, porcine PGRPs are involved in antimicrobial peptide expression (Sang et al., 2005), and human PGRPs were reported to kill both Gram-positive and Gram-negative bacteria by synergistic effects with antibacterial peptides (Cho et al., 2005; Wang et al., 2007). Different from insect and mammalian PGRPs, it was found that gcPGRP6 was not involved in the expression of two antibacterial peptides. The insignificant change of TNF-α in the expression of two antibacterial peptides. The insignificant change of TNF-α, NOD1, perforin as well as two antibacterial peptides (Hepcidin and lysozyme) in CIK cells overexpressed with gcPGRP6 may imply that these molecules are unrelated with gcPGRP6 mediated immune response.

In conclusion, a long PGRP, homologous to PGRP6 was identified in grass carp. The induced expression of gcPGRP6 by various microbial components suggests that gcPGRP6 is an important innate molecule in antibacterial immune response of teleost fish. It is also confirmed that gcPGRP6 play roles in activating NF-κB activity, in binding to Lys-PGN and Dap-PGN, in inhibiting the growth of intracellular E. tarda, and then in regulating innate immune genes. However, further research is needed to understand the cooperative mechanism between gcPGRP6 and gcNOD2 in antibacterial immune response of grass carp.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.devimm.2013.09.014.

References


