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Molecular cloning, expression analyses of polymeric immunoglobulin receptor gene and its variants in grass carp (*Ctenopharyngodon idellus*) and binding assay of the recombinant immunoglobulin-like domains

Chao Pei, Xiaoying Sun, Yi Zhang, Li Li, Yan Gao, Li Wang, Xianghui Kong*

College of Fisheries, Henan Normal University, Xinxiang, Henan, 453007, China

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ABSTRACT

The Polymeric Immunoglobulin Receptor (*pIgR*) gene has been proved to play an important role in transporting polymeric immunoglobulin (Ig) in the mucosal tissues of mammals. *pIgR* gene also exists in teleost, but the genetic diversity and functions of this gene still need to be further explored. We obtained seven grass carp pIgR splicing transcripts, a full-length pIgR (*CipIgR-1*) and six truncated variants (*CipIgR-2* to *CipIgR-7*). The full-length pIgR contained two immunoglobulin-like domains (ILD), a transmembrane domain (TMD) and a cytoplasmic domain (CyD). The *CipIgR-2* lacked a small part in CyD, and *CipIgR-3* lost TMD and CyD. Partial cDNA sequences of the other four grass carp pIgR variants (*CipIgR-4* to *CipIgR-7*) were also cloned. The total expression levels of *CipIgR* and its variants in different tissues were detected by real-time quantitative PCR. The highest expression was found in the intestine, followed by the spleen and the skin. The function of the two extracellular ILDs of *CipIgR* was investigated based on its combining capacity with grass carp immunoglobulin M (IgM) and aquatic pathogenic bacteria. The cDNA sequences of two ILDs were cloned and expressed in *Escherichia coli* BL21 (DE3). Recombinant ILDs protein was purified and incubated with different bacteria respectively. Results of Western blot showed the recombinant protein could combine *Bacillus subtilis*, *Vibrio parahaemolyticus*, and *Escherichia coli*. In addition, binding activity of rILDs with grass carp IgM was detected. Collectively, these results indicated that multiple variants of *pIgR* gene in grass carp might be involved in the antibacterial immunity.

1. Introduction

In vertebrates, epidermal tissue construct the first line of defense against microbial invasion, among which, mucosal tissue plays an important role [1]. Mucosal tissue is covered with mucus that contains various kinds of antimicrobial materials, such as lysozyme and secretory Ig [2,3]. Dimeric IgA in mammals, tetrameric IgM and dimeric IgT in fish are found in the mucus [4–10]. These polymeric immunoglobulins (pIg) are produced by epithelial plasma cells and transported into apical surfaces by the polymeric immunoglobulin receptor (pIgR). The pIgR on the basolateral membrane of epithelial cells can bind pIg and mediate their transportation via transcytosis [11–13]. Once arriving at the apical surface of cells, pIgR undergoes a proteolytic cleavage event, and the secretory component (SC) of pIgR together with the pIg is released into the mucus [14,15]. The secretory pIg is vital for the host to resist pathogenic invasion and maintain homeostasis [16,17].

Mammal pIgR protein is a single transmembrane protein that is composed of an extracellular region, a transmembrane domain, and an intracellular region. The extracellular region has five immunoglobulin-like domains (ILD), called ILD1-ILD5 respectively. ILD1 shows high homology to variable region of Ig, and is assumed to be the major domain that binds pIg [18,19]. Point mutation of ILD will weaken the binding ability of pIgR with IgA [20]. The *pIgR* genes, being analogous to mammalian pIgR, have been cloned in many teleost fish, such as fugu (*Takifugu rubripe*), common carp (*Cyprinus carpio*), zebrafish (*Danio rerio*), rainbow trout (*Oncorhynchus mykiss*), orange-spotted grouper (*Epinephelus coioides*), olive flounder (*Paralichthys olivaceus*), turbot (*Scophthalmus maximus*), Atlantic salmon (*Salmo salar*), Atlantic cod (*Gadus morhua*), sea bass (*Lateolabrax japonicus*), crucian carp (*Carassius auratus*), and dojo loach (*Misgurnus anguillicaudatus*) [16,21–31]. The *pIgR* gene is found to be widely expressed in different tissues and organs, but especially highly expressed in mucosal tissues and immune organs [24,25,29–31]. However, fish pIgR have only two ILD domains, which are supposed to be homologous to ILD1 and ILD5 of mammalian pIgR respectively [19]. Interestingly, a single *pIgR* gene and a large

* Corresponding author. College of Fisheries, Henan Normal University, No. 46, Jianshe Road, Xinxiang city, Henan Province, 453007, China.
E-mail address: xhkong@htu.cn (X. Kong).

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multigene family consisting of 29 pIgR-like (PIGRL) genes have been found in *Danio rerio* [23,32]. These results suggest that fish pIgR is unique and may have diversified functions in immunology.

In some previous studies, fish pIgR has been proved to perform functions in binding pIg, such as in fugu, common carp, rainbow trout, orange-spotted grouper, olive flounder, and sea bass [9,22,24,25,29]. In rainbow trout and sea bass, pIgR can bind to commensal bacteria and pathogens [16], and pIgR in dojo loach and crucian carp exhibited an obvious response to bacterial challenge [30], indicating that fish pIgR also plays an important role in maintaining microbial communities and defending against pathogenic bacteria invasion on mucosal surface.

Grass carp (*Ctenopharyngodon idellus*) is an important economic fish that accounts for the highest production in freshwater aquaculture in China, but is severely threatened by many fish diseases, such as septicemia, rotten gill disease, red skin disease, and enteritis caused by *Aeromonas hydrophila*, *Aeromonas veronii*, and other bacteria [33,34]. Those diseases can seriously damage the mucosal organ. In consideration of the pivotal role of pIgR in the mucosal immunity, the aim of the present study was to ascertain the characteristics and function of grass carp *pIgR* gene, in order to help understand its role in immune defense.

2. Materials and methods

2.1. Cloning and sequence analysis of grass carp pIgR

Healthy grass carp weighing about 50 g were purchased from a local fish farm. After the fish were anaesthetized and dissected, liver, intestine, and spleen were quickly sampled respectively. Total RNAs were extracted with RNAiso plus (TaKaRa, Japan) according to the manufacturer's instructions. The first strand cDNA was synthesized with M-MLV Reverse Transcriptase (Promega, USA) using Oligo (dT) primers. A partial *pIgR* cDNA fragment was amplified by PCR with a pair of primers (GpIgR-F and GpIgR-R) designed based on consensus sequence of fish pIgR cDNA sequences in GenBank (fugu, common carp, zebrafish, rainbow trout, orange-spotted grouper, and olive flounder). 3′ and 5′ RACE were performed to obtain the full-length cDNA sequence. In the progress of 3′ RACE, the first strand cDNA was used as template, primer pIgR 3′-1 and 3′ RACE Olig(T)-Adaptor were adopted for the first round PCR, and primer pIgR 3′-2 and 3′ RACE Adaptor for the second round PCR. For 5′ RACE, a ploy(A) tail was added to the first strand cDNA with TDT enzyme and purified as template, primer pIgR 5′-1 and 5′ RACE Olig(T)-Adaptor were used for the first round PCR, pIgR 5′-2 and 5′ RACE Adaptor for the second round. The DNA fragments obtained from RACE were sequenced and assembled with partial cDNA fragment amplified previously. Based on the assembled cDNA sequence, the full length of grass carp pIgR was verified with nest PCR by primers All-F and All-R1/All-R2/All-R3 (first round) targeting the terminal sequences, ORF-F (targeting the initiation codon) and All-R1/All-R2/All-R3 (second round). Open reading frame (ORF) was verified with primers All-F and 3′ RACE Olig(T)-Adaptor for the first round, and ORF-F and 3′ RACE Adaptor for the second round (Table 1).

The grass carp cDNA sequences were subjected to BLAST program of NCBI for homology research (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The open reading frame (ORF) was identified using EditSeq in DNAStar. The protein domains were predicted using the SMART program (http://smart.embl-heidelberg.de/). Signal peptide and transmembrane region of the protein were predicted using SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) and TMHMM2.0 (http://www.cbs.dtu.dk/services/TMHMM/), respectively. Multiple alignments of the amino acid sequences were generated by Clustal X (http://www.clustal.org/clustal2/). On the basis of the amino acid sequences of pIgR from vertebrates, a phylogenetic tree was constructed using the neighbor-joining method in MEGA 7.0 with a bootstrap repetition number of 1000. The intron/exon structures of genomic sequences were determined by alignment of the full-length cDNA sequence to genomic sequence using Splign (https://www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi).

Table 1
Primers used in this study.

Primers	Sequences (5′-3′)
G-pIgR F	CAGCABGTGTTCACNGTVAVCATG
G-pIgR F	GTYNCCRSYSCGACACCACYKCTTC
pIgR 3′-1	TATGAACCTCAGGTCCAGTGAGAAGCG
pIgR 3′-2	AGTGGGAACTGGAACTCCTGCGTGTC
pIgR 5′-1	CCCACTGCGACACCACCGCTTCT
pIgR 5′-2	CGCTTCTCACTGGACCTGAGGTTCATA
3′ RACE Olig(T)-Adaptor	CTGATCTAGAGGTACCGGATCCTTTTTTTTTTTTTT
3′ RACE Adaptor	CTGATCTAGAGGTACCGGATCC
5′RACE Olig(T)-Adaptor	GACTCGAGTCGACATCGATTTTTTTTTTTTTTT
5′RACE Adaptor	GACTCGAGTCGACATCG
All-F	GGCAGAAATATTCCTTCTCGCAGCAG
ORF-F	ATGGCTCTTCCGCTGCTTCTAACCATT
All-R1	ACCTTTGTTTGCCTTTTCTTGCCTCT
All-R2	GTGTTTAATAAATGCTGTTTATTGGTA
All-R3	GGTGGTAACAAGCTTCCATAGTGATGC
QpIgR-F	TCTGAAGGAATGTTCAGCAGTAG
QpIgR-R	AGCCCGAGTCTCTCATCTC
β-actin-F	CGTGACATCAAGGAGAAG
β-actin-R	GAGTTGAAGGTGGTCTCAT
pILD-F	TCCGAATTCACAGTGACCACTGTAGGAG
pILD-R	GGCAAGCTTTTAGACTGACACATGAAC

2.2. Detection of total mRNA expression of CipIgR in normal condition

The different tissues of healthy grass carp including muscle, gill, liver, intestine, spleen, kidney, head kidney, and skin, were sampled, and total RNA was extracted as described above. The first-strand cDNA was synthesized with PrimeScript™ II 1st Strand cDNA Synthesis Kit (TaKaRa, Japan) after digestion with recombinant DNase I (RNase free; TaKaRa, Japan) to eliminate genomic contamination. TB Green™ Premix Ex Taq™ II (Takara, Japan) and LightCycler® 96 Real-Time PCR system (ROCHE, Switzerland) were used for quantitative real-time PCR (qRT-PCR). The qRT-PCR amplification for each sample was performed in a 20 μl reaction volume consisting of 2 μl of cDNA template. The primers QpIgR-F and QpIgR-R (Table 1) were used to amplify a 100 bp fragment of pIgR. Primers β-actin-F and β-actin-R (Table 1) were used to amplify gene *β-actin* as the reference for internal standardization. The cycle profile was performed as follows: 95 °C for 5 min, followed by 40 cycles at 95 °C for 10 s and at 60 °C for 30 s. Each sample was examined in triplicate. The relative mRNA expression of pIgR was calculated using the comparative threshold cycle method ($2^{-\triangle\triangle Ct}$).

2.3. Expression, purification, and refolding of recombinant pIgR-ILD

The ILD1 and ILD2 domains of *CipIgR* were amplified by the primers of pILD-F and pILD-R (Table .1) being used for prokaryotic expression. Restriction enzyme cutting sites of *EcoR* I and *Hind* III were designed in primers for the insertion of targets gene into plasmid expression vector pET-32a (Novagen). The identity of insert was verified by sequencing, and the plasmid *pET-32a/pIgR-ILD* was constructed and transformed into *E. coil* BL21 (DE3), which were cultured in 300 mL LB medium containing 0.1 mg/ml ampicillin at 37 °C at 200 rpm until OD_{600} reached about 0.6. The expression of recombinant pIgR-ILD was induced after isopropyl-β-*d*-thiogalactoside (IPTG) was added into the culture at a final concentration of 1 mM. After further incubation at 37 °C for 6 h, the bacterial cells were harvested by centrifugation at 8000 g at 4 °C for 10 min, re-suspended in ice-cold 20 ml binding buffer (0.1 M Trise, 5 mM imidazole, pH 8.0), and broken by ultrasonic in ice bath for 60 min. The inclusion bodies were collected by centrifugation at 10,000 g at 4 °C for 10 min. To remove additional cellular proteins, the inclusion bodies were washed twice with binding buffer (0.1 M Trise, 5 mM imidazole, 2 M urea, pH 8.0). Subsequently, the inclusion

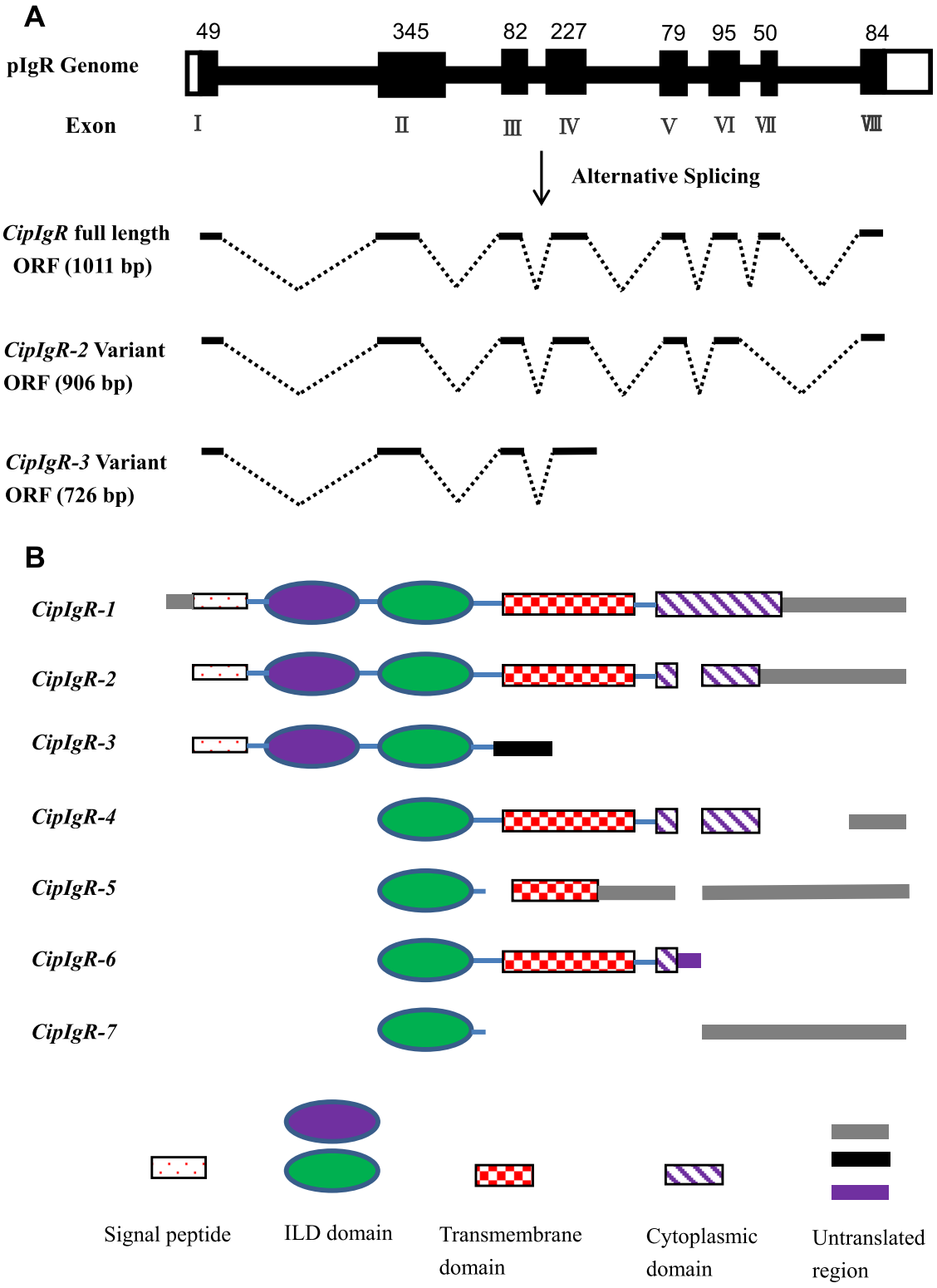

Fig. 1. Identification of splicing transcripts of grass carp *pIgR* gene. (A) Schematic diagrams of exon-intron arrangements of grass carp pIgR gene and generation of three pIgR transcripts by exon skipping. The filled boxes and the thick lines indicate the exons that are differentially selected to assemble three splicing transcripts. (B) Schematic representation of the structure of seven grass carp pIgR mRNA. CipIgR-1 represents the complete structure. Gaps in the other isoforms show the missing part compared with CipIgR-1, and untranslated regions with different terminal sequence are shown in different colors. (C) Amino acid sequence alignments of CipIgR-1, CipIgR-2, and CipIgR-3 showing the localization of domains including signal peptide, ILD1, ILD2, transmembrane region, and intracellular region.

C

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                 < Signal peptide  > <   ILD1
CipIgR-1  : -MALPLLLTILVLGVLPGSHCTVTTVGDLAVLEGQSVTVPCHYNPQYISHVKYWCQGRMIDFCSSLARTDDPES   73
CipIgR-2  : -MALPLLLTILVLGVLPGSHCTVTTVGDLAVLEGQSVTVPCHYNPQYISHVKYWCQGRMIDFCSSLARTDDPES   73
CipIgR-3  : -MALPLLLTILVLGVLPGSHCTVTTVGDLAVLEGQSVTVPCHYNPQYISHVKYWCQGRMIDFCSSLARTDDPES   73
                                                                  ILD1  >  <   ILD2
CipIgR-1  : TPQSKGRVTIADDPTQHVFTVSMQNLTVGDSGWYWCGVELGGMWVADSTASLYISVIQGMSVVSSMVSADEGSSVT  149
CipIgR-2  : TPQSKGRVTIADDPTQHVFTVSMQNLTVGDSGWYWCGVELGGMWVADSTASLYISVIQGMSVVSSMVSADEGSSVT  149
CipIgR-3  : TPQSKGRVTIADDPTQHVFTVSMQNLTVGDSGWYWCGVELGGMWVADSTASLYISVIQGMSVVSSMVSADEGSSVT  149

CipIgR-1  : VQCRYSMNLRSSEKRWCRSGNWNSCVSTDSEGMFSSKNVLIHDDKNSLFTVTLMQLEMRDSGWYWCGAGQQHVAVH  225
CipIgR-2  : VQCRYSMNLRSSEKRWCRSGNWNSCVSTDSEGMFSSRNVLIHDDKNSLFTVTLMQLEMRDSGWYWCGAGQQHVAVH  225
CipIgR-3  : VQCRYSMNLRSSEKRWCRSGNWNSCVSTDSEGMFNSRNVLIHDDKNSLFTVTLMQLEMRDSGWYWCGAGQQHVAVH  225

                 ILD2 >                    <  Transmembrane region    >  <
CipIgR-1  : VSVTPQATTVVTASSVQNLKTTIMTSSVMSSNDPHSRPVWESPLVVCGVVLLVMTAFLAVWKLRKQCKKKQKHQRT  301
CipIgR-2  : VSVTPQATTVVTASSVQNLKTTIMTSSVMSSNDPHSRPVWESPLVVCGVVLLVMTAFLAVWKLRKQCNVSMERRRL  301
CipIgR-3  : VSVTPQATTGMLHRAYS----------------------------------------------------------  242

                 Intracellular regio              >
CipIgR-1  : NEMNDNLTMCPWREGDYKNTSVIFLNTPAQQLQML      336
CipIgR-2  : ----------------------------------
CipIgR-3  : ----------------------------------
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Fig. 1. (*continued*)

bodies were solubilized in denaturing buffer (0.1 M Trise, 5 mM imidazole, 6 M urea, pH 8.0) overnight in ice, and insoluble ingredients were removed by centrifugation at 12,000 g at 4 °C for 30 min. The recombinant proteins in supernatant were purified through a Ni-IDA resin column (Sangon biotech, China) according to the manufacturer's instructions. The column was successively washed with PBS containing 8 M urea and 20 mM imidazole (pH7.4), and then eluted with PBS containing 8 M urea and 200 mM imidazole (pH7.4). The purified proteins were dialyzed with PBS for 48 h at 4 °C to remove imidazole and urea. The purity of eluted protein was analyzed by a 12% SDS-PAGE and stained with Coomassie brilliant blue R-250. Protein concentration was detected by BCA protein assay kit (Solarbio, China).

2.4. Assay for binding activity of recombinant pIgR-ILD to grass carp IgM

Grass carp IgM was extracted from blood and purified for binding assay. Blood was drawn from vein in the tail of 3 grass carp weighed about 2 kg. The blood was placed at 4 °C overnight, and serum was separated from blood cells by centrifugation at 3000 g for 10 min. A volume of 20 ml serum was added with 2 ml saturated ammonium sulfate solution (pH 7.0) to precipitate other unwanted proteins, and these proteins were removed by centrifugation at 10,000 g for 10 min. Another 10 ml saturated ammonium sulfate was added to the supernatant with and incubated at 4 °C overnight. The precipitate was collected by centrifugation at 10,000 g for 10 min and dissolved in 10 ml PBS. Dialysis was performed to remove the salt. The immunoglobulin was further purified by Protein A sefinose 1 (Pre-Packed Gravity Column, Sangon biotech, China) following the manufacturer's instructions. The purity of protein was analyzed by SDS-PAGE and its concentration was determined by BCA method.

ELISA was used to assay the binding activity of rpIgR-ILD to grass carp immunoglobulin. Immunoglobulin was diluted to a concentration of 100 μg/ml, and 50 μl was applied to each well of a 96-well microplate and incubated overnight at 4 °C. Each wells was blocked with 200 μl of 3% BSA in PBS for 2 h at 37 °C. After washing four times with 200 μl of PBST (10 mM PBS supplemented with 1% Tween-20), 50 μl of two-fold serial dilutions of rpIgR-ILD solutions (1.7, 3.3, 7.5, 15, 31, 62.5, 125 and 250 μg/ml) was added into the wells respectively. After incubation at 25 °C for 3 h, each well was rinsed four times with 200 μl of PBST. Then 100 μl of mouse anti-6x His tag antibody (ABM, Canada, 1:2000 diluted in 3% BSA) was added to the wells as the first antibody. After incubation at 37 °C for 1 h, the plate was washed again four times, and 100 μl of peroxidase-conjugated goat anti-mouse IgG secondary antibodies (ABM, Canada, 1:5000 diluted in 3% BSA) was added as the second antibody, and incubated at 37 °C for 1 h. The wells were washed as above, and reacted with 0.01% 3, 3′, 5, 5′-tetramethylbenzidine (Solarbio, China). The reaction was stopped by adding 50 μl of 2 M H_2SO_4 per well, and the absorbance was measured at 450 nm. In the control, rTrx was processed similarly. The experiments were performed in triplicate.

2.5. Assay for binding activity of recombinant pIgR-ILD to bacteria

Gram-positive bacteria *Bacillus subtilis*, *Bacillus megaterium*, and *Micrococcus lysodeikticus*, and gram negative bacteria *Vibrio anguillarum*, *Vibrio parahaemolyticus*, *Escherichia coli*, *Aeromonas hydrophila*,

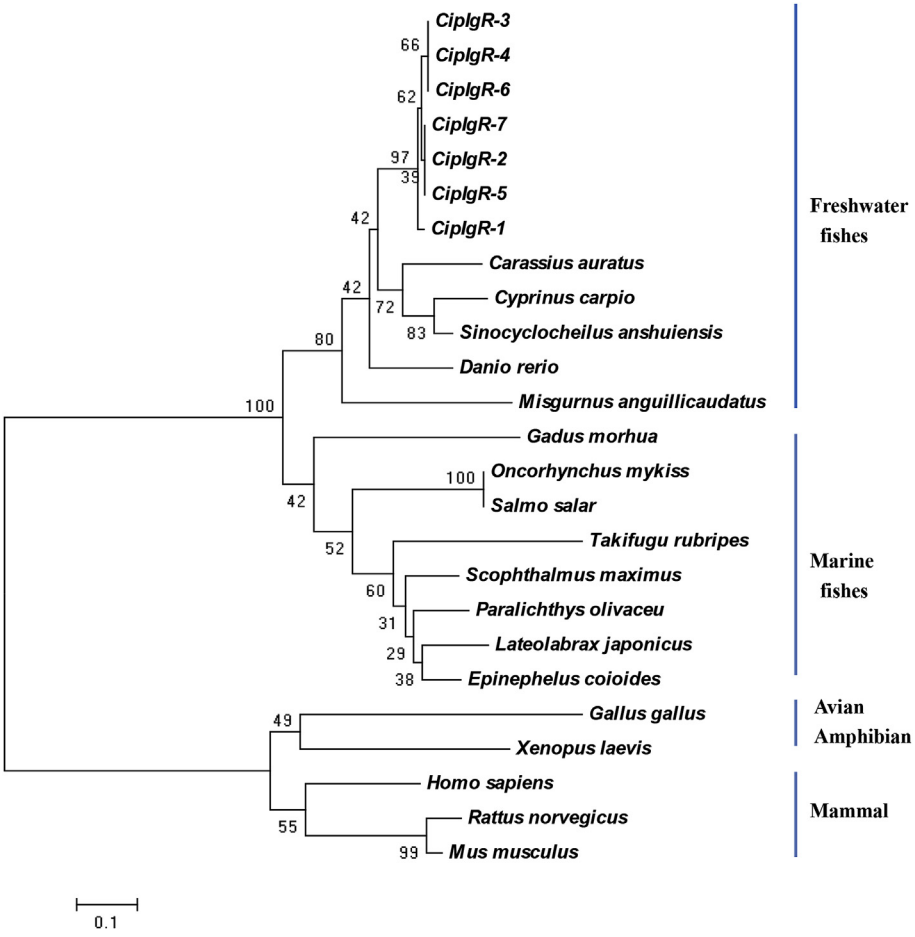

Fig. 2. Phylogenetic tree constructed with the neighbor-joining method based on the amino acid sequences of pIgR from CipIgR and other vertebrates. The scale bar (0.1) represents the genetic distance. The numbers marked near the nodes indicate the bootstrap test scores. GenBank accession numbers: *Carassius auratus*, KY652915; *Cyprinus carpio*, ADB97624; *Sinocyclocheilus anshuiensis*, XP_016298944; *Danio rerio*, ABQ10652; *Misgurnus anguillicaudatus*, MG431994; *Gadus morhua*, AIR74929; *Oncorhynchus mykiss*, ADB81776; *Salmo salar*, ACX44838; *Takifugu rubripes*, NP_001266944; *Scophthalmus maximus*, AGN54539; *Paralichthys olivaceus*, ADK91435; *Lateolabrax japonicas*, ANZ03107; *Epinephelus coioides*, ACV91878; *Gallus gallus*, AAP69598; *Xenopus laevis*, ABK62772; *Homo sapiens*, CAA51532; *Rattus norvegicus*, EDM09843; *Mus musculus*, AAA67440.

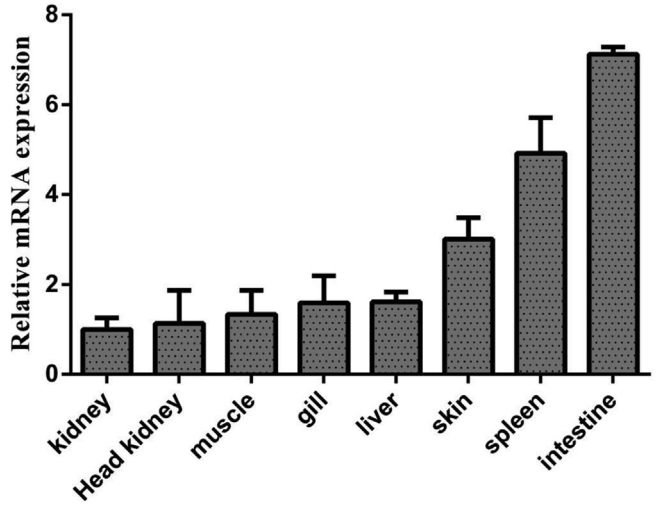

Fig. 3. Relative expression levels of total CipIgR mRNA in different tissues. For the convenience of comparison, the expression level in kidney was chosen as a calibrator. The relative expression of CipIgR mRNA was presented as fold change to the calibrator. Data are shown as means ± S.E. (n = 3).

Aeromonas sobria, and *Aeromonas veronii* were employed to investigate the binding activities of recombinant pIgR-ILD to the bacteria as described previously by Yang et al. [29]. The bacteria were cultured in Luria-Bertani medium at 37 °C and collected at logarithmic-phase by centrifugation at 6000 *g* for 5 min, and washed twice with PBS. The bacteria were re-suspended in PBS to a concentration of 1×10^8 cells/ml. Aliquots of 150 μl of each bacterium suspension (containing 1×10^7 cells) were mixed with 300 μl of 100 μg/ml rpIgR-ILD in PBS, and the mixtures were incubated at 25 °C for 2 h. After centrifugation at 5000 *g* at room temperature for 6 min, the cell pellets were washed six times and re-suspended in PBS. They were then electrophoresed on a 12% SDS-PAGE gel and Western blot was performed to detect the rpIgR-ILD. Protein was transferred to the NC membrane (Merck, USA) and the membrane was blocked with 5% skimmed milk. Mouse anti-6 × His tag antibody (ABM, Canada) was used as primary antibody and alkaline phosphatase conjugated horse anti-mouse IgG (ZSGB-BIO, China) as secondary antibody. Western Blue® Stabilized Substrate for Alkaline Phosphatase (Promega, USA) was used for developing for 10 min. The purified rpIgR-ILD was used as positive control, and the microbial cells incubated with BSA were conducted as negative control.

3. Results

3.1. Cloning and molecular analysis of grass carp pIgR cDNA

Using a pair of degenerate primers designed based on conserved sequence of other fish *pIgR* genes, and 3′ and 5′ RACE, three complete and four partial cDNA sequences (3′ end) of grass carp *pIgR* were cloned. These grass carp transcripts appear to be generated by alternative splicing from a single *pIgR* gene in the chromosome, which is composed of 8 exons and 7 introns (Fig. 1A). The full-length transcripts *CipIgR-1* includes all 8 exons and encodes a 336 aa-protein; *CipIgR-2* encoding a 301 aa protein is slightly shorter than *CipIgR*-1 that lacks of exon 7; while *CipIgR-3* is much shorter with exons 5–7 truncated, encoding a 242 aa protein. Analysis of the deduced amino acid sequence showed that *CipIgR-1* was comprised of a signal peptide, an extracellular region, a transmembrane region (TMD) and an intracellular

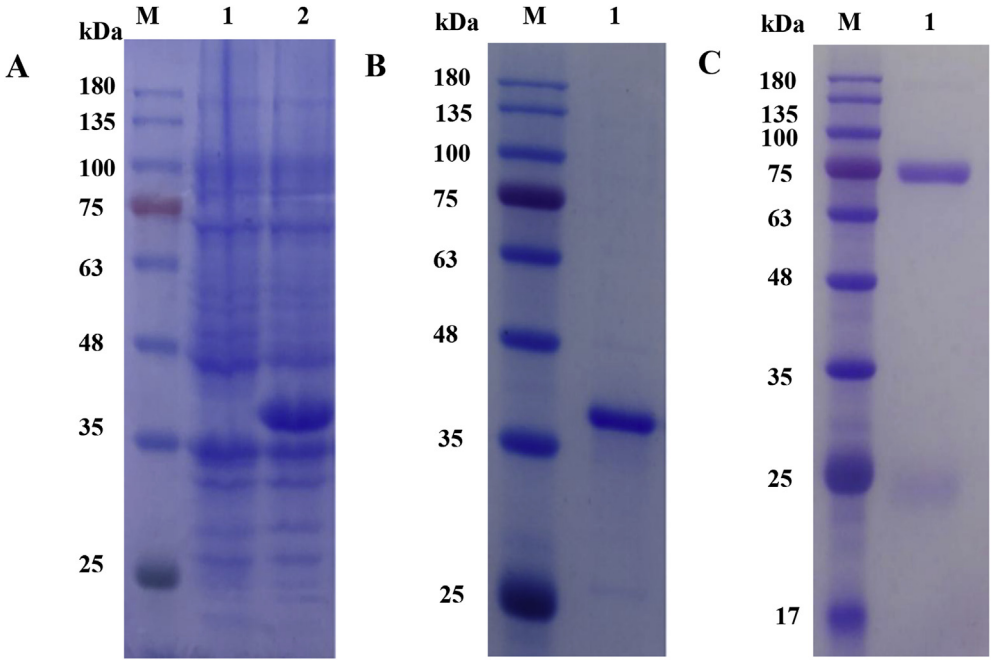

Fig. 4. (A) Prokaryotic expression of pIgR-ILD. M, Molecular mass standards; Lane 1, Total cellular extracts from *E. coli* BL21 (DE3) containing expression vector without IPTG induction; Lane 2, Total cellular extracts from *E. coli* BL21 (DE3) containing expression vector with IPTG induction; (B) Purification of pIgR-ILD. 1, purified rpIgR-ILD. (C) Purification of grass carp IgM. 1, purified grass carp IgM by protein A column.

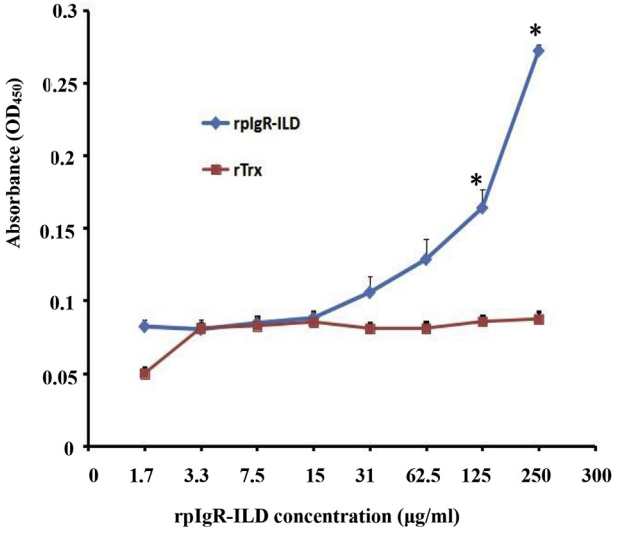

Fig. 5. Interaction between rpIgR-ILD and grass carp IgM by ELISA analysis. IgM dissolved in PBS (pH7.4) was applied to wells of a 96-well microplate and incubated with the rpIgR-ILD or rTrx. Anti-His antibody was applied as the primary antibody and horse anti-mouse IgG as the secondary antibody. Asterisk indicates the ratio of absorbance (OD_{450}) of rpIgR-ILD to rTrx ≥ 2, and was considered as positive. Data were expressed as mean values ± SEM (n = 3).

region (CyD). The extracellular region contained two immunoglobulin-like domains (ILD), which was in accordance with the results reported in other fish (Fig. 1B and C). *CipIgR-2* lacks a small part of the intracellular region, and *CipIgR-3* lost the transmembrane region and intracellular region. Moreover, 3′ end of *CipIgR-4* is similar to *CipIgR-2*, except a different 3′ UTR; so is *CipIgR-7* and *CipIgR-3*. *CipIgR-6* lacks the part encoded by exon 8 compared with *CipIgR-2* (Fig. 1B). The cDNA sequences of *CipIgR* and its variants were submitted to GenBank database (Accession No. MH624161-MH624167).

In order to evaluate the evolutionary relationship of *CipIgR* with pIgRs from other vertebrates, a phylogenetic tree was constructed using the neighbor-joining method based on the amino acid sequences of pIgRs from 18 species. As shown in Fig. 2, the species from teleost fell into one cluster, which can be further divided into two small clusters, which respectively represented the freshwater fish and marine fish. In the teleost pIgR clade, *CipIgR* shared the closest relationship with the pIgRs of *Carassius auratus* and *Cyprinus carpio*.

3.2. Total CipIgR expression in different tissues

All of the *CipIgR* genes containing the ILD2 domain (Fig. 1B), so primers targeting the ILD2 sequence were designed for detection of total expression of *CipIgR* mRNA in different tissues by qRT-PCR. The highest expression was found in the intestine, followed by the spleen and the skin. The lowest expression was observed in the kidney and head kidney (Fig. 3).

3.3. Recombinant expression and purification of pIgR-ILD

The cDNA sequence of two ILD domains of *CipIgR* was cloned by PCR, and then was constructed into pET-32a plasmid. The plasmid *pET-32a/pIgR-ILD* was transformed into *E. coli* BL21 (DE3) and induced to express rpIgR-ILD by IPTG. An additional protein band about 38 kDa was observed in IPTG induced *E. coli* compared with un-induced bacteria by SDS-PAGE analysis (Fig. 4A), which was consistent with the predicted molecular mass of pIgR-ILD (about 20 kDa) plus an 18 kDa of Trx-His-tag. The rpIgR-ILD was expressed as inclusion body. The recombinant protein was refolded, purified and renatured. The purified rpIgR-ILD was identified by using 12% SDS-PAGE (Fig. 4B).

3.4. Grass carp immunoglobulin interacts with rpIgR-ILD

Grass carp immunoglobulin was purified from grass carp serum and analyzed by SDS-PAGE. As shown in Fig. 4C, the molecular mass of heavy chain of grass carp immunoglobulin was about 75 kDa, and the light chain was about 23 kDa, indicating that the grass carp IgM protein was successfully extracted. The binding affinity of rpIgR-ILD with IgM was detected by ELISA. The result showed a dose-dependent binding activity, and the rpIgR-ILD could bind to IgM when the concentration was higher than 125 μg/ml, but no obvious binding activity was observed when lower than 125 μg/ml (Fig. 5). The absorbance value was only about 0.3 even if the concentration of rpIgR-ILD was at a high level (250 μg/ml), indicating that the binding activity of rpIgR-ILD with grass carp IgM was very weak.

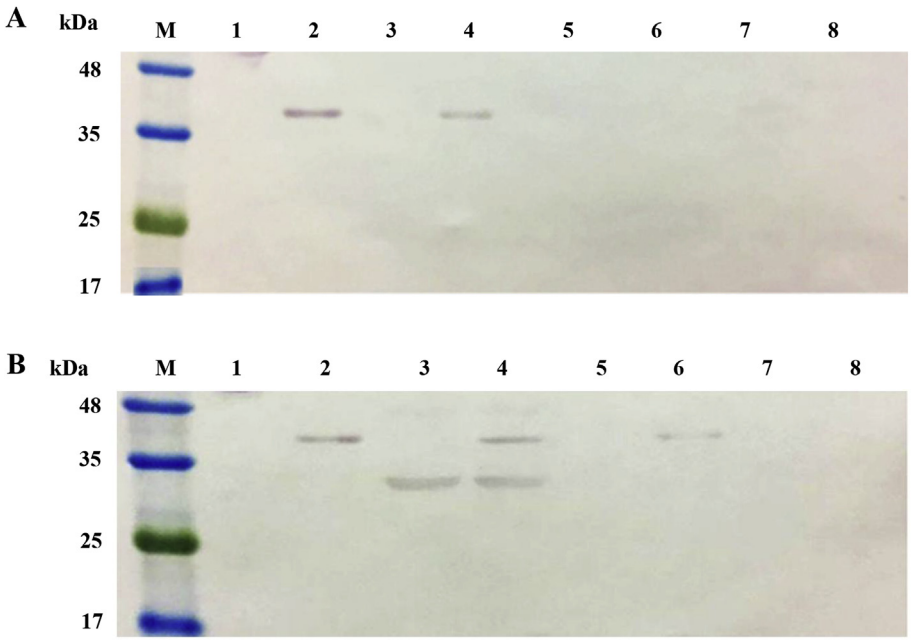

Fig. 6. The bacteria binding assay of rpIgR-ILD by western blot. (A) rpIgR-ILD incubated with gram-positive bacteria; Lane 1, BSA; Lane 2, Purified rpIgR-ILD; Lane 4, *Bacillus subtilis* incubated with rpIgR-ILD; Lane 6, *Bacillus megaterium* incubated with rpIgR-ED; Lane 8, *Micrococcus lysodeikticus* incubated with rpIgR-ED; Lane 3, 5, 7, corresponding bacteria incubated with BSA. (B) rpIgR-ILD incubated with gram-negative bacteria; Lane 1, BSA; Lane 2, Purified rpIgR-ILD; Lane 4, *Escherichia coli* incubated with rpIgR-ILD; Lane 6, *Vibrio parahaemolyticus* incubated with rpIgR-ED; Lane 8, *Vibrio anguillarum* incubated with rpIgR-ED; Lane 3, 5, 7, corresponding bacteria incubated with BSA.

3.5. Recombinant pIgR-ILD binds to bacteria

The binding of rpIgR-ILD to various Gram-negative and positive bacteria was tested by Western blot. As shown in Fig. 6, rpIgR-ILD could bind to the Gram-positive bacteria *B. subtilis*, and the Gram-negative bacteria *E. coli* and *V. parahaemolyticus*, but could not bind to the Gram-positive bacteria *B. megaterium*, *M. lysodeikticus*, and the Gram-negative bacteria *V. anguillarum* (Fig. 6). Meanwhile, we also detected the binding ability of rpIgR-ILD to the fish pathogenic bacteria of *A. hydrophila*, *A. sobria*, and *A. veronii*, which were Gram-negative bacteria, however, binding activity was not detected (data not shown). These results indicated that grass carp pIgR could interact with certain kinds of Gram-negative and positive bacteria, rather than all kinds of bacteria.

4. Discussion

One grass carp *pIgR* gene and six truncated variants were amplified in this study, among which three complete transcripts were obtained. The *pIgR* gene has been identified in many fish species, but only a single transcript of this gene is found in most of the fishes. In this study, alternatively splicing was found in grass carp *pIgR* gene. The variety of *pIgR* gene was also found in zebrafish, which has a single *pIgR* gene on chromosome 2 along with a large multigene family consisting of 29 *pIgR*-like genes [23,32]. Generally, fish pIgR contains two ILD domains, which are similar with the corresponding fragments of ILD1 and ILD5 domain in mammalian and avian pIgR. In our results, *CipIgR-1*, *CipIgR-2*, and *CipIgR-3* also contain two ILD domains like most fish *pIgR* gene, but the complete cDNA sequences of the other four grass *pIgR* transcripts were not obtained by primers targeting the ORF, which might be identical to *CipIgR-1*, *CipIgR-2*, and *CipIgR-3*. The reason is maybe because the overlap of the 5' ends of these genes with *CipIgR-1* and relatively low mRNA expression made it difficult to amplify [23].

The detection of total mRNA of grass carp pIgR showed that it was expressed in various tissues of healthy fish, and the expression was most abundant in intestine, spleen, skin, and gill. This result was similar to the previous studies in carp, flounder, sea bass, and crucian carp [22,25,29,31]. The mucosal tissues in intestine, skin, and gill constructed the first protective barrier against invading organisms. The high expression levels of total *pIgR* mRNA in mucosal tissues suggested that *CipIgR* may play an important role in mucosal immunity.

Mammalian pIgR carried out functions in combining and transporting polymeric immunoglobulin in mucosal tissues. The binding and transporting activities of fish pIgR for pIg were also reported in fugu, common carp, and rainbow trout [9,22,24], indicating that fish pIgR had a similar function as mammalian pIgR. However, in this experiment, the grass carp rpIgR-ILD could only bind to IgM at high concentration and the binding activity was rather weak. This may be due to the lack of glycosylation in the process of prokaryotic expression of recombinant protein. In addition, several reports indicated that teleost mucosal IgM was produced independently to systemic Abs [35–37]. Besides the important function of transporting the secreting Ig, pIgR was also found to play an important role in recognizing bacteria in recent studies. Rainbow trout pIgR can bind to commensal bacteria, such as *V. anguillarum* and *E. ictaluri* [16]. Sea bass pIgR can bind to *E. coli*, *A. hydrophila*, and *B. subtilis* [29]. In this study, grass carp rpIgR-ILD also displayed binding activity with Gram-negative and Gram-positive bacteria. Furthermore, the rpIgR-ILD exhibited different binding abilities to different bacteria. A relatively strong affinity to rpIgR-ILD was observed with *B. subtilis* and *E. coli*, compared with *V. parahaemolyticus*. But the combination of grass carp rpIgR-ILD with certain kinds of bacteria cannot be detected, such as *A. hydrophila* and *V. anguillarum*. This maybe because the content and structure of the bacterial protein that interact with rpIgR-ILD was different in different kinds of bacteria. This result was different from that of the rainbow trout and sea bass pIgR. There were possibilities that the recognition spectrum of bacteria was different among different fish pIgRs.

In summary, in this study, *pIgR* and its variants were cloned and characterized in grass carp, and showed sequence difference and motif variety among them. Total gene expression of *pIgR* was found in different tissues and was relatively high in immune tissues. Grass carp recombinant rpIgR-ILD was achieved by prokaryotic expression system, which showed combining capacity with grass carp IgM and certain kinds of bacteria. Therefore, based on the findings in this study, it was suggested that grass carp pIgRs were involved in mucosal immunity against bacterial infection and could play an important role in immune defense. Our report also helps to understand the diversity of grass carp pIgR gene and to further study their functions in immunity in the future.

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