Fish and Shellfish Immunology 87 (2019) 524–533

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Molecular cloning of polymeric immunoglobulin receptor-like (pIgRL) in flounder (*Paralichthys olivaceus*) and its expression in response to immunization with inactivated *Vibrio anguillarum*

Susu Liu[a], Yang Du[a], Xiuzhen Sheng[a,*], Xiaoqian Tang[a], Jing Xing[a], Webin Zhan[a,b]

[a] Laboratory of Pathology and Immunology of Aquatic Animals, KLMME, Ocean University of China, 5 Yushan Road, Qingdao, 266003, PR China
[b] Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Qingdao, 266071, PR China

ARTICLE INFO

Keywords:
Polymeric immunoglobulin receptor-like (pIgRL)
Flounder (*Paralichthys olivaceus*)
Gene cloning
Immune response
Mucosal immunity

ABSTRACT

In the present work, the polymeric immunoglobulin receptor-like (pIgRL) from flounder (*Paralichthys olivaceus*) was firstly cloned and identified. The full length cDNA of flounder pIgRL was of 1393 bp including an open reading frame of 1053 bp, and the deduced pIgRL sequence encoded 350 amino acids, with a predicted molecular mass of 39 kDa. There were two immunoglobulin-like domains in flounder pIgRL. In healthy flounder, the transcriptional level of pIgRL was detected in different tissues by real-time PCR, showing the highest level in the skin and gills, and higher levels in the spleen and hindgut. After flounders were vaccinated with inactivated *Vibrio anguillarum* via intraperitoneal injection and immersion, the pIgRL mRNA level increased firstly and then declined in all tested tissues during 48 h, and the maximum expression levels in the gills, skin, spleen and hindgut in immersion group, or in the spleen, head kidney, skin and gills in injection group, were higher than in other tested tissues. In addition, recombinant protein of the extracellular region of flounder pIgRL was expressed in *Escherichia coli* BL21 (DE3), and rabbit anti-pIgRL polyclonal antibodies were prepared, which specifically reacted with the recombinant pIgRL, and a 39 kDa protein confirmed as natural pIgRL by liquid chromatography-mass spectrometry in skin mucus of flounder. Co-immunoprecipitation assay and western-blotting demonstrated that the pIgRL, together with IgM, could be immunoprecipitated by anti-pIgRL antibody in gut, skin and gill mucus of flounder, suggesting the existence of pIgRL-IgM complexes. These results indicated that the flounder pIgRL was probably involved in the mucosal IgM transportation and played important roles in mucosal immunity.

1. Introduction

The mucosal immune system acts as the first line of immune defense, playing a pivotal defense role against a variety of pathogens [1–3]. The secretory immunoglobulins (SIgs) are considered as the main player to neutralize antigens and bind on the mucosal bacteria in mucosal defenses [4]. To exert its protective effect, the SIgs is transported across epithelial cells to mucus by polymeric immunoglobulin receptor (pIgR). In mammals, pIgR is mainly expressed on the basolateral surface of epithelial cells and could ensure efficient secretion of polymeric IgA and IgM, which are produced by local plasma cells [5,6]. The pIgR mediates transport of SIgs across the glandular and mucosal epithelial cells, at the apical surface, the extracellular ligand-binding region of pIgR undergoes a cleavage event in which the secretory component (SC) of pIgR is released as a free form or bound to IgA as the secretory IgA complex (SIgA), protecting SIgs from proteolytic degradation [7]. These have expanded our view of the immunobiology of pIgR that bridges innate and adaptive immune defense.

Up to know, pIgR cDNA has been cloned in all vertebrates, including mammals [8], birds [7], reptiles [9], amphibians [10]and fish [11]. In mammals, pIgR encodes five immunoglobulin-like domains (ILDs: D1-D5), while avian, reptilian and amphibian pIgR consists of four ILDs (D1-D3-D4-D5) [7,12]. However, only two ILDs have been reported in teleost fish, including dojo loach (*Misgurnus anguillicaudatus*), carp (*Cyprinus carpio*), flounder (*Paralichthys olivaceus*) and rainbow trout (*Oncorhynchus mykiss*) [13–16]. The two ILDs of teleost are homologous to mammal pIgR ILD1 and ILD5, respectively [7]. In teleost fish, the IgM and IgT are detectable at the protein level in the gut, skin and gill mucus. IgM is a predominant isotype in teleost body fluids, responding to pathogenic challenge in systemic and mucosal compartments of many fish species including turbot (*Scophthalmus maximus*), sea bream (*Sparus aurata*) and flounder [16–19]. While IgT was recently

* Corresponding author.
E-mail address: xzsheng@ouc.edu.cn (X. Sheng).

https://doi.org/10.1016/j.fsi.2019.01.039
Received 11 October 2018; Received in revised form 19 January 2019; Accepted 25 January 2019
Available online 31 January 2019

Table 1
Primers used in the present study.

Primer name	Primer sequence (5′-3′)
Core segment PCR	
pIgRL-F	5′-GGATGYGCHGGNGGNTGG
pIgRL-R	5′- TTDARDCCRCACCARTA
RACE PCR	
pIgRL 3′-RACE 1st	5′-AATACAAGAAATGACAAGTGGGAAAGAT-3′
pIgRL 3′-RACE nested	5′-CTCAGAGTCCAACGTCAAGTTCTTC-3′
pIgRL 5′-RACE 1st	5′-GCCGCTTGTTGTGATTGTGAGTGAG-3′
pIgRL 5′-RACE nested	5′-TCACAGGTGTCACTATTATTTTTGCAG-3′
3′-CDS	5′-AAGCAGTGGTATCAACGCAGAGTAC(T)30VN-3′
Oligo dT	5′-TTTTTTTTTTTTTTT-3′
UPM-long	5′-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3′
UPM-short	5′-CTAATACGACTCACTATAGGGC-3′
NUP	5′-AAGCAGTGGTATCAACGCAGAGT-3′
5-AP-DG	5′-AAGCAGTGGTATCAACGCAGAGTACGCGGGGGGGGGG-3′
RT-PCR	
pIgRL-eF1	5′-TAAAGAAGGGGATGCTGGG-3′
pIgRL-eR1	5′-TCGGAGGCCATTCACTGTA-3′
qRT-PCR	
pIgRL-qF1	5′- GCGGCTTCAACGTTTCCAT-3′
pIgRL-qR1	5′- GCCGATGCAGGAGTTGGTG-3′
β-ActinF	5′-AGAGCAAGAGAGGCATCCTGAC-3′
β-ActinR	5′-CGATGGGTGATGACCTGTCC-3′
18sRNA-F	5′-GGTCTGTGATGCCCTTAGATGTC-3′
18sRNA-R	5′-AGTGGGGTTCAGCGGGTTAC-3′
Recombinant primes	
pIgRL-rF1	5′-CGGGATCCGAGTCTGTCGTGAGGGGATGTG-3′
pIgRL-rR1	5′-CCCTCGAGTCCTTGATTCTCAGCCGATGC-3′

considered as a new Ig class in mucosal surfaces in fish and may play role in mucosal immunity. The pIgR has been reported to able to transport secretory IgM in fugu (*Takifugu rubripes*), carp and flounder [11,15,20], and IgT in rainbow trout [21], also, mediate the immune excretion of mucosal IgM-antigen complexes across intestinal epithelium into gut mucus in *P. olivaceus* [22], playing an essential role in mucosal immune response and effective maintenance of immune homeostasis.

Recently, pIgR homologs have been identified in Atlantic salmon, zebrafish and carp [23–25], these pIgR-like (pIgRL) molecules contain two ILDs, sharing structural similarities with teleost pIgR. Evidences indicate that the two-domain containing teleost pIgR may function similarly [11,14,16,26], and the homolog of the pIgR has a similar role in teleost [23–25]. In Atlantic salmon, high pIgRL expression is found in mucosal tissues such as skin and gills, and also positively responds to the infection by *Lepeophtherirus salmonis* [23]. Zebrafish pIgRL is highly expressed after bacterial infection and can bind phospholipids [24]. While the carp pIgRL is secreted upon immune stimulation as a soluble immune-type receptor (SITR) [25]. However, the knowledge about teleost pIgRL is still limited compared to the pIgR.

In the present study, the flounder pIgRL gene was firstly cloned and characterized, and its expression in flounder tissues was detected before and after immunized with inactivated *V. anguillarum* by injection and immersion. Moreover, the recombinant pIgRL (rpIgR) protein was expressed and the polyclonal antibody was developed, and reactions between the developed polyclonal antibody and the rpIgRL protein, as well as the native pIgRL molecule in skin mucus and serum, were analyzed. In addition, the binding capability of the flounder pIgRL to IgM in gut, skin and gill mucus was investigated by co-immunoprecipitation assay and western-blotting. These studies could help to elucidate the function of pIgRL in mucosal immunity.

2. Materials and methods

2.1. Fish and antibodies

Healthy flounders with length of 10–15 cm were purchased from a fish farm in Qingdao of Shandong province, China. Flounders were maintained in aerated running seawater at 21 ± 1 °C and fed with commercial diet twice daily. After acclimation for 2 weeks, flounders were employed for experiments. In this study, the methods used in the animal experiments were approved by the Committee on the Ethics of Animal Experiments of Ocean University of China (permit number: 20150101).

Mouse monoclonal antibody (MAb) 2D8 against flounder IgM which could recognize the heavy chain of serum [27] and mucus IgM [19], were previously produced in our laboratory.

2.2. Cloning of the full-length pIgRL of flounder

Three healthy flounders were anesthetized with 100 mg/L Tricaine methanesulfonate (MS-222), and the spleen, skin, gills, stomach, pyloric caeca, foregut, midgut, hindgut, liver, heart, trunk kidney, head kidney and peripheral blood leucocytes were isolated, immediately frozen in liquid nitrogen and then stored at −80 °C prior to use.

The spleen of flounder was homogenized with TRIzol reagent (Invitrogen, USA), and total RNA was extracted using the E.Z.N.A HP Total RNA Kit according to the manufacturer's instructions (Omega BioTek, USA). The RNA concentration and integrity were measured using NanoDrop ND-1000 (NanoDrop Technologies, USA) and agarose gel electrophoresis. Subsequently, the first strand cDNA was synthesized using SuperScript Reverse Transcriptase (Omega BioTek, USA) following the manufacture's protocol. For the isolation of the flounder pIgRL cDNA fragment, multiple alignments of the amino acid sequences of several other flounders were performed using the BioEidt multiple sequence alignments program. Degenerate primers were designed based on the highly conserved sequences of *Monopterus albus* pIgRL (XP_020444821), *Lates calcarifer* pIgRL (XP_018558444) and *Larimichthys crocea* pIgRL (XP_019122446). A cDNA fragment of the flounder pIgRL was amplified using degenerate primers pIgRL-F and pIgRL-R. Then rapid amplifications of cDNA ends (RACE) were performed using the SMART RACE cDNA amplification Kit (BD Bioscience, USA) to get the full-length of pIgRL cDNA sequences by the specific primers which were designed based on the obtained partial sequence. All primers mentioned above were listed in Table 1.

2.3. Sequence analysis

The homology search of nucleotide and protein sequence of flounder pIgRL was conducted with BLAST program (http://www.ncbi.nlm.gov/blast) and then multiple alignments of amino acid sequences were performed by the BioEidt multiple sequence alignments program [28]. Moreover, the structural features of protein sequence were predicted by SMART (http://smart.embl-heidelberg.de/). The open reading frame (ORF) was identified using an ORF Finder frames [29]. Phylogenetic tree of flounder pIgRL based on the neighbor-joining (NJ) method were constructed by the MAGE7.0 [30]. In case, bootstrap values were performed based on 1000 replicates. The potential N-glycosylation sites and the molecular weight of the deduced pIgRL proteins were calculated by the EXPERT Protein Analysis System [31].

2.4. Quantitative analysis of pIgRL mRNA in healthy flounder

Total RNA of the spleen, skin, gills, stomach, pyloric caeca, foregut, midgut, hindgut, liver, heart, trunk kidney, head kidney and peripheral blood leucocytes were isolated as mentioned above. The cDNA templates were prepared as previously described.

The expressions of pIgRL mRNA in detected tissues of healthy flounder were determined by RT-PCR. Specific primers (pIgRL-eF1 and pIgRL-eR1) were used to amplify pIgRL gene fragment. While β-ActinF and β-ActinR were applied to amplify actin gene fragment as internal control. The PCR products were gel-extracted, and the cloned amplicons were determined by the specific-sized single application. The primers used for RT-PCR were listed in Table 1.

2.5. Fish immunization and sampling

Healthy flounders were divided into two groups and transferred into tanks (30 fish/tank). The inactivated *V. anguillarum* was prepared as described previously [32,33]. Group A was vaccinated with formalin-killed *V. anguillarum* (1.0×10^8 CFU/mL) by immersion for 60 min, and then removed to fresh seawater; group B was immunized with formalin-killed *V. anguillarum* (0.2 mL; 1.0×10^8 CFU/mL) by intraperitoneal (IP) injection; The gills, skin, spleen, head kidney, liver, hindgut, stomach, and muscle were randomly collected from three flounders from each group before treatment (0h) and at 12 h, 24 h and 48 h post immunization, all tissue samples were immediately stored at −80 °C and used to extract total RNA for qRT-PCR assay.

For serum and gut, skin and gill mucus collection, three flounders were randomly sampled at 21 d after immersion immunization with formalin-killed *V. anguillarum* (1.0×10^8 CFU/mL). The blood was collected by venipuncture, and allowed to clot at room temperature and kept at 4 °C overnight. The serum was obtained by centrifugation at 3000 × *g* for 20 min and then stored at −80 °C for next use. The skin, gut and gill mucus were collected as described by previous studies [16,21,34]. Briefly, flounder skin mucus was gently scraped from the skin surface, and transferred into an Eppendorf tube and vigorously vortexed. After blood drawing, gill arches were excised and rinsed with PBS three times to remove the remaining blood and vortexed vigorously in sample buffer containing 1 mM phenylmethylsulfonyl fluoride, 0.5% BSA and 10 mM PBS (pH 7.2). For the collection of gut mucus, the flounder hindgut was collected and opened longitudinally, and 0.5 mL sample buffer was added onto its surface. Afterwards, approximately same volume of mucosal fluid from each flounder was gently scraped from the inner surface of the gut and transferred to a centrifuge tube, followed by vigorous vortexing. All sampled mucus was centrifuged at 12,000 × *g* for 10 min at 4 °C, and the mucus supernatant was collected and stored immediately at − 80 °C until use.

2.6. qRT-PCR assay

Total RNA and cDNA were prepared from tissue samples collected before treatment (0h), and at 12 h, 24 h and 48 h post immunization as described above. qRT-PCR was performed using a Roche480 real-time PCR system (LightCycler480, CA, USA). A fragment of flounder pIgRL gene, about 160 bp, was amplified by the specific primers pIgRL-qF1 and pIgRL-qR1 (Table 1), and two primers 18sRNA-F and 18sRNA-R (Table 1) of flounder were used to amplify 18sRNA gene fragment as internal controls.

2.7. Production of flounder pIgRL recombinant proteins and antibodies

The extracellular region of the flounder pIgRL was amplified using specific primers (pIgRL-rF1 and pIgRL-rR1). The PCR product was digested with restriction enzymes BamH I and Xho I after sequencing, and then ligated into a modified pET-28a plasmid with an N-terminal 6 × His-Tag (Novagen) to construct pET-28a-pIgRL. The recombinant plasmid (pET-28a-pIgRL) was transformed into *Escherichia coli* BL21 (DE3) (Novagen). Positive clones were confirmed by PCR and sequencing, and then inoculated into LB medium containing 50 μg mL^{-1} of kanamycin. After incubation at 37 °C with shaking to a mid-logarithmic phase of growth, the production of recombinant pIgRL (rpIgRL) was induced by adding 0.5 mM isopropyl β-D-1-thiogalactosidase (IPTG) and incubated for 6h. The *E. coli* BL21 (DE3) containing pET-28a-pIgRL without induction was selected as a negative control under the same conditions. The rpIgRL was purified by His Trap™ HP Ni-Agarose (GE healthcare, China) as described in the manufacturer's instruction. The induced bacteria lysate and purified rpIgRL were analyzed by SDS-PAGE and visualized with Coomassie Brilliant Blue R-250. The concentration of rpIgRL was quantified according to the Bradford method [35].

The purified rpIgRL was used to immunize New Zealand white rabbits to produce the rabbit anti-pIgRL polyclonal antibody. The immunization procedure was performed as previously described [27]. The titer of the polyclonal antibody against pIgRL was determined by indirect enzyme-linked immunosorbent assay (ELISA). Briefly, the wells of 96-well plates (Costar, USA) were coated with purified rpIgRL (20 μg/well) in 100 μL phosphate buffered saline (PBS) and incubated overnight at 4 °C. Subsequently, wells were washed thrice with PBS containing 0.05% Tween-20 (PBST) and blocked with 3% bovine serum albumin (BSA) in PBS for 1 h at 37 °C. After washing as above, wells were incubated with 100 μL rabbit anti-pIgRL polyclonal antibodies diluted at the ratios of 1:1000, 1:2000, 1:4000, 1:8000, 1:16000, 1:32000, 1:64000, and 1:1280000 with PBS for 1 h at 37 °C. Following a further washing, 100 μL of goat anti-rabbit Ig-alkaline phosphatase conjugate (Sigma, USA) diluted 1:4000 in PBS was added and incubated for 1 h at 37 °C. After a final washing, 100 μL 0.1% (w/v) *p*-nitrophenyl phosphate (*p*NPP, Sigma, USA) in 50 mM carbonate-bicarbonate buffer (1% diethanolamine, 0.5 mM $MgCl_2$, pH 9.8) was added to each well and incubated for 20 min at room temperature in dark. The reaction was stopped with 50 μL/well of 2 M NaOH and absorbance was measured with an automatic ELISA reader at 405 nm (Molecular Devices, USA). As negative control, the non-immune rabbit serum was used as the first antibody. Each experiment was conducted in triplicate.

2.8. Western blotting

In order to characterize the polyclonal antibody specificity, the reactivity of the recombinant proteins, serum and skin mucus to the polyclonal antibody was detected. The induced bacteria lysate, purified rpIgRL, serum and skin mucus were subjected to SDS-PAGE, and then the samples were transferred onto a PVDF membrane (Millipore, USA). The membrane was blocked with 3% BSA in PBS for 1 h, and incubated with rabbit anti-pIgRL polyclonal antibodies (diluted 1:5000 in PBS) for 1 h at 37 °C and washed thrice with PBST. Thereafter, the membrane was incubated with goat-anti-rabbit Ig-alkaline phosphatase conjugate (Southern Biotech) diluted 1:4000 in PBS for 1 h at 37 °C, followed by washing three times with PBST. Finally, the bands were stained with

fresh substrate solution (100 mM NaCl, 100 mM Tris and 5 mM $MgCl_2$, pH 9.5) containing nitroblue tetrazolium (NBT, Sigma) and 5-bromo-4-chloro-3-indolyphosphate (BCIP, Sigma) for 5 min, and stopped by washing with distilled water. The non-immune rabbit serum was used as negative control. The immune-reactive proteins from skin mucus was excised from polyacrylamide gels, and then analyzed by liquid chromatography-mass spectrometry (LC-MS/MS) assay system (Applied Biosystems, USA).

2.9. Co-immunoprecipitation

Co-immunoprecipitation experiment was performed to confirm the association between the flounder pIgRL and mucus IgM [36]. Briefly, 20 μL Protein G agarose (Santa Cruz Biotechnology) reacted with 5 μL rabbit anti-pIgRL polyclonal antibodies in 1 mL PBS for 4h at room temperature, and then washed three times with PBST by centrifugation at 1000 × *g* for 10 min. Afterwards, 1 mL skin, gut and gill mucus was added and incubated for 4 h at 22 °C, respectively. Non-immune rabbit serum and PBS instead of rabbit anti-pIgRL polyclonal antibodies were performed as negative control and blank control, respectively. Subsequently, co-immunoprecipitation samples were subjected to SDS-PAGE in duplicates. One was stained with Coomassie blue R-250, and another went through western blotting analysis by using mouse anti-serum IgM Mab 2D8 as primary antibody and goat anti-mouse IgG as secondary antibody. Non-immune mouse serum instead of anti-serum IgM Mab 2D8 served as negative control.

2.10. Statistics

Differences were analyzed with one-way analysis of variance (ANOVA) and Dunn's multiple comparison tests. The results were expressed as mean ± standard error of mean. In all cases, the difference level was defined as $P < 0.05$.

3. Results

3.1. Molecular characterization of flounder pIgRL

The pIgRL gene was identified from flounder by RACE method and submitted to NCBI under GenBank accession number KY436367. The full length of flounder pIgRL cDNA was of 1393 bp, including an ORF of 1053 bp, a 5′ untranslated region (UTR) of 20 bp, and a 3′ UTR of 320 bp. The 3' UTR had a putative polyadenylation signal sequence AATAAA located upstream of the poly (A) tail (Fig. 1A). The deduced pIgRL amino acid sequence encoded 350 amino acids, which possessed a signal peptide, an extracellular region containing two immunoglobulin-like domains, a transmembrane region and an intracellular region (Fig. 1B). In addition, DxGxYWC motifs were highly conserved in the flounder pIgRL (Fig. 1B). The molecular mass of the flounder pIgRL was about 39 kDa, and six N-glycosylation residues on the extracellular region were predicted.

3.2. Multiple sequences alignment and phylogenetic analysis

The BLAST analysis showed that the deduced amino acid sequence of flounder pIgRL shared significant identity with other reported pIgRL. Flounder pIgRL exhibited 41% amino acid sequence identity with *M. albus* pIgRL, 39% with *L. bergylta* pIgRL and 38% with *L. calcarifer* pIgRL. Protein sequence analysis and BioEdit multiple alignments revealed that the two immunoglobulin-like domains (ILDs) of flounder pIgRL resembled other fish (Fig. 2). Moreover, as shown in phylogenetic tree, the homologous pIgRL of amphibian, avian and mammalian species grouped together, while the flounder pIgRL was clustered in teleost (Fig. 3).

3.3. Tissue distribution of pIgRL in healthy flounder

The pIgRL mRNA expression was analyzed in flounder tissues by RT-PCR, and the results showed that pIgRL expression levels were the highest in skin and gills, and higher in spleen and hindgut, followed by foregut, peripheral blood leucocytes, muscle, liver, heart, pyloric caeca, trunk kidney, midgut, head kidney and stomach (Fig. 4).

3.4. pIgRL expression in response to inactivated V. anguillarum

The expression of flounder pIgRL mRNA in all tested tissues were significantly upregulated after IP injection and immersion immunization with inactivated *V. anguillarum* as compared with before immunization (0h) (Fig. 5), showing a similar change trend that increased firstly and then declined. The pIgRL expression peaked at 12 h in spleen of injection group and in gills, skin and hindgut of immersion group, and at 24 h in other tissues of the two groups. Compared with before immunization, the peak value of pIgRL expression was 22.5-fold in spleen, 15.5-fold in head kidney, 15.2-fold in skin, 14.3-fold in gills, 11.0-fold in muscle, and 10.8-fold in hindgut, 10.5-fold in stomach, 9.8-fold in liver in injection group, while in immersion group, which was 16.8-fold in gills, 16.0-fold in skin, 15.8-fold in spleen, 13.7-fold in hindgut, 11.6-fold in liver, 9.6-fold in head kidney, 9.2-fold in stomach and 7.9-fold in muscle respectively. The maximum expression levels of pIgRL in gills, skin, spleen and hindgut in immersion group, or in spleen, head kidney, skin and gills in injection group, were higher than in other tissues.

3.5. Purification of recombinant pIgRL protein

A distinct band at 28.4 kDa was observed in SDS-PAGE, showing that the extracellular region of the flounder pIgRL was successfully expressed in *E. coli* BL21 (DE3) with pET-28a system after IPTG induction, which was in accordance with the predicted molecular mass of fusion rpIgRL containing approximately 7 kDa His-tag. After purification with the Ni^{2+} affinity chromatography, the rpIgRL protein with high purity was obtained (Fig. 6A, lane 3).

3.6. Specificity of anti-pIgRL antibody and pIgRL detection in mucus

The ELISA results showed that the rabbit anti-pIgRL antibodies diluted 1:64000 still had positive signals (P/N = 2.4) (data not shown). Western-blotting result indicated that the rabbit anti-pIgRL antibody could specifically react with rpIgRL protein in the induced transformed *E. coli* and the purified rpIgRL (Fig. 6A, lane 5 and 6), but no positive band was detected in the cells without IPTG induction (Fig. 6A, lane 4), revealing anti-flounder pIgRL antibody had good specificity.

The flounder pIgRL was detected in skin mucus (Fig. 6B, lane 3) but not in the serum (Fig. 6B, lane 4) by rabbit anti-pIgRL polyclonal antibody. The molecular mass of immune-reactive band was about 39 kDa, which was near the theoretical mass obtained from the sequence of the flounder pIgRL. LC-MS/MS results demonstrated that this 39 kDa protein was identified to be the native pIgRL of flounder with 40% coverage of amino acid sequences (Fig. 7).

3.7. Mucus IgM-pIgRL binding analysis

In order to determine if the flounder pIgRL was able to bind mucus IgM, the skin, gill and gut mucus of the flounder were analyzed by co-immunoprecipitation assay using rabbit anti-pIgRL antibody. The SDS-PAGE results demonstrated that five protein bands at 74 kDa, 50 kDa, 39 kDa, 25 kDa and 24 kDa were specifically precipitated in skin, gill and gut mucus of flounder (Fig. 8A, B, C, lane 1), while the 50 and 25 kDa protein bands were also found in the control using non-immune rabbit serum replacing rabbit anti-pIgRL antibody (Fig. 8A, B, C, lane 2), but no band was observed in the control using PBS instead of rabbit

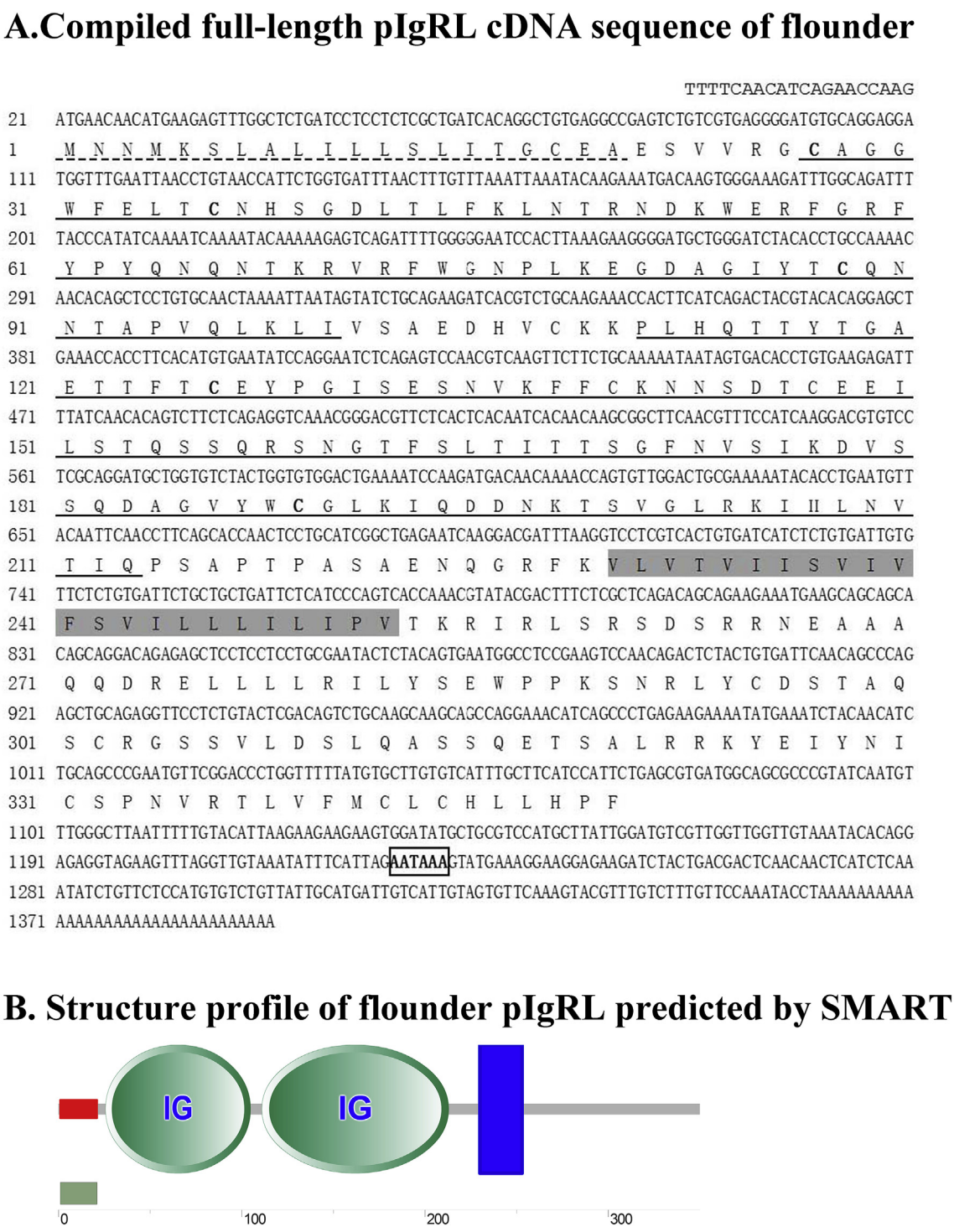

Fig. 1. **(A)** Nucleotide and deduced amino acid sequences of flounder pIgRL (GenBank accession number KY436367). The polyadenylation signal (AATAAA) in the 3′-UTR was boxed. The signal peptide sequence was indicated by the dotted line. The two ILDs were underlined. The transmembrane region was shaded in dark grey. **(B)** Structure profile of flounder pIgRL predicted by SMART. Red: signal peptides; blue: transmembrane segments; IG: Ig-like domain. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

anti-pIgRL antibody (Fig. 8A, B, C, lane 3), indicating that the 50 and 25 kDa proteins were the heavy and light chain of rabbit IgG, and the 74 kDa, 39 kDa and 24 kDa were in accordance with the molecular mass of the heavy chain of flounder IgM, the pIgRL and light chain of flounder IgM, respectively. Thereafter, western-blotting was carried out by using anti-IgM Mab 2D8 as first antibody, and the results showed that the 74 kDa protein in skin, gill and gut mucus were recognized by anti-IgM MAb 2D8 (Fig. 8A, B, C, lane 4), revealing that the IgM in skin, gill and gut mucus of flounder could be immunoprecipitated together with the pIgRL by anti-pIgRL polyclonal antibody.

4. Discussion

In teleost, four mucosa-associated lymphoid tissues (MALT) have been described: the gut-associated lymphoid tissue, the skin-associated lymphoid tissue, the gill-associated lymphoid tissue and the recently

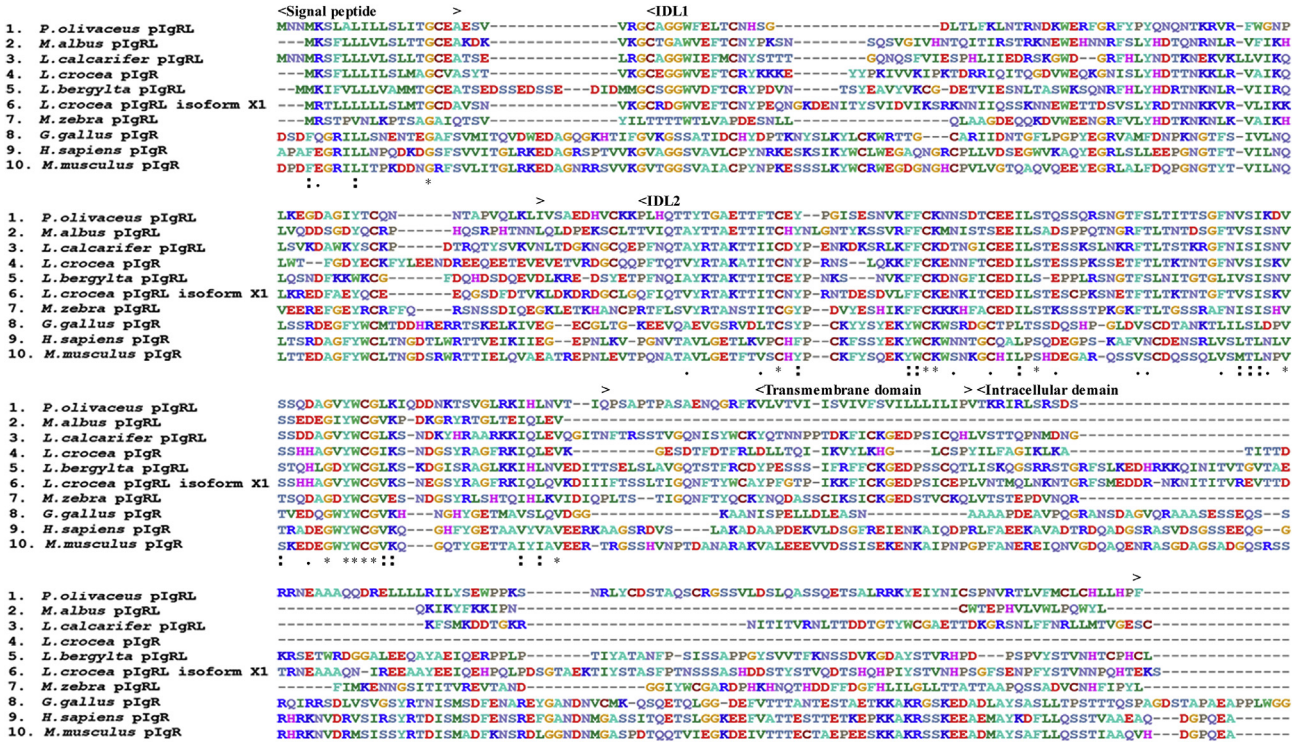

Fig. 2. Multiple alignments of flounder pIgRL with other known pIgRL and pIgR deduced amino acid sequences: *Monopterus albus* pIgRL (XP_020444821), *Lates calcarifer* pIgRL (XP_018558444), *Larimichthys crocea* pIgR (KKF22826), *Labrus bergylta* pIgRL (XP_020515087), *Larimichthys crocea* pIgRL (XP_019122446), *Maylandia zebra* pIgRL (XP_012776701), *Gallus gallus* pIgR (AAP69598), *Homo sapiens* pIgR (NP_002635) and *Mus musculus* pIgR (AAA67440). Residues identical in all sequences and highly conserved sequences were indicated by a star (*) and a colon (:), respectively.

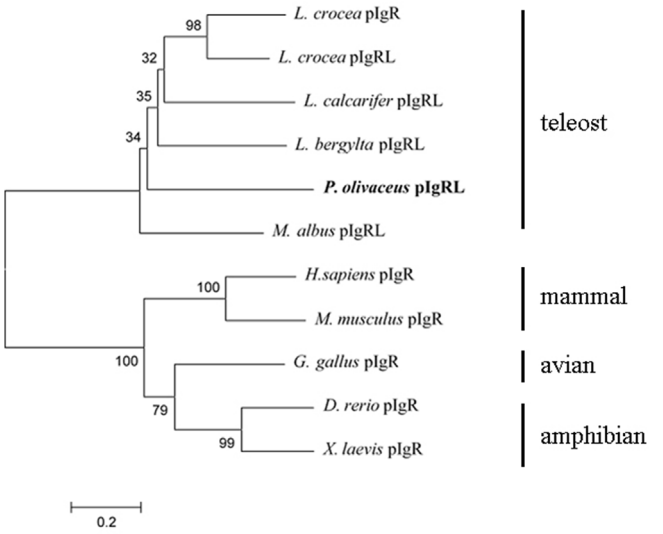

Fig. 3. Neighbor-join in phylogenetic tree of pIgRL mature peptide from flounder and other vertebrates. The tree was constructed using MEGA 7.0 software. Aligned sequences were bootstrapped 1000 times and the numbers at the forks indicated the bootstrap proportions. A scale bar was shown below the tree. The accession number of *Xenopus laevis* (ADB97624), *Danio rerio* (XP_694833) and other accession numbers used were as in Fig. 3.

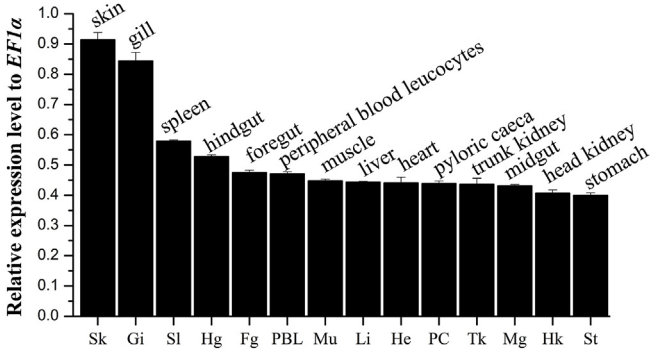

Fig. 4. RT-PCR analysis of the pIgRL mRNA transcripts in tissues of healthy flounder. Relative expression of the flounder pIgRL was detected in different tissues (n = 3).

discovered nasopharynx-associated lymphoid tissue, which are the first line of defense against pathogens [37]. The pIgR, expressed by lymphoid organs including mucosal tissues [11,14,23,26], mediates polymeric immunoglobulin transcytosis from the basolateral to the luminal surface of the epithelial cells, playing a pivotal role in mucosal immunity [2,21,34]. Previously, we have cloned the flounder pIgR [15] and found flounder pIgR can mediate immune excretion of mucosal IgM-antigen complexes across intestinal epithelium [22]. The homolog of the teleost pIgR (pIgRL) has been reported in several teleost species, which also plays a crucial role in mucosal immune response during infection with different pathogens [23,24], but no data on flounder pIgRL is found. In this study, we firstly characterized the nucleotide and protein sequence of the flounder pIgRL, analyzed its expression pattern in different tissues in healthy flounder and the upregulated expression in response to immunization with inactivated *V. anguillarum* via IP injection and immersion, and determined that the pIgRL-IgM complexes existed in skin, gill and gut mucus, our results indicated that the pIgRL also involved in mucosal immunity in flounder.

The mammalian pIgR contains five ILDs [38] and avian and amphibian pIgR consists four ILDs [12], while the teleost pIgR contains only two ILDs [11,14,26]. Sequence analysis between teleost pIgR and mammalian pIgR shows that these two ILDs may be the essential parts for successful binding to IgM [7]. The pIgRL has been identified in some

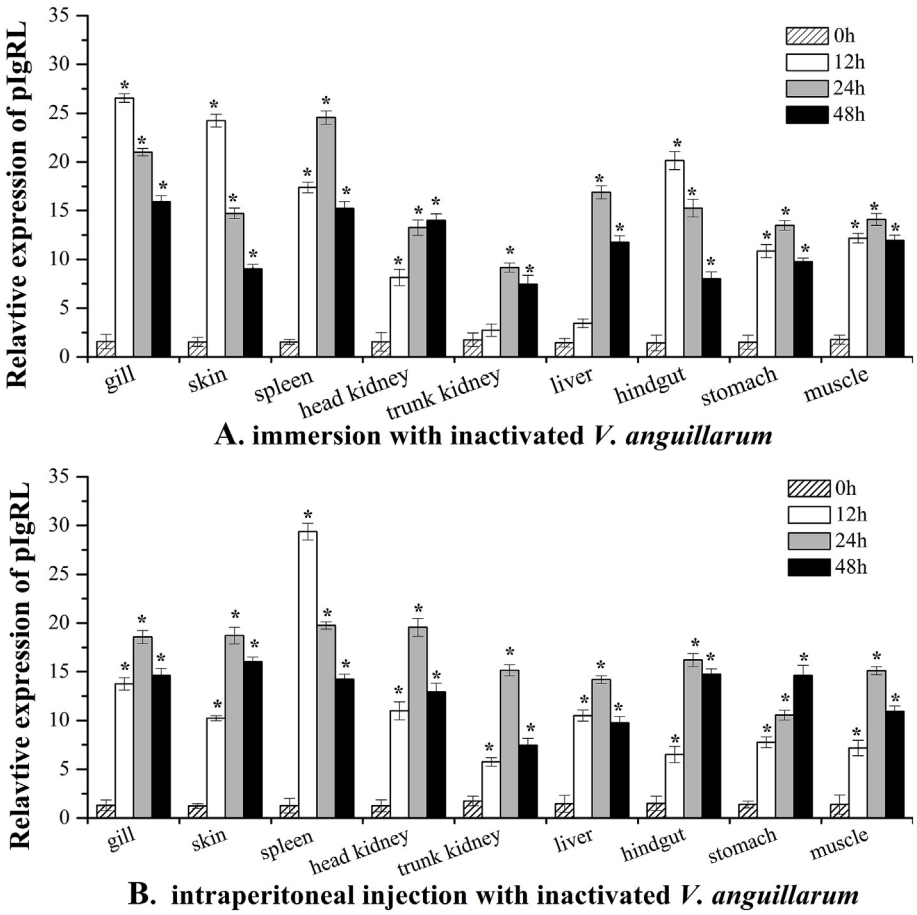

Fig. 5. The flounder pIgRL mRNA expression level in different tissues in response to formalin-killed *V. anguillarum* by qRT-PCR. **(A)** Different tissues of flounder after immersion with formalin-killed *V. anguillarum* bacteria (1.0×10^8 CPU/mL for 60 min). **(B)** Different tissues of flounder after IP injection with formalin-killed *V. anguillarum* bacteria (0.2 mL; 1.0×10^8 CPU/mL per fish). Values were present as means ± error of mean (n = 3). *$P < 0.05$.

teleost, which is similar to the reported putative pIgR polypeptides (thus termed pIgR-like) in many aspects, i.e. it is composed of an extracellular region encoding two ILDs, a connecting peptide, a transmembrane and a cytoplasmic region [23]. In the present study, we found a new molecule in flounder is similar to other teleost pIgRLs and pIgRs, which composed of two ILDs. Moreover, the new molecule had typical conserved amino acid residues, DxGxYxC motifs (x stands for any amino acid), in accordance with other known pIgRs and pIgRLs. There were the common DxGxYxC motifs in the ILD1 of the vertebrate pIgRs [15]. The deduced amino acids sequence of the new molecule shared identity with the reported pIgRL including *M. albus* pIgRL (41%), *L. bergylta* pIgRL (39%) and *L. calcarifer* pIgRL (38%), therefore, we termed this new molecule as the flounder pIgRL. However, the flounder pIgRL just shared low degree (10%) of sequence similarity to flounder pIgR, so further research about the function difference resulting from the difference of their gene sequence was needed.

According to the previous reports, pIgR is one important player of mucosal defenses, mucosal tissues including skin, gill and intestine also showed relative high expression levels of pIgR in dojo loach and flounder [13,15]. In this study, different expression levels of pIgRL mRNA transcripts could be detected in mucosal and lymphoid organs of the healthy flounder, especially higher in the skin, gills, spleen and hindgut, which was consistent with the report in Atlantic salmon and zebrafish [23,24], implying that the flounder pIgRL probably involved in mucosal defense like pIgR. In order to determine the role of flounder pIgRL in the systemic and mucosal immune regulation, the flounder were vaccinated with inactivated *V. anguillarum* by IP injection and immersion, respectively, and the result showed that the pIgRL mRNA expressions were up-regulated in all detected tissues, with a similar dynamic tendency that increased firstly and then decreased within 48h,

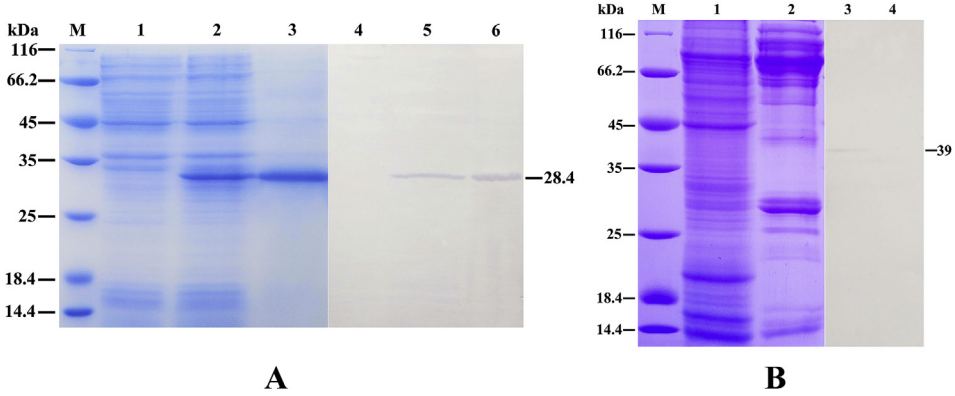

Fig. 6. Recombination pIgRL preparation and the anti-pIgRL polyclonal antibody specificity. **(A) M**: marker (kDa)**; Lane 1**: negative control without IPTG induction; **lane 2**: *E. coli* transfected with pET-28a-rpIgRL by IPTG; **lane 3**: purified rpIgRL of flounder; **lane 4**: Samples of lane 1 reacted with anti-pIgRL polyclonal antibody; **lane 5**: Samples of lane 2 reacted with anti-pIgRL polyclonal antibody; **lane 6**: Samples of lane 3 reacted with anti-pIgRL polyclonal antibody; **(B) M**: marker (kDa); **Lane 1**: skin mucus of flounder; **Lane 2**: serum of flounder; **Lane 3**: Skin mucus of flounder reacted with anti-pIgRL polyclonal antibody; **lane 4**: Serum of flounder reacted with anti-pIgRL polyclonal antibody.

A

MNNMKSLALILLSLITGCEAESVVRGCAGGWFELTCNHSGDLTLFKLNTRNDKWERFGRFYP
YQNQNTKRVRFWGNPLKEGDAGIYTCQNNTAPVQLKLIVSAEDHVCKKPLHQTTYTGAETT
FTCEYPGISESNVKFFCKNNSDTCEEILSTQSSQRSNGTFSLTITTSGFNVSIKDVSSQDAGVYW
CGLKIQDDNKTSVGLRKIHLNVTIQPSAPTPASAENQGRFKVLVTVIISVIVFSVILLLILIPVTK
RIRLSRSDSRRNEAAAQQDRELLLLRILYSEWPPKSNRLYCDSTAQSCRGSSVLDSLQASSQET
SALRRKYEIYNICSPNVRTLVFMCLCHLLHPF

B

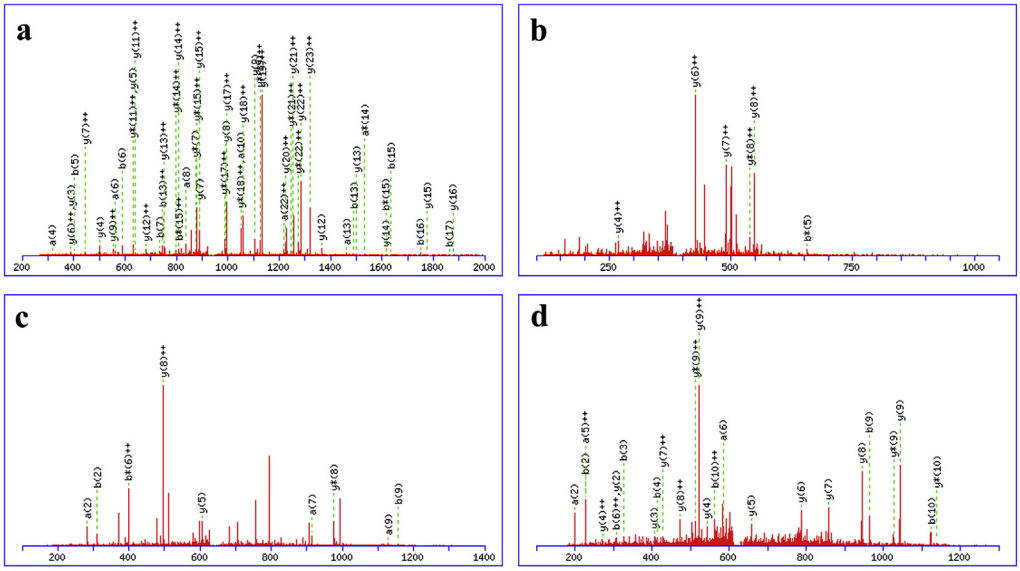

Fig. 7. Identification of the 39 kDa protein recognized by rabbit anti-pIgRL polyclonal antibodies in skin mucus using mass spectrometry. (A) The four peptides (a, b, c, d) that most matched the flounder pIgRL sequence were underlined. (B)The mass spectrometric results of the four peptides.

and the higher expression levels in gills, skin, spleen and hindgut in immersion group, or in spleen, head kidney, skin and gills in injection group, were higher than in other tissues, which indicated both injection and immersion routes all could simultaneously induce flounder pIgRL response in systemic and mucosal tissues. Similar results have been reported in Atlantic salmon and zebrafish that the pIgRL not only participated in mucosal immunity, but also systemic immunity [23,24]. As shown in dojo loach, pIgR mRNA expression is up-regulated after bathing with bacteria (*Aeromonas hydrophila*) and parasite (*Ichthyophthirius multifiliis*) infection, not only in skin and gills, but also in kidney and spleen [13]; while the pIgR mRNA expression of crucian carp (*Carassius auratus*) is also up-regulated in intestine, skin, gills, spleen, liver and head-kidney after *Aeromonas hydrophila* infection [39]. Moreover, the peak values of pIgRL mRNA in gills, skin and hindgut were obviously higher in immersion than injection group, but higher in spleen and head kidney in injection than immersion group, meanwhile, the pIgRL transcriptional levels in gills, skin and hindgut peaked more quickly in immersion than injection group, whereas they peaked earlier

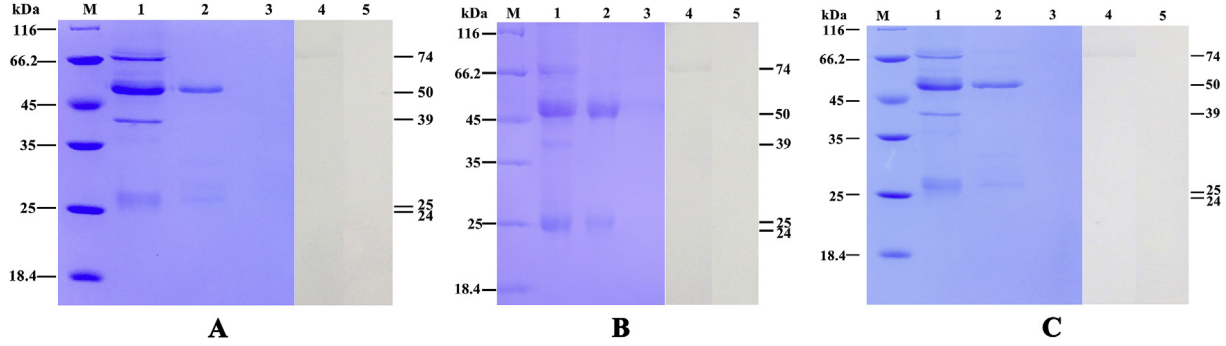

Fig. 8. The pIgRL associated IgM complexes in skin, gill and gut mucus. **(A) M**: Marker; **Lane 1**: SDS-PAGE under reducing condition analyzed the samples from co-immunoprecipition of skin mucus with rabbit anti-pIgRL polyclonal antibodies; **Lane 2**: non-immune rabbit serum instead of rabbit anti-pIgRL polyclonal antibodies as negative control; **Lane 3**: PBS instead of skin mucus as blank control. **Lane 4**: Samples of lane 1 of (A) reacted with anti-IgM MAb 2D8 analyzed by western blotting. **Lane 5**: Samples of lane 1 of (A) reacted with non-immune mouse serum. **(B) M**: Marker; **Lane 1**: SDS-PAGE under reducing condition analyzed the samples from co-immunoprecipitation of gill mucus with rabbit anti-pIgRL polyclonal antibodies; **Lane 2**: non-immune rabbit serum instead of rabbit anti-pIgRL polyclonal antibodies as negative control; **Lane 3**: PBS instead of gill mucus as blank control. **Lane 4**: Samples of lane 1 of (B) reacted with anti-IgM MAb 2D8 analyzed by western blotting. **Lane 5**: Samples of lane 1 of (B) reacted with non-immune mouse serum. **(C) M**: Marker; **Lane 1**: SDS-PAGE under reducing condition analyzed the samples from co-immunoprecipitation of gut mucus with rabbit anti-pIgRL polyclonal antibodies; **Lane 2**: non-immune rabbit serum instead of rabbit anti-pIgRL polyclonal antibodies as negative control; **3**: PBS instead of gut mucus as blank control. **Lane 4**: Samples of lane 1 of (C) reacted with anti-IgM MAb 2D8 analyzed by western blotting. **Lane 5**: Samples of lane 1 of (C) reacted with non-immune mouse serum.

in spleen in injection than immersion group. All these results illustrated that IP injection and immersion route could induce different pIgR response in the same tissue. The immersion route may stimulate the mucosal immune system earlier and much stronger local immune responses in mucosa-associated lymphoid tissues, and the local responses in skin, gills and guts could be working without yet an activation of the central organs, because of the surfaces of fish skin, gill and guts directly in contact with inactivated *V. anguillarum* in the water that might activate the local receptors and generate a first response, while after injection a response in the mucosal compartment must await systemic processing of the antigen and initiation of a systemic response in the systemic compartment, i.e., head kidney, spleen [40–42]. Collectively, our results revealed that route of vaccination would determine the location of the primary immune response, and that a disparity in pIgRL response would be observed when the same antigen was administered by different route. Interestingly, the flounder pIgRL mRNA expression was higher in the spleen regardless of the route of administration, it might be because of the lymphocytes there, since it was reported that the pIgRL expression possibly originated from lymphocytes which were the predominant cells in lymphoid organs in Atlantic salmon [23], and in carp, intraepithelial lymphocytes and lymphoid cells in the lamina propria and the thymus could express the pIgRL [25].

In teleost, evidences have revealed that pIgR can bind polymeric Igs and mediate their transcytosis [1,43]. In fugu and rainbow trout, the mucosal IgM or IgT need to bind with a pIgR for transport into the skin mucus, while the IgM or IgT in serum is free of the secretory component. We have recently found that flounder pIgR can transport IgM-antigen complexes into gut mucus [22]. However, it is not known whether the teleost pIgRL can bind IgM or IgT and how the pIgRL play a role in mucosal immunity. Previous study has found a single pIgR gene and a multigene family of 29 pIgRL genes in chromosome 2 of zebrafish, and confirms that certain pIgRL can encode secreted and membrane receptors and bind lipids and phospholipid extracts [24]. While the carp pIgRL is secreted as a soluble immune-type receptor from macrophages after infection with a parasite [25]. In the present study, the natural flounder pIgRL was confirmed to exist in skin mucus by LC-MS/MS, but not in serum, which was consistent with previous results of flounder pIgR [15]. Moreover, the binding between the flounder pIgRL and IgM was detected by co-immunoprecipitation assay and western-blotting, it was found that the IgM in skin, gill and gut mucus could be precipitated by the anti-pIgRL antibodies, indicating the existence of pIgRL-IgM complexes, therefore, the flounder pIgRL might involve in the mucosal IgM transportation and maintenance of immune homeostasis, but further studies were needed. However, whether flounder pIgRL can bind IgT is not known because of the lack of anti-flounder IgT antibody, therefore, further studies about this are needed in the future. Additionally, it was interesting that the pIgRL in skin, gill and gut mucus, recognized by the antibodies against recombinant protein of extracellular region of flounder pIgRL, was 39 kDa, similar to the predicted molecular mass of flounder pIgRL, which differed from the pIgR that was considered in the form of polymeric Ig-SC complexes in external secretions, thus more work was worth doing to make it clear.

In conclusion, a two domain containing flounder pIgRL was identified and characterized in this study, which was highly expressed in skin, hindgut, gills and spleen in healthy flounder. Upon immunization by immersion and IP injection, pIgRL mRNA expression was differentially up-regulated in different tissues, and a higher immune response was induced in gills, skin, hindgut and liver by immersion route, and in spleen and head kidney by injection route. Moreover, the flounder pIgRL was confirmed to associate with IgM in skin, gill and gut mucus by co-immunoprecipitation assay using rabbit anti-pIgRL antibodies, suggesting the existence of pIgRL-IgM complexes. All these results collectively indicated that the flounder pIgRL was involved in the mucosal IgM transportation and played important role in mucosal immunity.

Acknowledgements

This work was supported by the National Natural Science Foundation of China [grant numbers: 31730101, 31472295 and 31672685], the Fundamental Research Funds for the Central Universities [grant number: 201822015], and Taishan Scholar Program of Shandong Province.

References

[1] P. Brandtzaeg, Basic mechanisms of mucosal immunity - a major adaptive defense system, Immunologist 3 (3) (1995) 89–96.
[2] C.S. Kaetzel, The polymeric immunoglobulin receptor: bridging innate and adaptive immune responses at mucosal surfaces, Immunol. Rev. 206 (1) (2005) 83–99.
[3] J.M. Woof, J. Mestecky, Mucosal immunoglobulins, Immunol. Rev. 206 (1) (2005) 64–82.
[4] C. Kelly, F. Takizawa, I. Salinas, et al., Rainbow trout (*Oncorhynchus mykiss*) secretory component binds to commensal bacteria and pathogens, Sci. Rep. 7 (2017) 417–453.
[5] B. Per, P. Reinhard, Let's go mucosal: communication on slippery ground, Trends Immunol. 25 (2004) 570–577.
[6] P. Brandtzaeg, H. Kiyono, R. Pabst, et al., Terminology: nomenclature of mucosa-associated lymphoid tissue, Mucosal Immunol. 1 (1) (2008) 31–37.
[7] S. Akula, S. Mohammadamin, L. Hellman, Fc receptors for immunoglobulins and their appearance during vertebrate evolution, PLoS One 9 (5) (2014) e96903.
[8] W.H. Wieland, D. Orzáez, A. Lammers, et al., A functional polymeric immunoglobulin receptor in chicken (*Gallus gallus*) indicates ancient role of secretory IgA in mucosal immunity, Biochem. J. 380 (3) (2004) 669–676.
[9] S. Magadán-Mompó, C. Sánchez-Espinel, F. Gambón-Deza, IgH loci of American alligator and saltwater crocodile shed light on IgA evolution, Immunogenetics 65 (7) (2013) 531–541.
[10] R. Braathen, V.S. Hohman, P. Brandtzaeg, et al., Secretory antibody formation: conserved binding interactions between J chain and polymeric Ig receptor from humans and amphibians, J. Immunol. 178 (3) (2007) 1589–1597.
[11] K. Hamuro, H. Suetake, N.R. Saha, et al., A teleost polymeric Ig receptor exhibiting two Ig-like domains transports tetrameric IgM into the skin, J. Immunol. 178 (9) (2007) 5682–5689.
[12] B.M. Stadtmueller, Z. Yang, K.E. Hueytubman, et al., Biophysical and biochemical characterization of avian secretory component provides structural insights into the evolution of the polymeric Ig receptor, J. Immunol. 197 (4) (2016) 1408–1414.
[13] Y.Y. Yu, Y.Z. Liu, H.L. Li, et al., Polymeric immunoglobulin receptor in dojo loach (*Misgurnus anguillicaudatus*): molecular characterization and expression analysis in response to bacterial and parasitic challenge, Fish Shellfish Immunol. 73 (2018) 175–184.
[14] J.H. Rombout, V.D.T. Sj, G. Yang, et al., Expression of the polymeric immunoglobulin receptor (pIgR) in mucosal tissues of common carp (*Cyprinus carpio* L.), Fish Shellfish Immunol. 24 (5) (2008) 620–628.
[15] G.J. Xu, W.B. Zhan, B.J. Ding, et al., Molecular cloning and expression analysis of polymeric immunoglobulin receptor in flounder (*Paralichthys olivaceus*), Fish Shellfish Immunol. 35 (3) (2013) 653–660.
[16] Y.A. Zhang, I. Salinas, J. Li, et al., IgT, a primitive immunoglobulin class specialized in mucosal immunity, Nat. Immunol. 11 (9) (2010) 827–835.
[17] J.D. Hansen, E.D. Landis, R.B. Phillips, et al., Discovery of a unique Ig heavy-chain isotype (IgT) in rainbow trout: implications for a distinctive B cell developmental pathway in teleost fish, Proc. Natl. Acad. Sci. 102 (19) (2005) 6919–6924.
[18] I. Estensoro, J.A. Calduch-Giner, S. Kaushik, et al., Modulation of the IgM gene expression and IgM immunoreactive cell distribution by the nutritional background in gilthead sea bream (*Sparus aurata*) challenged with Enteromyxum leei (*Myxozoa*), Fish Shellfish Immunol. 33 (2) (2012) 401–410.
[19] X.Z. Sheng, G.J. Xu, X.Q. Tang, et al., Monoclonal antibodies recognizing mucus immunoglobulin and surface immunoglobulin-positive cells of flounder (*Paralichthys olivaceus*), Vet. Immunol. Immuno. 145 (1–2) (2012) 143–150.
[20] F.M. Zhang, D. Liu, L. Wang, et al., Characterization of IgM-binding protein: a pIgR-like molecule expressed by intestinal epithelial cells in the common carp (*Cyprinus carpio* L.), Vet. Immunol. Immuno. 167 (1–2) (2015) 30–35.
[21] Z. Xu, F. Takizawa, D. Parra, et al., Mucosal immunoglobulins at respiratory surfaces mark an ancient association that predates the emergence of tetrapods, Nat. Commun. 7 (2016) 10728.
[22] X.Z. Sheng, X.Y. Qian, X.Q. Tang, et al., Polymeric immunoglobulin receptor mediates immune excretion of mucosal IgM - antigen complexes across intestinal epithelium in flounder (*Paralichthys olivaceus*), Front. Immunol. 9 (2018) 1562.
[23] T.M. Tadiso, A. Sharma, I. Hordvik, Analysis of polymeric immunoglobulin receptor- and CD300-like molecules from Atlantic salmon, Mol. Immunol. 49 (3) (2011) 462–473.
[24] A.N. Kortum, I. Rodrigueznunez, J. Yang, et al., Differential expression and ligand binding indicate alternative functions for zebrafish polymeric immunoglobulin receptor (pIgR) and a family of pIgR-like (PIGRL) proteins, Immunogenetics 66 (4) (2014) 267–279.
[25] C.M. Ribeiro, S. Bird, G. Raes, et al., A novel soluble immune-type receptor (SITR) in teleost fish: carp SITR is involved in the nitric oxide-mediated response to a protozoan parasite, PLoS One 6 (1) (2011) 79–89.
[26] L.N. Feng, D.Q. Lu, J.X. Bei, et al., Molecular cloning and functional analysis of polymeric immunoglobulin receptor gene in orange-spotted grouper (*Epinephelus*

coioides), Comp. biochem. Physiol. B 154 (3) (2009) 282–289.
[27] Q. Li, W.B. Zhan, J. Xing, et al., Production, characterisation and applicability of monoclonal antibodies to immunoglobulin of Japanese flounder (*Paralichthys olivaceus*), Fish Shellfish Immunol. 23 (5) (2007) 982–990.
[28] T. Muth, J.A. Garcíamartín, A. Rausell, et al., JDet: interactive calculation and visualization of function-related conservation patterns in multiple sequence alignments and structures, Bioinformatics 28 (4) (2012) 584–586.
[29] I.T. Rombel, K.F. Sykes, S. Rayner, et al., ORF-FINDER: a vector for high-throughput gene identification, Gene 282 (1–2) (2002) 33–41.
[30] S. Kumar, G. Stecher, K. Tamura, MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets, Mol. Biol. Evol. 33 (7) (2016) 1870–1874.
[31] E. Gasteiger, A. Gattiker, C. Hoogland, et al., ExPASy: the proteomics server for in-depth protein knowledge and analysis, Nucleic Acids Res. 31 (13) (2003) 3784–3788.
[32] X.Q. Tang, L. Zhou, W.B. Zhan, Isolation and characterization of pathogenic *Listonella anguillarum* of diseased half-smooth tongue sole (*Cynoglossus semilaevis* Günther), J. Ocean Univ. China 7 (3) (2008) 343–351.
[33] Y.L. Gao, X.Q. Tang, X.Z. Sheng, et al., Immune responses of flounder *Paralichthys olivaceus* vaccinated by immersion of formalin-inactivated *Edwardsiella tarda* following hyperosmotic treatment, Dis. Aquat. Organ. 116 (2) (2015) 111–120.
[34] Z. Xu, D. Parra, I. Salinas, et al., Teleost skin, an ancient mucosal surface that elicits gut-like immune responses, Proc. Natl. Acad. Sci. 110 (32) (2013) 13097–13102.
[35] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1–2) (1976) 248–254.
[36] M. Wang, X.Z. Sheng, J. Xing, et al., Identification of a 27.8 kDa protein from flounder gill cells involved in lymphocystis disease virus binding and infection, Dis. Aquat. Organ. 94 (1) (2011) 9–16.
[37] I. Salinas, Y.A. Zhang, Mucosal immunoglobulins and B cells of teleost fish, Dev. Comp. Immunol. 35 (2011) 1346–1365.
[38] R. Braathen, V.S. Hohman, P. Brandtzaeg, et al., Secretory antibody formation: conserved binding interactions between J chain and polymeric Ig receptor from humans and amphibians, J. Immunol. 178 (3) (2007) 1589–1597.
[39] L. Wang, J. Zhang, X.H. Kong, et al., Molecular characterization of polymeric immunoglobulin receptor and expression response to Aeromonas hydrophila challenge in Carassius auratus, Fish Shellfish Immunol. 70 (2017) 372–380.
[40] S.H. Lin, G.A. Davidson, C.J. Secombes, et al., Use of a lipid-emulsion carrier for immunisation of dab (*Limanda limanda*) by bath and oral routes: an assessment of systemic and mucosal antibody responses, Aquaculture 181 (1) (2000) 11–24.
[41] P.G. Jenkins, A.B. Wrathmell, J.E. Harris, et al., Systemic and mucosal immune responses to enterically delivered antigen in Oreochromis mossambicus, Fish Shellfish Immunol. 4 (4) (1994) 255–271.
[42] D. Parra, F.E. Reyes-Lopez, L. Tort, Mucosal immunity and B cells in teleosts: effect of vaccination and stress, Front. Immunol. 6 (2015) 354.
[43] K.D. Cain, D.R. Jones, R.L. Raison, Characterisation of mucosal and systemic immune responses in rainbow trout (*Oncorhynchus mykiss*) using surface plasmon resonance, Fish Shellfish Immunol. 10 (8) (2000) 651–666.