Fish and Shellfish Immunology 87 (2019) 82–86

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Short communication

Identification and expression analysis of Fc receptor-like proteins in Japanese flounder (*Paralichthys olivaceus*)

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ARTICLE INFO

Keywords:
Fc receptor-like proteins
Japanese flounder
Immunoglobulin

ABSTRACT

Fc receptors (FcRs) are specific to the Fc portion of immunoglobulin (Ig) molecules. Here, four Fc receptor-like proteins, JF-FcR-like protein 1-4, were identified in Japanese flounder. Their open reading frames encoded 358, 255, 519 and 441 amino acid residues, respectively. JF-FcR-like protein mRNAs were mainly detected in kidney and spleen of healthy fish. Injection of formalin-killed cells (FKCs) of *Edwardsiella tarda* significantly increased the spleen mRNA levels of JF-FcR-like protein 1 but not the other JF-FcR-like proteins. Injection of FKC of *Streptococcus iniae* did not significantly affect any of the JF-FcR-like protein mRNAs. These findings suggest that the FcR-like proteins have different involvements in pathogen responses.

1. Introduction

Receptors specific to the fragment crystallizable (Fc) portion of immunoglobulin (Ig) molecules are known as Fc receptors (FcRs). FcRs are expressed broadly on the membranes of various immune cells [1,2]. In mammals, the interaction of FcR with the Fc region on the heavy chain of Ig molecules results in activation and/or inhibition of immune responses such as mediation of antibody-dependent cell-mediated cytotoxicity, cell proliferation and antibody secretion [3,4]. The family of FcRs specific to different Ig isotypes and subclasses has been well described in human and mouse [5]. The family of Fc receptor specific to human IgG consists of high-affinity FcRs (FcγI) and low-affinity FcRs (FcγII and FcγIII). Both high- and low-affinity receptors bind IgG isotypes with different binding affinities [6,7]. FcεR is known as a receptor for the IgE isotype, which is also present in human and mouse [8]. Additionally, an FcR that binds to both IgM and IgA, Fcα/μR, is found in all placental mammalian species [9].

Mammalian Fc receptors contain extracellular immunoglobulin-like domains which are known as D domains. D1 and D2 domains are crucial for binding to the Fc portion of Ig molecules [10]. Certain amino acid residues at specific positions in human FcRs, especially in D1 and D2, that are required to interact with the constant domain 2 (CH2) and/or 3 (CH3) interface in the Fc region of the Ig heavy chain have been widely described in human [6,10,11]. Mutagenesis and crystal structure analyses of mammalian FcR-Ig complexes have revealed the positions in both proteins that are involved in the interaction [6,12].

In teleosts, an Fc receptor homolog of mammalian FcRs was identified and characterized in channel catfish (*Ictalurus punctatus*) [2]. Although mammalian FcRs appear to function as Ig receptors, they have not yet been well identified in teleost. To fulfill understanding of teleost immune system in response to pathogen infection, FcRs are needed to be identified and characterized in teleost. In this study, we identified the coding sequences of four Fc receptor-like proteins (FcR-like protein 1-4) in Japanese flounder. We also analyzed their mRNA expressions in various tissues of healthy fish, and fish injected with formalin-killed *Edwardsiella tarda* and *Streptococcus iniae* cells.

2. Materials and methods

2.1. Experimental fish and sampling

Japanese flounder with an approximate size of 8–10 cm in total length were reared at 20–22 °C and daily fed with commercial diet. Fish tissues including brain, gills, heart, intestine, kidney, liver, muscle, skin and spleen were collected from healthy Japanese flounder for RNA extraction using RNAiso plus (Takara Bio, Japan). One μg of RNA was used for cDNA synthesis using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) following the manufacturer's instructions.

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https://doi.org/10.1016/j.fsi.2019.01.002
Received 19 September 2018; Received in revised form 14 December 2018; Accepted 3 January 2019

Table 1
List of primers.

Primer name	Sequence (5′-3′)	Application
JF-FcR-like1-F	AATATATGTAACCCCCTCGG	Confirmation of coding sequence
JF-FcR-like1-R	GGAGTTACTTGAGTCTCACC	
JF-FcR-like2-F	AGACAAACAACCTGGGGA	
JF-FcR-like2-R	CATCGTGCTGTTTGGTTCGT	
JF-FcR-like3-F	TGTGACCATGCGGTACGA	
JF-FcR-like3-R	TCGTACCGCATGGTCACA	
JF-FcR-like4-F	CATTGGTCATTAGGAACATTCAAC	
JF-FcR-like4-R	ACAACCAAACAACGTGAGAGAGA	
JF-FcR-like1-RT-F	CTGGAAGACCAGGGGATGTA	RT-PCR
JF-FcR-like1-RT-R	TGAGACTGGGGGAACAAAAG	
JF-FcR-like2-RT-F	GAGTGCAGTAACGCCATCAA	
JF-FcR-like2-RT-R	GTGCAAGATGATCAGCAGGA	
JF-FcR-like3-RT-F	CTGCTCTGTCTGGGGAAACT	
JF-FcR-like3-RT-R	CCGCTGTCTCTGGAAGTGAT	
JF-FcR-like4-RT-F	CACAGCAGCAACCCTGTCAA	
JF-FcR-like4-RT-R	GAGACACGGACAGAGACACT	
JF-FcR-like1-qF	AACAACAAGCGACTGGGAAC	qPCR
JF-FcR-like1-qR	GATGAGACTGGGGGAACAAA	
JF-FcR-like2-qF	GGTGCTTCTCTGCCAATACA	
JF-FcR-like2-qR	TAGAGACCTTCGTCGGACCT	
JF-FcR-like3-qF	ATGTGAGTCGGCAGTAGGGA	
JF-FcR-like3-qR	CCACCAACTGAGACAACTTC	
JF-FcR-like4-qF	GAGACGCCCACACACCAAAT	
JF-FcR-like4-qR	ATGAGGGAGTGTTGGATGGA	
EF-1α-F	CATGGTCGTGACCTTCGCTC	RT-PCR and qPCR
EF-1α-R	CTCGGGCATAGACTCGTGGT	

2.2. Identification and sequence analyses of JF-FcR-like proteins

The predicted open reading frames (ORFs) of JF-FcR-like protein sequences were obtained from our RNA-sequence data generated by next-generation sequencing [13] using the reported mammalian FcRs sequences as queries. The JF-FcR-like protein coding sequences were confirmed by reverse transcription (RT-PCR) using specific primers (Table 1). The DNA fragments were amplified using Ex Taq DNA polymerase (Takara Bio, Japan), cloned into pGEM T-easy vector (Promega, Japan) and used for sequencing. The protein domains were analyzed with the Simple Modular Architecture Research Tool (SMART) program [14]. Signal peptides were predicted by the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/). The amino acid identities of JF-FcR-like proteins to FcR of other fish species were analyzed by BlastX. Analysis of JF-FcR-like proteins in Japanese flounder genome [15] was performed by Blastn. A phylogenetic tree was constructed based on the ClustalX alignments of amino acid sequences using the Mega6 program [16].

2.3. mRNA expression analyses of JF-FcR-like proteins

The mRNAs of JF-FcR-like proteins were detected in nine tissues (brain, gills, heart, intestine, kidney, liver, muscle, skin and spleen) of three fish by RT-PCR with Ex Taq DNA polymerase (Takara Bio, Japan) using the specific primer sets shown in Table 1. Elongation factor-1α (EF-1α) was used as an internal control gene. RT-PCR was performed under the following conditions: 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 55–60 °C for 30 s, 72 °C for 30 s and the final extension was carried out at 72 °C for 5 min. RT-PCR products were analyzed by agarose gel electrophoresis. The gels were stained with ethidium bromide and observed under UV light.

The effects of formalin-killed cells (FKCs) of *E. tarda* and *S. iniae* on the mRNA expressions of JF-FcR-like proteins were investigated by qPCR. FKCs were prepared as previously described [17]. Fish were intraperitoneally injected with FKCs of *E. tarda* at 2.3×10^8 cells per fish or FKCs of *S. iniae* at 1.9×10^8 cells per fish. Spleens from five fishes were collected at 0 (prior to injection), 6, 12, 24 and 72 h post injection (hpi). The mRNA levels of JF-FcR-like proteins were examined with Thunderbird SYBR qPCR mix (Toyobo, Japan) and specific primers (Table 1), following the manufacturer's instructions. Relative mRNA expressions were calculated by the $2_{T}^{-\Delta\Delta C}$ method and EF-1α was used as the internal reference gene. Statistical analyses were done with GraphPad Prism v 6.0 (La Jolla, CA, USA). The significance of differences between normal fish (0 h) and FKCs of *E. tarda*- or *S. iniae*-treated Japanese flounder at each time point (3, 12, 24 and 72 hpi) was evaluated with one-way ANOVA followed by Dunnett's test.

3. Results and discussion

3.1. Identification of Japanese flounder FcR-like proteins

The cDNAs of JF-FcR-like protein 1 (LC369763), 2 (LC369764), 3 (LC369765) and 4 (LC369766) consist of 1074, 765, 1557 and 1323 bp, respectively, encoding 358, 255, 519 and 441 amino acid residues, respectively (Supplementary Fig. 1). The amino acid sequences of the JF-FcR-like proteins showed 47–67% identities to the FcRs of other fish species (Supplementary Table 1). Conserved domains prediction of all the JF-FcR-like proteins revealed the presence of Ig, Ig-like and Igc2 domains (Fig. 1A). Interestingly, only JF-FcR-like protein 1 lacks a transmembrane domain (Fig. 1A). A channel catfish FcR homolog was also found to lack a transmembrane region [2]. This suggests that the channel catfish FcR homolog and JF-FcR-like protein 1 are secreted and/or intracellularly expressed. Ig and Ig-like domains are well conserved in the FcRs of Japanese flounder, channel catfish, mouse and human (Fig. 1A). The Ig domain is a structural domain in the immunoglobulin superfamily (IgSF) [18]. The FcRs encoding mammalian immune receptors are composed of Ig

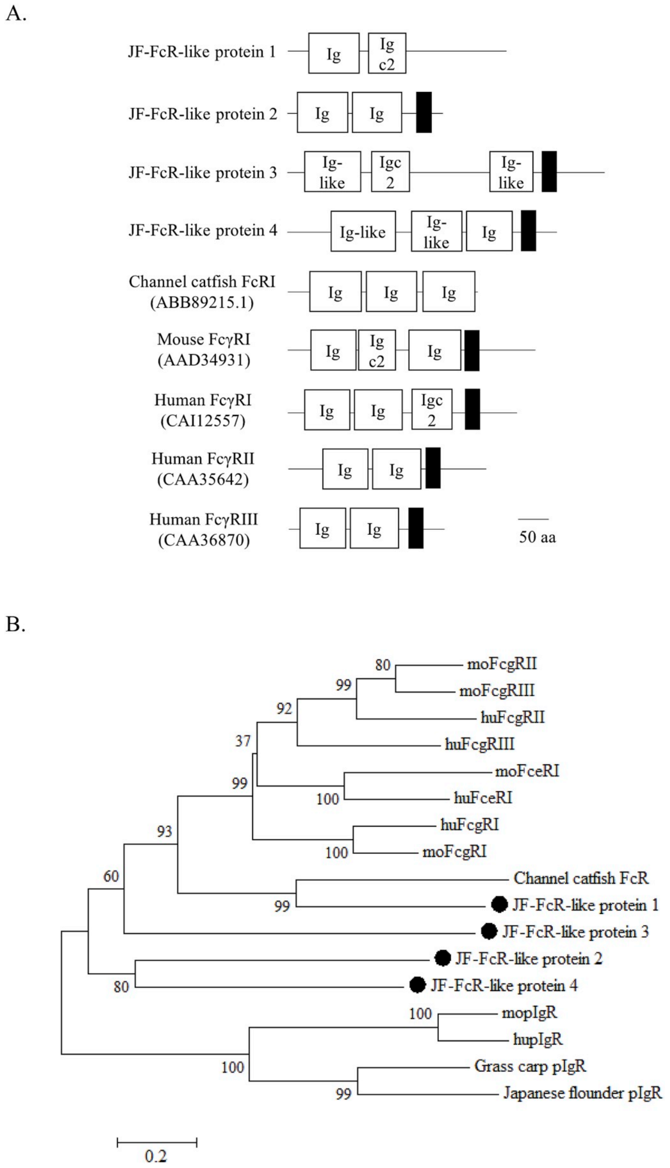

Fig. 1. (A) Schematic diagrams of the domain organizations of Japanese flounder FcR-like proteins and other vertebrates FcRs. The domains were predicted by SMART software. The transmembrane domain is indicated by black rectangular boxes. (B) phylogenetic analysis of Japanese flounder FcR-like proteins. The tree was constructed by the neighbor-joining method and was bootstrapped 10,000 times. The accession numbers of the sequences used for the analysis are as follows; mouse (mo) FcγRI (AAD34931), moFcγRII (AAA37608), moFcγRIII (NP_034318), moFcεRI (NP_034314), mopIgR (AAH13556.1), human (hu) FcγRI (CAI12557), huFcγRII (CAA35642), huFcγRIII (CAA36870), huFcεRI (AAH05912), hupIgR (AAI10495.1), Channel catfish FcR (DQ286290), Japanese flounder pIgR (ADK91435.1), Grass carp pIgR (ALX37964.1).

and Ig-like domains that play crucial role in interacting with the Fc region of Ig molecules [6,10]. Our results are in agreement with the finding that the FcR homolog identified in channel catfish also contains Ig-like domains that are structurally related to the mammalian D domains [2]. These findings demonstrate that the Ig domain has an important role in Ig receptors that it is structurally conserved in vertebrate immune receptors.

The phylogenetic tree showed that all four JF-FcR-like proteins were clustered with the mammalian FcRs and catfish FcR homolog (Fig. 1B). Teleost polymeric immunoglobulin receptors (pIgRs), which contain Ig domains that are similar to those in FcRs [19], were separately grouped from the FcRs in the tree (Fig. 1B), indicating that the four FcR-like proteins belong to the FcR family. JF-FcR-like protein 1 grouped with channel catfish FcR and mammalian FcR, whereas the other JF-FcR-like proteins formed another clade (Fig. 1B). Blastn searches of JF-FcR-like proteins showed the identity and position of annotated sequences in the Japanese flounder genome (Supplementary Table 2).

3.2. mRNA expression analyses of JF-FcR-like proteins

The mRNA expressions of JF-FcR-like proteins were mainly detected in lymphoid tissues of healthy Japanese flounder such as kidney and spleen (Fig. 2A). A representative gel in Fig. 2A showed mRNA expressions of JF-FcR-like proteins ($n = 3$). The mRNAs of JF-FcR-like proteins 1–3 were mainly detected in gills, kidney and spleen with less amounts in other tissues. The mRNA of JF-FcR-like protein 4 was detected in all tissues examined (Fig. 2A). In channel catfish, the expression of an FcR homolog was also detected in spleen, kidney and peripheral blood leukocytes [2]. Similarly, the expressions of mammalian FcRs were primarily detected in kidney and spleen [9,20].

Although FcRs are involved in several mechanisms of vertebrate immunity, little is known about their mRNA levels in teleosts after stimulation with different immunogens. The mRNA levels of JF-FcR-like proteins were determined in spleen of Japanese flounder stimulated by FKCs of *E. tarda* and *S. iniae*. These bacterial species cause severe diseases in Japanese flounder [21,22]. Since the expression of several genes in PBS-injected group are comparable to the normal fish [23,24], normal fish was used as a control group for determination of JF-FcR-like proteins mRNA expression by qPCR in this study. Moreover, it was previously determined that injection of Japanese flounder with an approximate size of 8–10 cm with bacterial FKC at 10^8 cells per fish is enough to stimulate fish immune responses as mentioned and used in previous studies [23,24]. The JF-FcR-like protein 1 mRNA level was significantly increased from 3 h to 72 h after injection of FKCs of *E. tarda*. The mRNA levels of JF-FcR-like proteins 2, 3 and 4 were not significantly changed at any of the time courses examined after stimulation with FKCs of *E. tarda*. The mRNA expression levels of all four JF-FcR-like proteins were not significantly changed in response to FKCs of *S. iniae* at any of the time course examined (Fig. 2B). The finding that JF-FcR-like protein 1 transcripts were significantly increased by FKCs of *E. tarda* (Fig. 2B) suggests that JF-FcR-like protein 1 has a role in response to Gram negative bacterial infections. Furthermore, in mammals, the mRNA and protein expressions of FcRs are involved in different activation states of immune cells and/or subpopulations of leukocytes during the stimulation period [2,25]. Although we reported the mRNA expression of JF-FcR-like proteins after PAMP stimulations, it remains unclear how FcRs respond to pathogens in teleosts. Further studies are needed to better understand the signaling pathway of teleost FcRs.

In conclusion, four FcR-like proteins were identified in Japanese flounder. The Ig domains of previously reported FcRs are structurally conserved in the FcR-like proteins. JF-FcR-like protein transcripts were constitutively expressed in lymphoid tissues. The mRNA levels of the four FcR-like proteins responded differently to FKCs of *E. tarda* but not FKCs of *S. iniae*, indicating that the FcR-like proteins have different involvements in pathogen responses.

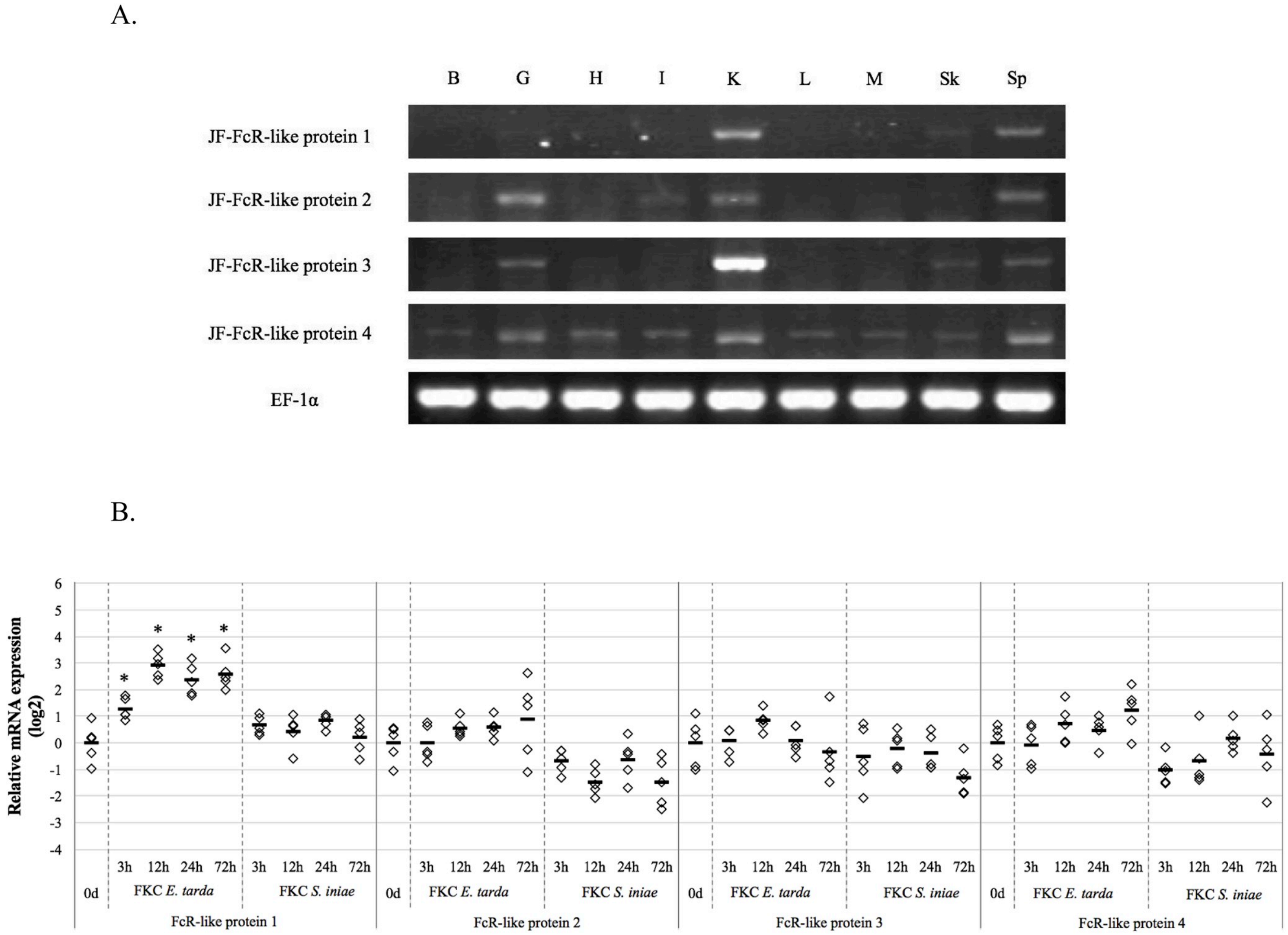

Fig. 2. (A) RT-PCR analysis of the expression of JF-FcR-like proteins in healthy Japanese flounder tissues. B; brain, G; gills, H; heart, I; intestine, K; kidney, L; liver, M; muscle, Sk; skin, Sp; spleen, EF-1α was used as an internal control gene. A representative gel represents RT-PCR products of JF-FcR-like protein ($n = 3$). (B) Relative mRNA levels of JF-FcR-like proteins in Japanese flounder spleen after stimulation with FKCs of *E. tarda* and *S. iniae*. The relative values are expressed as log2 values normalized to EF-1α. Black hyphens indicate the average expression values of each group. One-way ANOVA followed by Dunnett's test were used for statistical analyses. Significant difference of expression values of each time-point were compared to normal fish (0 h). Asterisks represent statistical significance between normal fish (0 h) and FKCs of *E. tarda*- or *S. iniae*-treated Japanese flounder at each time point (3, 12, 24 and 72 hpi) ($p < 0.005$).

Acknowledgements

This research was supported in part by grants from JSPS KAKENHI Grant Number 25292125 and Kyoritsu Seiyaku Corporation, Japan.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.01.002.

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