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Short communication

Molecular delineation of a caspase 10 homolog from black rockfish (*Sebastes schlegelii*) and its transcriptional regulation in response to pathogenic stress

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ABSTRACT

Caspase 10 is an initiator caspase in death cascades of death receptor mediated apoptotic signaling. We identified and molecularly characterized a novel homolog of caspase 10 from black rockfish (*Sebastes schlegelii*) and designated as RfCasp10. The complete coding region of RfCasp10 was found to consist of 1659 bps, encoding a 553 amino acid protein with a predicted molecular mass of 61.7 kDa. The characteristic caspase family domain architecture, including death effecter domains (DEDs), was clearly identified in RfCasp10. Moreover, the RfCasp10 gene was found to contain 13 exons. Our pairwise sequence alignment confirmed the prominent sequence similarity of RfCasp10 with its fish homologs, and phylogenetic reconstruction affirmed its homology and substantial evolutionary relationship with known caspases 10 similitudes, in particular with those of teleosts. As detected by qPCR, RfCasp10 was markedly expressed in blood tissues under physiological conditions, whereas its expression was found to be upregulated under pathogenic stress, elicited by *Streptococcus iniae* and polyinosinic:polycytidylic acid in blood, liver, and spleen tissues. Collectively, our study suggests the plausible elicitation of RfCasp10 mediated apoptosis in immune relevant tissues of black rockfish as a host immune response to a bacterial or viral infection.

1. Introduction

Apoptosis can be characterized by prominent morphological changes in cells, such as cell shrinkage, apoptotic body formation, and chromatin condensation (Broker et al., 2005). It can be involved in regulation of biological functions in immune cells, including maturation and homeostasis (Krammer, 2000). In particular, apoptosis can be involved in elimination of potentially harmful cells such as pathogen infected cells from host organisms (White and Steller, 1995; Sun and Shi, 2001), playing a significant role in cellular immunity as an immune response against infectious pathogens. Infections, particularly those caused by microorganisms including viruses, are known to trigger apoptosis through different mechanisms, such as an increase in the production of pore-forming proteins and molecules directing the endogenous death machinery in the damaged or infected cell (Everett and McFadden, 1999; Weinrauch and Zychlinsky, 1999). Apoptotic signaling is known to be a highly conserved feature among a wide array of taxa. It comprises common signaling factors, such as BCL-2 family members, caspases, tumor necrosis factors (TNF), and inhibitor of apoptosis (IAP) family proteins, which are thoroughly interconnected with other cell signaling pathways (Danial and Korsmeyer, 2004).

Caspases are cysteine aspartate specific proteinases and predominantly involved in cellular apoptotic cascades (Nicholson, 1999). To date, thirteen caspases have been identified in mammals and categorized into two main groups, namely "inflammatory caspases" (caspase 1, 4, 5, 11, 12, and 14) and "apoptotic caspases" (caspase 2, 3, 6, 7, 8, 9,

Abbreviations: DED, death effector domain; PCR, polymerase chain reaction; qPCR, quantitative real-time PCR; BCL-2, B-cell lymphoma 2; TNF, tumor necrosis factor; IAP, inhibitor of apoptosis; N-termini, amino termini; FADD, FAS-associating death domain-containing protein; HIV, human immunodeficiency virus; RIN, RNA integration score; bp, base pair; cDNA, complementary deoxyribose nucleic acid; RNA, ribose nucleic acid; mRNA, messenger ribonucleic acid; BLAST, Basic Local Alignment Search Tool; ExPASy, Expert Protein Analysis System; MEGA, Molecular Evolutionary Genetics Analysis software; BAC, bacterial artificial chromosome; gDNA, genomic DNA; NCBI, National Center for Biotechnology Information; PBS, phosphate buffered saline; Poly I:C, polyinosinic:polycytidylic acid; MIQE, minimum information for publication of quantitative real-time PCR experiments; RfCasp10, Rock bream caspase 10; RfEF1A, black rockfish elongation factor 1-alpha; SD, standard deviation; UTR, untranslated region; NK cells, natural killer cells; CTL, cytotoxic T lymphocyte; CFU, colony forming units; μl, microliter; dNTPs, deoxynucleotide triphosphates; °C, degrees of Celsius; ng, nanogram; rpm, revolutions per minute; h, hours; kDa, kiloDalton.

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and 10), based on their main function in the cell (Earnshaw et al., 1999). Caspases are synthesized as inactive proenzymes (zymogenes) in preapoptotic cells. However, when the external or internal stimulus triggers an apoptotic cascade in the cell, caspases can be activated by proteolytic cleavage on the C-terminal side of highly conserved aspartic acid residues, separating the protein into large and small subunits, which in turn can form the active mature protein (Fuentes-Prior and Salvesen, 2004). Apoptotic caspases can be further divided into two classes: initiator caspases (caspase 8, 9, 10) and effector, also known as executioner, caspases (caspase 3, 6, 7). Upstream initiator caspases can activate the zymogens of downstream effector caspases, eliciting apoptosis of the cell (Schulze-Osthoff et al., 1998; Earnshaw et al., 1999). However, according to recent studies, the conventional roles of effector and initiator caspases are debatable, because some effector caspases, such as caspase 6, were shown to activate so-called initiators (e.g., caspase 8) of the death cascade (Cowling and Downward, 2002; Monnier et al., 2011).

Among the initiator caspases identified to date, only caspase 8 and 10 carry a prodomain, consisting of two death effector domains (DEDs) at their N-termini (Fernandes-Alnemri et al., 1996; Li and Yuan, 2008), along with a more common active site, the pentapeptide motif QACQG (Cohen, 1997). DED is a protein interaction domain and highly conserved among the adaptor proteins involved in induction of apoptotic signals (Barnhart et al., 2003). Upon binding of death ligands such as Fas ligand or Fas antigen to their corresponding receptors on the cell surface membrane, the death receptor, FAS-associating death domain-containing protein (FADD), and procaspase 10 assemble to form a complex known as death inducing signal complex (Sprick et al., 2002), in which procaspase 10 is cleaved at specific aspartate residues by autoproteolysis, resulting in the mature caspase 10 (Wang et al., 2001). Active caspase 10 can then act upon effector procaspases and cleave them, resulting in biologically active forms (Stennicke et al., 1998). Caspase 10 plays an indispensable role in organisms. For instance, it is known to serve in immune responses triggered mainly through the apoptotic signals involving Fas ligand, and it is involved in death cascades that mediate the elimination of T-cells which respond to self-antigens (Shin et al., 2002). On the other hand, human immunodeficiency virus (HIV) was found to diminish apoptosis in infected cells, resulting from the suppression of caspase 10 expression, to propagate their infection in the host organism (Gibellini et al., 2005).

Documented studies on caspases of lower vertebrates, including teleosts, are relatively scarce. Among the reported studies, information on caspase 10 could only be found in *Channa striatus* (snakehead murrel) (Arockiaraj et al., 2013) and *Paralichthys olivaceus* (Japanese flounder) (Kurobe et al., 2007). Herein, we molecularly characterized another homolog of caspase 10 from black rockfish (*Sebastes schlegelii*; RfCasp10), which is a highly demanded delicacy in East and Southeast Asia. We analyzed its physiological expression and transcriptional modulation under pathogenic stress.

2. Materials and methods

2.1. Establishment of black rock fish cDNA data base

A database of black rockfish cDNA sequences was created by 454 GS-FLX™ sequencing (Droege and Hill, 2008). In brief, total RNA was extracted from blood, liver, kidney, gill, and spleen tissues of three fish. The extracted RNA was then cleaned by an RNeasy Mini Kit (Qiagen, USA) and assessed for quality and quantified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Canada), resulting in an RNA integration score (RIN) of 7.1. Subsequently, a GS-FLX™ 454 shotgun library was constructed, and the cDNA database was established using fragmented RNA (average size ~890 bp) from the aforementioned RNA samples (Macrogen, Korea).

2.2. In-silico characterization

Analysis of this cDNA database using the Basic Local Alignment Search Tool (BLAST) algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) led to the identification of the complete cDNA sequence of RfCasp10. The identified sequence was then characterized using different bioinformatics tools. The full-length putative coding sequence of RfCasp10 was identified and the corresponding amino acid sequence was derived using DNAsist 2.2 software. Domain architecture corresponding to the derived primary structure of RfCasp10 was predicted using the ExPASy Prosite database (http://prosite.expasy.org). Furthermore, some of the physicochemical properties of the derived protein sequence were determined by ExPASy ProtParam tool (http://web.expasy.org/protparam). Comparison of RfCasp10 with its homologs was carried out through pairwise and multiple sequence alignments, executed by Matgat software (Campanella et al., 2003) and ClustalW2 server (http://www.Ebi.ac.uk/Tools/clustalw2), respectively. The phylogenetic reconstruction of RfCasp10 was generated according to the neighbor-joining platform using Molecular Evolutionary Genetics Analysis software (MEGA 4.0) (Tamura et al., 2007).

2.3. Identification of genomic gene structure

A random shear bacterial artificial chromosome (BAC) genomic DNA library of black rockfish was custom constructed (Lucigen®, USA), using the genomic DNA extracted from blood tissues of healthy fish. Then, the BAC clone containing the RfCasp10 gene was identified by an iterative polymerase chain reaction (PCR) based genomic library screening approach, as mentioned in the vendor's protocol using pooled gDNA prepared by the vendor and a sequence specific primer pair (Table 1). Subsequently, the putative RfCasp10 bearing clone was sequenced with the 454 GS FLX™ system (Macrogen, Korea). After obtaining the sequence data, the exon–intron arrangement of the RfCasp10 gene was annotated by the National Center for Biotechnology Information (NCBI) Spidey online server (http://www.ncbi.nlm.nih.gov/spidey), using previously identified full-length RfCasp10 cDNA sequences.

2.4. Animal rearing and tissue collection

Healthy fish, pre-acclimatized to the laboratory conditions, were obtained from one of the aquariums of the Marine Science Institute of Jeju National University, Jeju Self-governing Province, Republic of Korea, and maintained in 400 L laboratory aquarium tanks filled with aerated seawater at 22 ± 1 °C. Five healthy fish with an average body weight of 200 g were sacrificed for the tissue collection. Before killing the fish, we collected ~1 ml of blood from each fish using sterile syringes coated with 0.2% heparin sodium salt (USB, USA), and the peripheral blood cells were separated by immediate centrifugation at 3000 ×*g* for 10 min at 4 °C. Other tissues including head kidney, spleen, liver, gill, intestine, kidney, brain, muscle, heart, and stomach were excised, snap-frozen in liquid nitrogen and stored at −80 °C.

2.5. Pathogen or chemical mediated immune stimulation

To analyze the transcriptional modulation of *Rf*Casp10 upon viral or bacterial invasion, healthy rockfish with an average body weight of 200 ± 20 g were used in time course immune stimulations. The gram-

Table 1
Oligomers used in the study.

Name	Purpose	Sequence (5′ → 3′)
RfCasp-qF	qPCR of *RfCasp10*	CTTCATCAAGGACGCCATGCTGTT
RfCasp-qR	qPCR of *RfCasp10*	TAGCCGTTCATGTGACGGAATCCA
RfEFA-F	qPCR for black rockfish EF1A	AACCTGACCACTGAGGTGAAGTCTG
RfEFA-R	qPCR for black rockfish EF1A	TCCTTGACGGACACGTTCTTGATGTT

positive live bacterial pathogen *Streptococcus iniae* (10^5 CFU/μl) as well as poly I:C (1.5 μg/μl), which resembles double-stranded viral RNA, was resuspended or dissolved in phosphate buffered saline (PBS), respectively, and used as immune stimulants. Fish were intraperitoneally injected with each stimulant in a total volume of 200 μl. For the injection control group, fish were injected with 200 μl PBS. The spleen and liver tissues were sampled from five individuals of each group at 3, 6, 12, 24, 48, and 72 h post-injection, as described above. Total RNA was then extracted from a pool of tissue samples (~40 mg from each fish) of five individual fish (both un-injected (represented by 0 h) and injected) using QIAzol® (Qiagen), following the vendor's protocol. Tissue samples collected from healthy as well as from immune stimulated fish were further purified using the RNeasy Mini Kit (Qiagen). RNA quality was examined by 1.5% agarose gel electrophoresis and the concentration was determined at 260 nm in a μDrop Plate (Thermo Scientific). First strand cDNA was synthesized in a 20 μL reaction

```
Rockfish                    ----------MDFQKLLLDVGKALRKDEVKALAFLCTDLLG 31
Three spined stickleback    ----------MHFQKLLFDIGKALSGDEVKALAFLCTDILG 31
Snakehead murrel            ----------MDFQRLLLDVGKALSKD--EALAFLCTDLLG 29
Japanese flounder           ----------MDFQKLLLDVGKALGTDEVKALIFLCTDILD 31
Black flying fox            MAAASQGLSMSSSSHDNGRENFRERLLDIDYNLGDLEVEQLKFLCQDLVS 50
human                       --MKSQGQHWYSSSDKNCKVSFREKLLIIDSNLGVQDVENLKFLCIGLVP 48
pig                         --MTAQGQSLSS--DDYRQMDFRGKLLIIDSNLGDQDVERLKFLCRDHIS 46
Frog                        ----------MDFNSMLLSIDDGLGREDIEALKFLCRDVLR 31
                                       *.  *: :.  *    : * *** . :

Rockfish                    -RNPNSVEMASDLFSRLADQDHLSAERPHLLTELLLIIQRTRLIRDFQLF 80
Three spined stickle back   -RNLSSVESASGLFSRLRDQGFLSAEQPHLLTELLIIIQRVRLTRDVDLP 80
Snakehead murrel            -RSLTSVESASDLFSRLMDQDYLSPESPQLLKELLNTVGRTRLVRELSLA 78
Japanese flounder           -RNPTSVKSANDLFSRLAERDHLSHERRDLLTELLRTIQRLDLVSAFNLN 80
Black flying fox            RKNLEKCSSASDIFEHLLARKLLSEEDPFLLAELLYIIKQNSLLKYLHYT 100
Human                       NKKLEKSSSASDVFEHLLAEDLLSEEDPFFLAELLYIIRQKKLLQHLNCT 98
pig                         SRSLERSNSAVDIFDQLMTEELLSEEDTFFLAELLYTIKQNSLLRHLHYT 96
Frog                        KNKLLSVRSGQELFQQLKTEDLISEDDFFLLAELLYIINHHSLLRDLGTN 81
                             ..      .  :*.:*  .  :* :   :* ***  : :  *   .

Rockfish                    DSAST----TGSLISPYRKLLYDLSEEITDEDLKSVKFLLNKDLPRRKLE 126
Three spined stickleback    AEPS-------SLISPYRKMLYNLSEELTADDLKDMKFLLHSYLPRRKLE 123
Snakehead murrel            DQES-------TRISPYRKLLYNLSQEITDDDLKNVKFLLNKTLPRRKLE 121
Japanese flounder           DREPT----TTSLISPYRKLLYNLYEEMTQEDLEEVKFLLYKTVPRRKLE 126
Black flying fox            KEQVENLLPTKRKVSLFRNLLYEMSENIDSEILKSMIFLQ-KESMPK--- 146
Human                       KEEVERLLPTRQRVSLFRNLLYELSEGIDSENLKDMIFLL-KDSLPK--- 144
pig                         KEQVACLLPTRRKVSLFRNLLYELSENISSENLKGMTFLL-RESIPR--- 142
Frog                        KENVQKDLPHQGKISSYRRMLYELSENVTGDDEKRILFLLPFQKKHK--- 128
                                      :* :*.:**:: : :  :  : : **       :

Rockfish                    DNVSTLEVFLEMEHMDLISDTNLHLLETIIQSVCPMLRDKINQFKALQVA 176
Three spined stickleback    ENVSTLEIWLEMEHKDLMNEHNLHLLEILISKVCPMLKDKINQLKAQQET 173
Snakehead murrel            ENVTTLEVFLEMEHMDLLSDTNLNELEKIIQSICPMLKEKITEFKALQVH 171
Japanese flounder           DNYSLLDVFLKMEHMDILSDTNLSELETIIYSVCPKMRGQINQFKTQQEA 176
Black flying fox            MQMTSLSFLGHLEKQAQIGEDDLKVLEDLCAKVAPSLGRKIEKYKKEKAF 196
Human                       TEMTSLSFLAFLEKQGKIDEDNLTCLEDLCKTVVPKLLRNIEKYKREKAI 194
pig                         VQMTSLSLLSYLEKQDQIDEDNLTLLEDVCKKIAPNLMRKIEKYKREKAS 192
Frog                        ENKTFLDVLCQLEKENSITEDNVGLLEDIFKKVSPDLLKIIEKYKERENN 178
                             : : *..   :*:   : : ::  ** :  .: * :   * : *  :

Rockfish                    HTSPAAQETSRLRSMS-NPSNPAPHSLTSERTFSCEIPEDFKLLEESLMN 225
Three spined stickleback10  PTSPATQESGRQGSLS-APNAPN-----------QVPQSLGAEGAGLFE 210
Snakehead murrel            HTSLITQETGRPRSMSYDFSLFQPTRSLDQETRASREFPGLQPLAESFRN 221
Japanese flounder           LQSSTQYSVDSSS-----------------------------------TVLN 193
Black flying fox            QVVTSPVDKETESLPQGKEEVFS--------------CSDVQQFLGALPA 232
Human                       QIVTPPVDKEAESY-QGEEELVS--------------QTDVKTFLEALPQ 229
pig                         QVVTPPSAEETESLPQGKEELFS--------------PSDIKHLLGSLQE 228
Frog                        LQPSAPPEYEHELINPPLSIQVS--------------SKNNESWNEETED 214

Rockfish                    TSNTSIDLPNVFPGGDECGALSLGLSDLNTGLSSVKSMRFDVLE-LPSQE 274
Three spined stickleback    MP-----DSPSGFNGGGDCAALSLGLSGH---------LKIEVVETMPPPK 247
Snakehead murrel            SSNTSLDYPDVLQSGGECGALPRRLSDLSTETSGFPSMKVRNDA-LEIQS 270
Japanese flounder           SS----------ESHLSSEMSSLSFSEHG--------LNSDTVS--VSQE 223
Black flying fox            SSLVRWQPFRIRSPSVGREGTSGEAEAPR--------------LALTEQN 268
Human                       ES---WQ-----NKHAGSNGNRATNGAP----------------SLVSRG 255
pig                         GSQQ--------DEHAGSNG---ETATP--------------------VSVE 249
Frog                        LI----------EHIGTIHEEAEKEDDK--------------SGNIDNQ 239
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Fig. 1. Multiple sequence alignment of vertebrate caspase 10 homologs including black rockfish caspase 10 (RfCasp10). Residues of predicted DED domains in RfCasp10 are depicted in bold letters. Caspase large and small subunits are indicated by solid black arrows and horizontal gray shading, respectively. Conserved residues in the substrate binding pocket and active sites are shaded in green and gray (vertical), respectively. Conserved caspase family histidine active sites are underlined and cysteine active sites including the pentapeptide motif (boxed) are bold and italicized. Residues conserved in all the aligned sequences are represented by asterisks (*), while partially conserved residues are marked with (.) or (:).

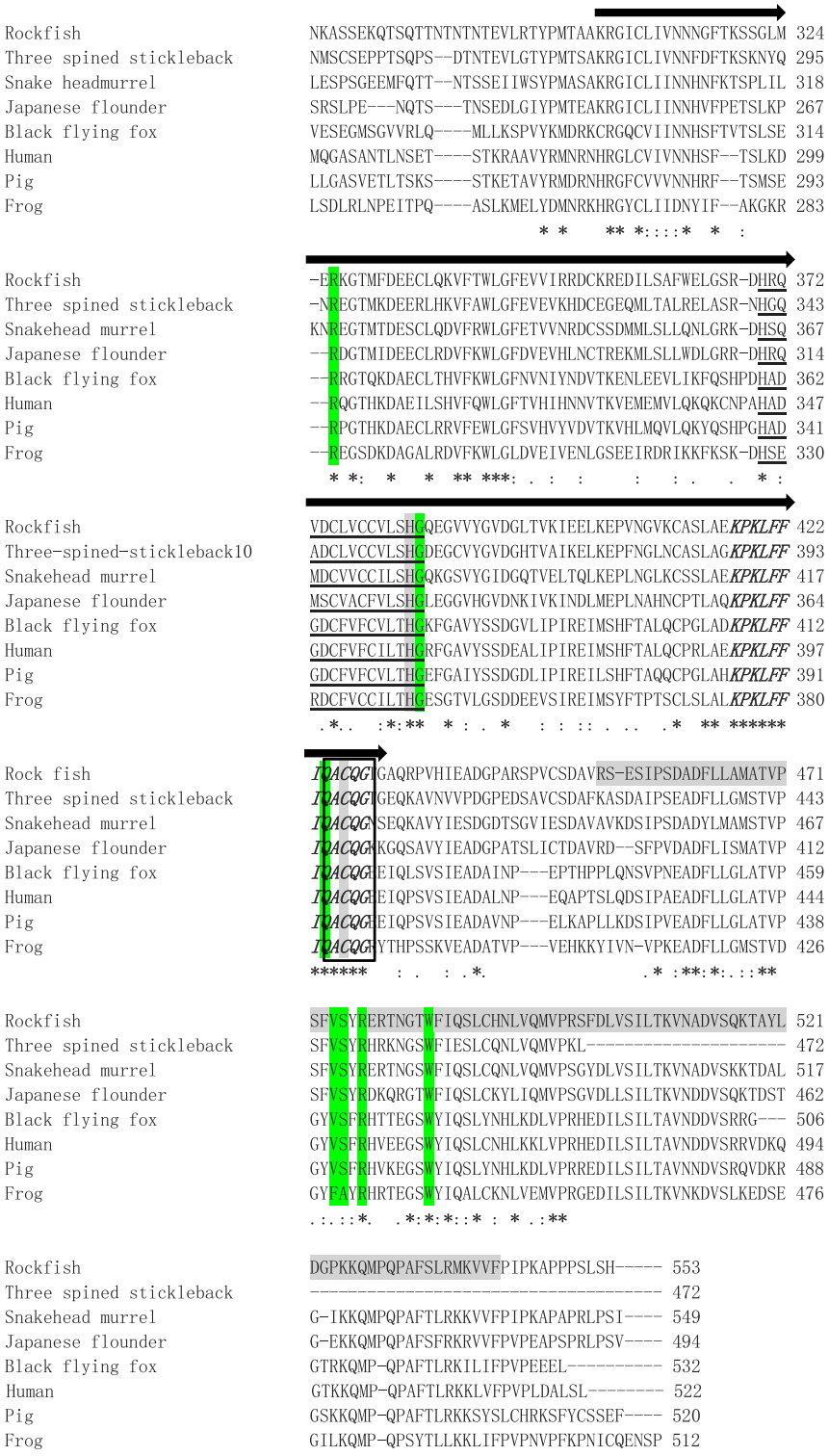

Fig. 1 (*continued*).

mixture containing 2.5 μg RNA with PrimeScript™ II 1st strand cDNA Synthesis Kit (TaKaRa). The synthesized cDNA was diluted 40-fold in nuclease free water and stored in a freezer at −80 °C until use.

2.6. Quantification of mRNA expression

In order to analyze the basal expression levels of RfCasp10 in the aforementioned dissected tissues of healthy fish and to monitor its transcriptional modulation in liver and spleen tissues of immune stimulated animals in response to the corresponding immune stimulations, qPCR was applied using previously prepared respective diluted cDNA samples as templates. qPCR was performed using the Dice™ Real Time System Thermal Cycler (TP800; TaKaRa, Japan) in a 10 μL reaction volume containing 3 μL of diluted cDNA from each tissue, 5 μL of 2× TaKaRa ExTaq™ SYBR premix, 0.4 μL of each primer (RfCasp-qF and RfCasp-qR; Table 1) and 1.2 μL of ddH_2O, according to the essential MIQE guidelines (Bustin et al., 2009). PCR cycling conditions were as follows: 95 °C for 10 s, 35 cycles of 95 °C for 5 s, 58 °C for 10 s, 72 °C for 20 s, and a final cycle

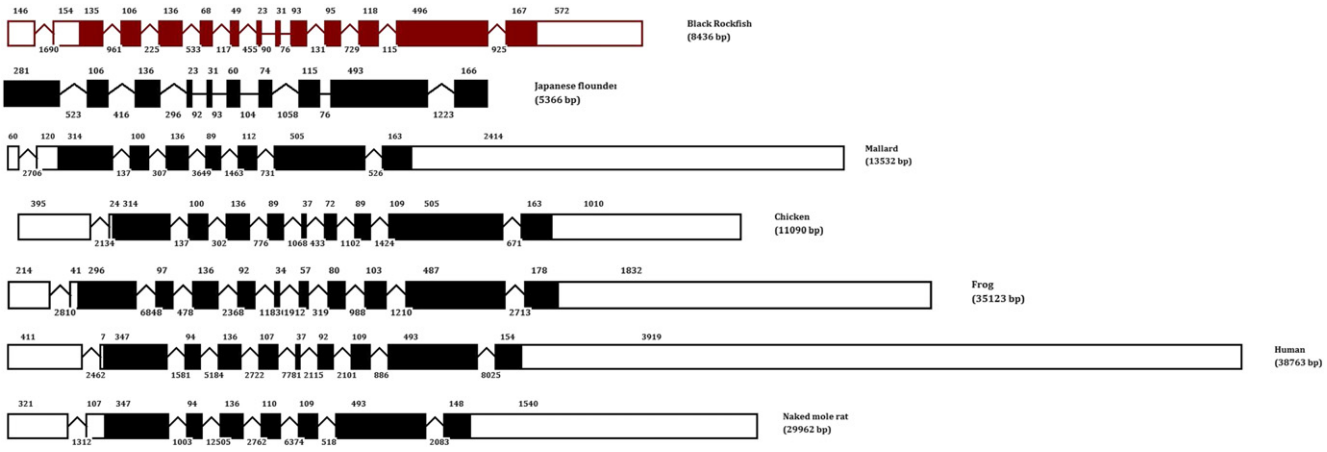

Fig. 2. Exon–intron organization of the RfCasp10 gene in comparison with other vertebrate caspase 10 gene architectures. Introns are indicated by lines and exons are depicted by boxes. UTRs are indicated by white regions whereas coding regions are filled with maroon or black, respectively. Introns greater than 150 bp are indicated as Λ. The corresponding DNA sequence lengths of introns and exons are indicated at the top and bottom of each structure, respectively. The genomic DNA sequence information of each gene was obtained from the NCBI-GenBank database under the following accession numbers or gene IDs: Japanese flounder—AB247499, mallard—101799731, chicken—424081, frog—548432, human—843, naked mole rat—101712620.

of 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s. Each assay was conducted in triplicates. The baseline was set automatically by the Dice™ Real Time System software (version 2.00). RfCasp10 relative expression was determined using the Livak ($2^{-\Delta\Delta CT}$) method (Livak and Schmittgen, 2001). Black rockfish elongation factor 1-alpha (RfEF1A) was used as the internal reference (GenBank ID: KF430623; Liman et al., 2013) and amplified under the same qPCR cycling parameters as applied for RfCasp10 amplification. The primers used for the internal reference are listed in Table 1. The data are presented as the mean ± standard deviation (SD) of the relative amount of mRNA determined in three PCR assays per sample. In the immune stimulation experiments, the transcription levels of RfCasp10 were calculated relative to those of RfEF1A. The expression fold values were then normalized to the corresponding PBS-injected controls at each time point. The relative transcription level in the un-injected control at the 0 h time point was used as the baseline reference. To determine the statistical significance ($P < 0.05$) of the difference between experimental and un-injected control groups, a two-tailed unpaired Student's *t*-test was carried out.

3. Results and discussion

A putative complete cDNA sequence of 2389 bp was identified from our black rockfish cDNA database, which shared substantial homology with caspase 10 sequences of other teleosts, and was designated as RfCasp10. *RfCasp10* consists of a 1659 bp coding sequence, encoding a 553 amino acid sequence with a predicted molecular mass of 61.7 kDa and a theoretical isoelectric point of 5.61, and of 5′ and 3′ untranslated regions (UTR) of 154 bp and 576 bp, respectively. Sequence data was deposited in the NCBI-GenBank sequence database under the accession number KP843661. As detected by our *in silico* study of the derived amino acid sequence, RfCasp10 harbors the typical domain structure of caspase family proteins, including large (p20: residues 305–430) and small (p10: residues 453–541) subunits, along with two N-terminal "DED" domains specific for caspases 8 and 10 (residues 2–79 and 92–159) (Fig. 1). Multiple sequence alignment of RfCasp10 with its closer homologs affirmed that some residues of the other active sites and residues forming the substrate binding pocket are thoroughly conserved in RfCasp10 (Fig. 1). Moreover, the caspase family histidine and cysteine active sites were also identified in RfCasp10. The latter was completely conserved among the caspase 10 homologs containing the active site pentapeptide motif (QACQG), compared in Fig. 1. As confirmed by our pairwise sequence alignment, RfCasp10 shared its highest sequence similarity (78.7%) and identity (64.7%) with caspase 10 of the teleost snakehead murrel (*Channa striata*). However, the similarity of RfCasp10 with other fish, mammalian, avian, or amphibian counterparts was found to be relatively low (Table S1). Collectively, our in silico

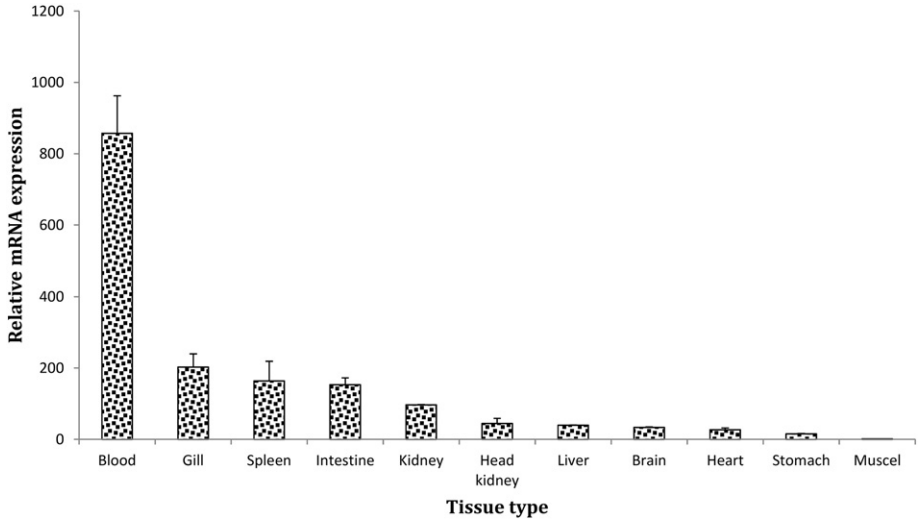

Fig. 3. Tissue specific transcript levels of RfCasp10 determined by qPCR. Fold changes in expression relative to the mRNA expression level in the muscle are shown. Error bars represent SDs (n = 3).

study provides evidence for the homology of RfCasp10 with known caspases 10.

As expected, phylogenetic reconstruction generated based on the evolutionary relationship of several initiator caspases clearly showed that respective caspase family members are clustered together, closely and independently (Fig. S1). However, house fly caspase 10 formed an out-group from the main cluster of caspase 10 and 8 similitudes, depicting an evolutionary distant relationship with those caspase similitudes. Validating the prominent sequence similarity detected in our pairwise sequence alignment, RfCasp10 was closely clustered with snake head murrel caspase 10 in the teleost clade, with a strong bootstrap support (75). The clustering pattern of the caspases affirms that caspases 10 and 8 as well as caspases 2 and 9 are evolutionarily more closely related, in good agreement with their known classification into initiator and effector caspases, respectively. Overall, our phylogenetic study confirms the vertebrate ancestral origin of RfCasp10, further validating its homology with teleostan caspase 10.

We could identify the complete gene of RfCasp10 using our BAC genomic DNA library. The gene consists of 8436 bps and comprises 13 exons and 12 introns, following the canonical AG-GT rule (Fig. 2). Its coding sequence spans 12 exons. However, the coding region of flounder caspase 10 is split into only 10 exons. Interestingly, some of the internal exons of flounder caspase 10 (exons 2, 3, 4, 5) were found to be conserved in RfCasp10. In a comparison of RfCasp10 genomic DNA with that of selected mammalian, avian and amphibian origin, marked deviation with respect to the number as well as the size of the corresponding exons could be detected. However, the 5′ UTR of those caspase 10 sequences was split into two exons, similar to RfCasp10. Whether this is true also for flounder caspase 10 remains to be determined, as information regarding its UTR arrangement in the genomic sequence is still lacking. Compared to all other caspase 10 sequences, RfCasp10 harbors the highest number of exons. The multi-exonic arrangement of caspases 10 in all the animals used in the study may suggest the existence of different spliced variants of caspase 10 (Keren et al., 2010).

As detected by qPCR, RfCasp10 was ubiquitously transcribed in the tissues examined, albeit in different magnitudes. Markedly abundant RfCasp10 transcription was observed in blood, and, in descending order, in gill, spleen, and intestine tissues, compared to the least abundant transcript level in muscle tissue (Fig. 3). Blood contains a pool of different cells, including natural killer cells (NK cells) and cytotoxic T lymphocytes (CTLs) which express Fas ligands (Arase et al., 1995; Suda et al., 1995). These cells can induce apoptosis via Fas ligand, which in turn can trigger the caspase 10 mediated apoptosis in target cells (Fowlkes and Schweighoffer, 1995; Baumler et al., 1996; Castro et al., 1996; Sprick et al., 2002). Because blood acts as the circulatory medium of animals including fish, infected cells in blood probably need to undergo rapid apoptosis to minimize the potential spread of pathogens to other tissues. Therefore, it is indeed likely to observe a pronounced level of expression of apoptotic mediators, including caspase 10, in blood cells, and in good agreement with our observation of RfCasp10 transcription. However, it is also important to note that RfCasp10 was poorly expressed in the kidney, although it is known to be involved in hematopoiesis in teleosts until adulthood (Zapata et al., 2006). However, in some teleosts, kidney cells mainly produce red blood cells and granulocytes, rather than cells which express Fas ligands, such as NK cells or lymphocytes (Uribe et al., 2011). Therefore, Fas ligand mediated apoptosis occurs at a low frequency in the blood cells in the kidney, which might explain the poor basal expression of *RfCasp10* in the kidney. Furthermore, as reported previously, lymphoid tissues can express prominent levels of caspase 10 (Vincenz and Dixit, 1997). In fish, the spleen is considered as the major lymphoid organ (Petrie-Hanson and Ainsworth, 2001), and tissues of gills and intestines are regarded as peripheral lymphoid tissues (Zapata and Cooper, 1990; Rombout et al., 1993), which also expressed substantial basal levels of RfCasp10 in black rockfish. Complying with our observations, prominent expression of caspase 10 in Japanese flounder was detected in peripheral blood leukocytes, gills, and spleen (Kurobe et al., 2007). Moreover, pronounced levels of snakehead murrel caspase 10 were also detected in gills and spleen, albeit blood showed a relatively minor basal expression level (Arockiaraj et al., 2013).

In order to prefigure the potential trigger of death receptor mediated apoptosis in black rockfish upon pathogen stress, modulation of RfCasp10 transcription was analyzed in several immune-relevant tissues after stimulation with bacterial (*S. iniae*) or viral (poly I:C) stimuli. According to the results of the qPCR assay, the basal RfCasp10 mRNA level in blood cells was found to be significantly elevated ($P < 0.05$) upon poly I:C stimulation, in an early phase (6 h and 12 h post stimulation; p.s.) as well as in a late phase (72 h p.s.) of the experiment, whereas it was continuously up-regulated from 12 h p.s. to 72 h p.s. by live *S. iniae* invasion (Fig. 4A). Similarly, poly I:C and *S. iniae* treatments elicited significant ($P < 0.05$) early and late phase transcriptional responses in spleen (Fig. 4B) and liver tissues (Fig. 4C), following the same pattern of expression, albeit the fold differences compared to the basal levels

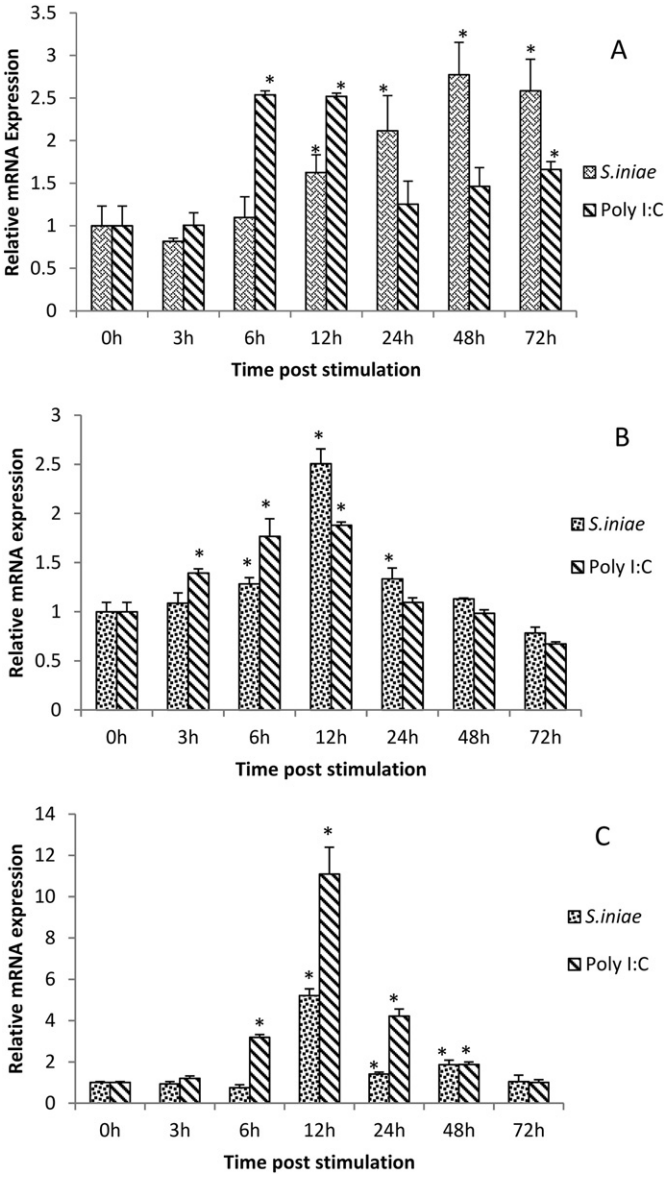

Fig. 4. Temporal modulation of RfCasp10 transcription in (A) blood, (B) spleen, and (C) liver tissues upon immune stimulation with *S. iniae* and poly I:C, as determined by qPCR. The relative expression was calculated by the $2^{-\Delta\Delta CT}$ method, using rockfish EF1A as the reference gene with respect to the corresponding PBS-injected controls at each time point. The relative fold-change in expression at 0 h post-injection was used as the basal line. Error bars represent SDs (n = 3); $^{*}P < 0.05$.

were dissimilar, depending on the tissue. However, the inductive expression pattern observed in the blood cells was different from that noted in the spleen or liver, in which early as well as late phase transcriptional responses showed marked fluctuation. This overall response might have been caused by the wide array of immune cells present in the blood, compared to the spleen or liver, elevating the *RfCasp10* expression at different time points in different magnitudes, in response to the induced pathogen stress, respectively. Compared to the spleen and blood, the liver showed strong inductive transcriptional responses after *S. iniae* and poly I:C treatments, relative to its basal level of RfCasp10 transcription at an early phase post-stimulation (5-fold and 11-fold, respectively, at 12 h p.s.). The poor basal expression of *RfCasp10* in the liver compared to that in the spleen or blood (Fig. 3) might explain this observation, since a higher increase of *RfCasp10* expression in the liver cells might require the compensation of the adequate level of RfCasp10 to trigger apoptosis against pathogen stress, compared to the fold increase required in the blood or spleen tissues. Here, high expression of RfCasp10 can adequately contribute to trigger the apoptosis upon pathogen stress. Moreover, as the liver is a potent immune organ in first line host defense (Seki et al., 2000), it is not unexpected to observe a noticeable expressional induction of caspases including caspase 10 in liver, which in turn facilitate the elicitation of apoptosis to eradicate infected cells. Altogether, our observations on inductive expression of RfCasp10 under pathogen stress suggest the potential induction of death receptor mediated apoptosis in immune relevant tissues of black rockfish, which in turn may also be the cause of mortality for bacteria or virus infected fish. However, further studies, especially on the functionality of RfCasp10, are necessary to elucidate its exact role in black rockfish physiology.

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