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Short Communication

Molecular cloning and characterisation of the rock bream, *Oplegnathus fasciatus*, Fas (CD95/APO-1), and its expression analysis in response to bacterial or viral infection

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ABSTRACT

Fas belongs to the tumour necrosis factor (TNF) receptor superfamily and can transmit a death signal leading to apoptosis. In the present study, we isolated the full-length cDNA for rock bream (*Oplegnathus fasciatus*) Fas (RbFas). The full-length RbFas cDNA was 1770 bp long and contained an open reading frame of 957 bp that encoded 319 amino acid residues with a predicted molecular mass of 35.1 kDa. The 319 amino-acid predicted RbFas sequence is homologous to other Fas sequences, contains three cysteine-rich domains and a death domain (DD) and two potential N-glycosylation sites. Expression of RbFas mRNA was detected in nine different tissues from healthy rock bream and was the highest in red blood cells. In analyses of mitogen-stimulated RbFas expression in peripheral blood leucocytes, expression of RbFas mRNA was observed between 1 and 36 h after stimulation with LPS, and 1 and 3 h stimulation with poly I:C. In the case of bacterial injection, the RbFas transcript peaked 6 h after injection in both the kidney and the spleen. Otherwise, the RbFas transcript peaked after 1 h in spleen and 6 h in kidney following injection with RSIV.

1. Introduction

Apoptosis, or programmed cell death, is the most common form of eukaryotic cell death, and it occurs during embryogenesis, metamorphosis, tissue atrophy and normal cell turnover [1]. Chemical agents and pathogenic infections accelerate apoptosis as it acts as an immune response in the host defence system [2]. The cytotoxicity of cytotoxic T lymphocytes (CTLs) or natural killer (NK) cells is mediated by apoptosis [3]. Apoptosis is characterised morphologically by cell shrinkage with nuclear fragmentation and biochemically by chromatin cleavage into nucleosomal oligomers [4]. Cell components and chromatin form apoptotic bodies and are removed efficiently by neighbouring macrophages and granulocytes [1,5,6]. Thus, apoptosis is regulated to maintain immunological homoeostasis.

During the selection of immature T cells in the thymus, CTLs induce apoptosis through the Fas ligand (FasL) system against cells that react as self-antigens or are not able to recognise self-MHC molecules [7–9]. The cells that react to self-antigens attack host tissues and cause autoimmune diseases [10]. Additionally, the affinity of the T-cell receptor for the MHC molecule is essential to recognise the presentation of antigens [11].

Fas belongs to the tumour necrosis factor (TNF) receptor superfamily and can transmit a death signal leading to apoptosis [12]. The interaction between Fas and FasL has been investigated in a variety of cell lines *in vitro*, and the findings of these studies suggest that the binding of FasL to Fas on the target cell induces a death signal that initiates apoptosis [13]. The intracellular portion of Fas contains a protein-interaction motif termed the death domain. Death domains are found in other pro-apoptotic receptors such as TNF receptor I and DR 3–5, as well as in their adaptor proteins such as FADD, TRADD, RIP and RAIDD [12]. During Fas-mediated apoptosis, FADD binds to the Fas death domain and recruits procaspase 8, which is an apical protease for inducing apoptosis [14,15].

Fas is constitutively expressed by a broad range of normal epithelial cells and various haematopoietic cells. Notably, some tumour cells such as those of adult T-cell leukaemia, acute myelogenous leukaemia, chronic lymphocytic leukaemia, hepatocellular carcinoma and colon carcinoma abnormally over- and under-express Fas [16–19] and some of these virus-infected cells are sensitive to

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Fas-mediated apoptosis. The expression of Fas appears to be induced by interferon γ and CD40, which indicates that the expression of Fas is controlled by certain mechanisms [20,21].

When CTLs recognise antigens derived from the virus by the MHC molecule, they express the FasL and induce apoptosis [22]. Thus, the FasL plays an important role in maintaining homoeostasis in the immune system by inducing apoptosis. Several members of the TNF superfamily and TNF superreceptors family have been cloned in fish [23–38]. Although a Fas molecule has been reported in zebrafish and medaka [29,30], expression analyses in rock bream has not been reported. Molecular cloning and characterisation of the Fas should contribute to elucidating the mechanism of innate immunity in rock bream. Here we report the molecular cloning and sequence analysis of a Fas gene from rock bream and its expression in relation to infection by *Streptococcus iniae* or iridovirus.

Table 1
Primers used in this study.

Primer name	Sequence (5′–3′)
For RACE PCR	
RbFas-5R	CCA TCC AAT CAC TTC TGC AAT
RbFas-3F	CAG CTG ATC CGG AGA GAC AG
For RSIV quantification	
MCPL sense	CCC TAT CAA AAC AGA CTG GC
MCPL anti-sense	TCA TTG TAC GGC AGA GAC AC
MCPS sense	CTG CGT GTT AAG ATC CCC TCC A
MCPS anti-sense	GAC ACC GAC ACC TCC TCA ACT A
For qRT-PCR amplification	
RbFas-F	GTT TCG TGC GTC GTT TAT CA
RbFas-R	CAA ACC TGC AGC ACA CAG ACA
β-actin F	GGA CAC GGA AAG GAT TGA CA
β-actin R	CGG AAT TAA CCA GAC AAA TC

2. Materials and methods

Fas cDNA was identified by analysing expressed sequence tags in the cold shock-stimulated rock bream erythrocytes library. The full-length cDNA of RbFas was obtained by 5′ RACE. The 5′ and 3′ ends of the RbFas cDNA were identified by the RACE cDNA amplification kit (Clontech, CA, USA) according to sequence information from the obtained fragment. A specific primer set (RbFas-5R and RbFas-3F) was designed according to the known EST sequences of RbFas (Table 1). Briefly, total RNA was extracted from red blood cells using Trizol Reagent (Invitrogen, USA) and first strand cDNA was synthesised using the protocol recommended by the SMART RACE cDNA Amplification Kit. The primer set of lRbFas-3F and a nested universal primer (supplied by Clontech) was used for 3′ RACE, and 5′ RACE was carried out with RbFas-5R and the nested universal primer. The generated PCR products were purified, cloned into pGEM T-easy vector system (Promega, USA) and subsequently sequenced. Thus the complete cDNA of RbFas was compiled by the 5′ and 3′ RACE DNA sequences.

The determined nucleotide and deduced amino acid sequences and multiple sequence alignments were analysed with GENETYX ver. 8.0 (SDC Software Development Co. Ltd., Tokyo, Japan). The signal peptide was predicted using the online SignalP 3.0 programme (http://www.cbs.dtu.dk/services/SignalP/) [31] and domain identification was analysed with the PROSITE and SMART programmes (http://smart.embl-heidelberg.de/). The phylogeny was inferred using the Mega 4 programme and distance analysis by the neighbour-joining (NJ) method [32]. The values supporting each node were derived from 2000 re-samplings.

The RbFas mRNA expression levels were analysed by quantitative real-time PCR using gene-specific primers (Fig. 1). β-Actin was amplified as a control using β-actin F and β-actin R primers [33]. Tissue-specific mRNA expression was analysed in healthy

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GGGGAGACGCGGTGAAGTTGTGATGTGAAGAACATGATGGCCGACTCAAAGAAGTTTCGTGCGTCGTTTATCACCTTTGTTCTGCACTCC   90
                                  M  M  A  D  S  K  K  F  R  A  S  F  I  T  F  V  L  H  S    19
TACCTTGTGTTAGTGGCGCTCTCCCAGACGGAAGCGACAAACCGAGGCCAGTGTCAGGATGGTACCTACAAACATGAAGGGAGGGAGTGT   180
 Y  L  V  L  V  A  L  S  Q  T  E  A  T  N  R  G  Q  C  Q  D  G  T  Y  K  H  E  G  R  E  C   49
TGTCTGTGTGCTGCAGGTTTGCATCTGGAGCAGCATTGCAGTACGAATCTAACGCAGGGAAAATGCAAGGCCTGTCCTAGTGAGATGTAC   270
 C  L  C  A  A  G  L  H  L  E  Q  H  C  S  T  N  L  T  Q  G  K  C  K  A  C  P  S  E  M  Y   79
AAGAGTCACCCCACCTCAGAAGTGCACTGCGAGCCCTGCACATCCTGCTCACAGCAAAATGCAAATCTAATGGAGGCTGAACCCTGCACC   360
 K  S  H  P  T  S  E  V  H  C  E  P  C  T  S  C  S  Q  Q  N  A  N  L  M  E  A  E  P  C  T   109
CCTGGCAAGGACAGGAAGTGTCGATGTAAAGAGGATCACTATTGCGACAGTGTTGTAGAAACCTGTACACTCTGTCAACCTTGTACAAAA   450
 P  G  K  D  R  K  C  R  C  K  E  D  H  Y  C  D  S  V  V  E  T  C  T  L  C  Q  P  C  T  K   139
TGTGGTGCTGAGGGCATCAAAGCAGACTGCACAGCCACGAGTAACAGAGTCTGCAATGACAAAATTCAAGAGGGAAATCACAGTGCAGGG   540
 C  G  A  E  G  I  K  A  D  C  T  A  T  S  N  R  V  C  N  D  K  I  Q  E  G  N  H  S  A  G   169
ACAATAGCCGCCATAACTGTCACAATTGTAATCATTGTTCTAGCAGCAATAGCTGCGTTAATTTGGAAAAAGAAAGAAGCGTGGAAGAGA   630
 T  I  A  A  I  T  V  T  I  V  I  I  V  L  A  A  I  A  A  L  I  W  K  K  K  E  A  W  K  R   199
AAACAGACAGCCCAAGAGAGCAATGGAAATGCAGCTGATCCGGAGAGACAGCCTCTTAGAGTTCCAGATGTGGATCTCCAGCCTCACATG   720
 K  Q  T  A  Q  E  S  N  G  N  A  A  D  P  E  R  Q  P  L  R  V  P  D  V  D  L  Q  P  H  M   229
TCTGACATTGCAGAAGTGATTGGATGGAAGGTGATGCAGGATGTAGCAATGCGTAGTAGCATACCTGACACTACTATTGAGTCTTGTCAA   810
 S  D  I  A  E  V  I  G  W  K  V  M  Q  D  V  A  M  R  S  S  I  P  D  T  T  I  E  S  C  Q   259
CTGGACCAACCTAATAACAGCCAGGAGCAGACACGCCAACTACTCAAGATCTTTGCGGAGATACAGGGCAGGGGAGCCTCAGAGAAGTTG   900
 L  D  Q  P  N  N  S  Q  E  Q  T  R  Q  L  L  K  I  F  A  E  I  Q  G  R  G  A  S  E  K  L   289
GTCGAAATCCTAGAAAATAGCGGCAGAAGGCGCACAGCAGAGAAGGTGGTAGATATATTGCATGTAGCAGCTAGATCAAATCTTCCTGCT   990
 V  E  I  L  E  N  S  G  R  R  R  T  A  E  K  V  V  D  I  L  H  V  A  A  R  S  N  L  P  A   319
TGAATGTGTCTGTTATGAGCACTTTTTAAATATTTGCAGTGCTGTCTACTGTATGATGTTTACATGTAGGATTGAACTATAAATATTGAT  1080
 *
TTAAAGGAGTAGGATTTTCCTAAAAAACAATGTATAGACTGATACAAAAATAGTCCCTCTCAGTCATCATTTATGATCCACTAGAAGTGT  1170
GTGGCGGTGTCTGTATCTGCAGAGACCCTGCCCTCTGCCTGTATTTTCTTATTTTGCTGTGCTCGGGACGTTTCTGGGTGTCAACCTTTG  1260
TGTCGCCCCCAGCCAATAACAGTGCGCAGGGTGTGAGGTGCGGGACTGTATAAAAACGTAGTGACCAGGCGCCAGATCGTAAGCACGACT  1350
CTCACTTTTGGTGTCGTTGAGCCGGGGAGGAGGGGCCAACTTTCAATGTTGTGTTTACAAACCGCAACCATTCCTACTACTCATTCTGCC  1440
TTTAAGCTTGTATGTGTATATTATGAGTCTTTCTGTCGTTACTTGAACTTGAAAGCAGAACTGCTGTGATCTACAGAGGAATGACAGGGA  1530
GAATCACTATTATCTGCTTCTCATTTTATTAATGGATTGAGAGGTCATCTGCCACTATTTCCTTCCAGTTTTTGGTGCGAAGGTGTTAAA  1620
AAAAGATCAGTCTGGATTACACCTCCCTTCCTGTGTTGATATCATGTTTCTGGAGAAAGTCTATATGCCATTTGCGAGTTATTAATAGCT  1710
CGCCACAGCTTGTATATTGTGCAATAAACCTCAATAAATCTGAAAAAAAAAAAAAAAAAA                                  1770
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Fig. 1. Nucleotide and deduced amino acid sequences of the rock bream Fas (RbFas). The active cysteine and other two cysteine residues, Cys^{71} and Cys^{75}, are in bold and the predicted signal peptide is underlined. The death domain predicted by the SMART programme is shaded and the polyadenylation signal AAUAAA is italicised.