Molecular cloning, characterization and expression analysis of the PKZ gene in rare minnow Gobiocypris rarus

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Received 3 February 2008; revised 7 March 2008; accepted 12 March 2008
Available online 18 March 2008

Abstract Double-stranded RNA-activated protein kinase (PKR) plays an important role in interferon-induced antiviral responses, and is also involved in intracellular signaling pathways, including the apoptosis, proliferation, and transcription pathways. In the present study, a PKR-like gene was cloned and characterized from rare minnow Gobiocypris rarus. The full length of the rare minnow PKR-like (GrPKZ) cDNA is 1946 bp in length and encodes a polypeptide of 503 amino acids with an estimated molecular mass of 57,355 Da and a predicted isoelectric point of 5.83. Analysis of the deduced amino acid sequence indicated that the mature peptide contains two Zalpha domains and one S_TKc domain, and is most similar to the crucian carp (Carassius auratus) PKR-like amino acid sequence with an identity of 77%. Quantitative RT-PCR analysis showed that GrPKZ mRNA expression is at low levels in gill, heart, intestine, kidney, liver, muscle and spleen tissues in healthy animals and up-regulated by viruses and bacteria. After being infected by grass carp reovirus, GrPKZ expression was up-regulated from 24 h post-injection and lasted until the fish became moribund (P < 0.05). Following infection with Aeromonas hydrophila, GrPKZ transcripts were induced at 24 h post-injection (P < 0.05) and returned to control levels at 120 h post-injection. These data imply that GrPKZ is involved in antiviral defense and Toll-like receptor 4 signaling pathway in bacterial infection.

Introduction

The double-stranded RNA-activated protein kinase (PKR) is a critical mediator of antiproliferation and a key component of the innate antiviral response exerted by interferons. PKR integrates signals in response to Toll-like
receptor (TLR) activation, growth factors, differentiation, apoptosis, and diverse cellular stresses [1–5]. PKR phosphorylation of the α-subunit of eukaryotic initiation factor 2 (eIF2α) results in a blockade on translation initiation. This prevents viral replication and inhibits normal cell ribosome function, killing both the virus and the host cell if the response is active for a sufficient amount of time [6]. PKR cannot block the translation of some cellular and viral mRNAs bearing special features in their 5' untranslated regions [7]. PKR also affects diverse transcriptional factors such as interferon regulatory factor 1, STATs, p53, activating transcription factor 3, and NF-κB [8,9]. The extent and strength of the antiviral action of PKR are clearly understood by the findings that unrelated viral proteins of animal viruses have evolved diverse strategies to inhibit PKR action [7,10]. Knock-out mouse studies confirmed a role for PKR in antiviral innate immunity [11]. Recently, PKR has been implicated in TLR signal transduction in response to bacterial cell wall components [12].

The PKR gene was first identified from humans in 1990 [13]. Now PKR sequence similarity genes have been discovered in many eukaryotes, such as mammals, birds, amphibians, fishes, insects, and yeast (searched by blastp). Induction of the gene with sequence similarity in yeast is in response to starvation and activated by uncharged tRNAs and the Gcn1 p-Gcn20 p complex (Accession No. AY293929; E-value = 6e–36). In insects, there are several similar sequences with E-value from 2e–34 to 3e–47. However, these sequence similarity genes in invertebrates may not have functional similarity, because of the lack of homologous interferon genes in several fully sequenced invertebrate genomes [14–16]. In fishes, PKR homologous genes are found in five species in GenBank, including crucian carp, zebrafish, Atlantic salmon, olive flounder and pufferfish (February 2008).

Fish appear to have a PKR-like protein that has Z-DNA binding (Zalpha) domain(s) instead of dsRNA binding (DSRM) domain(s) in the regulatory domain, and thus has been designated Z-DNA binding protein kinase (PKZ) [17]. Zalpha domain(s) specifically bind dsDNA and dsRNA in the left-handed Z conformation, often with high affinity [17,18]. PKZ is able to phosphorylate eIF2α [19] and may play a role, like PKR, in host defense against virus infection [17,19].

Grass carp (Ctenopharyngodon idellus) is an important aquaculture species in China, but great economic loss is often caused by Grass Carp Reovirus (GCRV), a dsRNA virus. A better understanding of the immune defense mechanisms of grass carp may contribute to the development of management strategies for disease control and long term sustainability of grass carp farming. The rare minnow Gobio cypris rarus, which is a small cyprinid species, has been recognized as a useful model. It is very sensitive to GCRV [20]. Moreover, Aeromonas hydrophila, a Gram-negative bacterium of the family Aeromonadaceae, is often found in association with hemorrhagic septicemia in cold-blooded animals including fish, reptiles and amphibians [21].

In this paper, we employed the rare minnow as a model fish to study the mechanism of GCRV disease. A full length cDNA sequence encoding a PKR-like protein (GrPKZ) was identified and characterized. We present GrPKZ mRNA expression profiles in different tissues and following GCRV and A. hydrophila infection.

Materials and methods

Animals, immune challenge and sample collection

Rear minnows (2–3 g body weight) were obtained from a laboratory-breeding stock and acclimatized to new laboratory conditions for one week in a quarantine area. They were maintained in 25 l aerated aquaria at 28 °C and fed twice a day with commercial diet (feed composition: protein 32%, starch 63%, fat 3%, additive 2%). Before experiments, the fish were apparently healthy.

For challenge experiments with GCRV (991 strain) or A. hydrophila (C1 strain), fish were injected with 10 µl PBS per gram body weight, 10 µl GCRV suspended in PBS (2 × 10⁶ PFU/ml) or 10 µl A. hydrophila resuspended in PBS (OD₆₀₀ = 0.1) intraperitoneally. Non-injected animals were used as a blank group. Three individuals were killed and tissues including gill, heart, intestine, kidney, liver, muscle, and spleen were collected at 0, 12, 24, 36, 48, 72, 96, 120, 144 and 168 h after injection. The samples were homogenized in TRIzol® LS reagent (Invitrogen) and total RNA was extracted according to the manufacturer’s instruction. Total RNA was incubated with RNase-free DNase I (Roche) to remove contaminated genomic DNA before reverse transcription into cDNA using random hexamer primers with SuperScript™ III Reverse Transcriptase (Invitrogen).

Amplification of cDNA and nucleotide sequence analysis

To clone PKZ cDNA from rare minnow, degenerate primers were designed based on the multiple alignment of known fish PKR-like sequences including Danio rerio (Accession No. AJ852024), Carassius auratus (Accession No. AY293929), Salmo salar (Accession No. DQ182560), Tetradon nigroviridis (Accession No. AJ544919). PCR was performed with primers F106a and R110a (Table 1) using the cDNA generated from liver. The PCR program was: 1 cycle of 94 °C /4 min, 35 cycles of 94 °C/30 s, 50 °C/30 s, 72 °C/45 s; 1 cycle of 72 °C/5 min. The PCR product was ligated into the pMD18-T easy vector, transformed into the competent E. coli TOP10 cells, and plated on the LBagar petri-dish. Positive clones containing inserts at the expected size were screened by colony PCR and plasmid prepared using an Axyprep™ plasmid miniprep kit (Axygen Biosciences). Three plasmid DNAs were sequenced by a commercial company (Shanghai Invitrogen Biotechnology Co., Ltd, China).

Rapid amplification of cDNA ends (RACE)

Using the BD SMART™ RACE cDNA amplification kit (BD Biosciences Clontech), first strand cDNA synthesis and RACE were performed on liver-derived RNA. To obtain the 3’ unknown region, primer pairs, F141/adaptor primer UPM and F142/adaptor primer NUP (Table 1), were used for the
primary PCR and the nested PCR, respectively. The amplified PCR products were cloned and sequenced as described above. Similarly, the 5' end of GrTLR3 was obtained by nested PCR using primer pairs R163/UPM and R164/NUP (Table 1). The full length cDNA sequences were confirmed by sequencing the PCR product amplified by primers F164a and R164b (Table 1) within the predicted 5' and 3' untranslated regions, respectively.

Sequence analysis and phylogeny

Sequence homology was obtained using BLAST program (http://www.ncbi.nlm.nih.gov/blast). The deduced amino acid sequences were analyzed with the Expert Protein Analysis System (http://www.expasy.org/) and the protein domain features were predicted by the Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/) [22]. Intra-domain features were predicted by a scan of the sequence against the PROSITE database (http://us.expasy.org/tools/scanprosite) [23]. Multiple sequence alignments were created using the ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html) and The sequence manipulation suite (http://www.bioinformatics.org/sms/) programs. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 [24] and optimized manually.

Quantification of gene expression

Expression of GrPKZ mRNA was assessed in different tissues and infection states using quantitative real-time RT-PCR (qRT-PCR) in an ABI Prism 7000 Sequence Detection System (Applied Biosystems). To check the invariant expressions of the putative house keeping gene β-actin under different situations, the expression of β-actin in 1 μg of total RNAs was tested in healthy fish and fish infected by viruses and bacteria. β-Actin was utilized as an internal control for cDNA normalization. The gene specific primers for β-actin were forward primer F86 and reverse primer R87 (Table 1). GrPKZ gene expression was detected using forward primer F126 and reverse primer R127 (Table 1). The qRT-PCR mixture consisted of 1 μl of cDNA sample, 8 μl nuclelease-free water, 10 μl of SYBR Green PCR master mix (Toyobo), and 0.5 μl of gene specific primers (5 μM). The PCR cycling conditions were: 1 cycle of 95°C/2 min, 40 cycles of 95°C/25 s, 60°C/30 s, and 72°C/1 min, followed by dissociation curve analysis to verify the amplification of a single product. The threshold cycle (CT) value was determined using the manual setting on the ABI Sequence Detection System and exported into a Microsoft Excel Sheet for subsequent data analysis where the PCR efficiency and relative expression ratio of target gene in treated groups versus that in control were calculated by Pfaffl equation [25]. The expression data obtained from three independent biological replicates were subjected to one-way analysis of variance (one-way ANOVA) followed by an unpaired, two-tailed t-test. P < 0.05 was considered statistically significant.

Nucleotide sequence deposition

The GrPKZ cDNA sequence was submitted to GenBank and has been designated accession number EF661570.

Results

Characterization of GrPKZ mRNA and the encoded protein

Initial amplification of GrPKZ gene sequences was conducted using liver cDNA as template and degenerate primers F106a and R110a. Of multiple amplified segments, a 506 bp product (trimmed vector sequence) was shown by blastx analysis to have significant homology (E-value = 4e-84) to the C-terminal region of C. auratus interferon-inducible and double-stranded-dependent eIF-2 kinase gene (namely PKZ) (Accession No. AAP49830). Based on the sequence of the amplified 506 bp segment, primers F141 and F142 were subsequently designed and used with adaptor primers UPM and NUP to amplify the downstream sequence. Alignment of sequences from all homologous clones yielded a 1034 bp consensus sequence. The clone included a poly(A) tail, suggesting that the expressed sequence tag (EST) represented the 3' region of the PKZ gene.

The 5' region of the gene was amplified by using 5'-RACE. A product of 1074 bp from nested PCR perfectly overlapped the initial 1034 bp consensus sequence. To confirm that the resulting 1946 bp consensus sequence represented a single

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**Table 1** Primers used in the study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>GrPKR</td>
<td></td>
</tr>
<tr>
<td>F106a (forward)</td>
<td>TRYYTVTVYATCAGATGGG</td>
</tr>
<tr>
<td>R110a (reverse)</td>
<td>GACAGCATYTTYMGATGATT</td>
</tr>
<tr>
<td>F141 (forward)</td>
<td>CAAAGAGAAGACAGAGAATC</td>
</tr>
<tr>
<td>F142 (forward)</td>
<td>TAGAGGCTCAAGAACAGAC</td>
</tr>
<tr>
<td>R163 (reverse)</td>
<td>ATAGTTTATCAACGCTAGT</td>
</tr>
<tr>
<td>R164 (reverse)</td>
<td>GTGAATGATTGTGAAGATGT</td>
</tr>
<tr>
<td>F164a (forward)</td>
<td>TGAAAATGTCTAGCAAAAGCTG</td>
</tr>
<tr>
<td>R164b (reverse)</td>
<td>ACCGAAGAATACCTGTGAAATCT</td>
</tr>
<tr>
<td>F126 (forward)</td>
<td>GGCAGGCTGAAACCAAATCA</td>
</tr>
<tr>
<td>R127 (reverse)</td>
<td>CAAACCATACGAGTCCAAGGG</td>
</tr>
<tr>
<td>β-Actin</td>
<td></td>
</tr>
<tr>
<td>F86 (forward)</td>
<td>GATGATGAAATGGCCGACTG</td>
</tr>
<tr>
<td>R87 (reverse)</td>
<td>ACAACCATGACACCTGATG</td>
</tr>
<tr>
<td>Universal adaptor primer</td>
<td></td>
</tr>
<tr>
<td>UPM</td>
<td>Long: CTAAATGACTCATAAGGGCA</td>
</tr>
<tr>
<td></td>
<td>Short: CTAAATGACTCATAAGGGC</td>
</tr>
<tr>
<td>NUP</td>
<td>AACGAGTGTCAACGCAAGT</td>
</tr>
<tr>
<td>5'-RACE adaptor</td>
<td></td>
</tr>
<tr>
<td>OligodG</td>
<td>AACGAGTGTCAACGCAAGT</td>
</tr>
<tr>
<td></td>
<td>TACGCGGG</td>
</tr>
<tr>
<td>3'-RACE primer</td>
<td></td>
</tr>
<tr>
<td>3'-CDS</td>
<td>AACGAGTGTCAACGCAAGT</td>
</tr>
<tr>
<td></td>
<td>TAC(T)30VN</td>
</tr>
</tbody>
</table>

Note: Y = C/T; M = A/C; R = A/G; W = A/T; V = A/G/C; N = A/G/C/T.
mRNA expressed in the liver of adult *G. rarus*, primers were designed within the 5' and 3' UTRs of the sequence. Using these primers, a single 1740 bp product was amplified from liver cDNA. Sequence analysis of the product confirmed that the full length transcript in *G. rarus* comprises 1946 nucleotides including the 5' untranslated region, coding sequence, 3' untranslated region and poly(A) tail (Fig. 1). The longest open reading frame commences at nucleotide 7 and terminates at nucleotide 1518, encoding a putative 503 amino acid polypeptide. Its 3' UTR contains a polyadenylation signal AATAAA at nucleotide positions 1752–1757 by polyadq software (http://rulai.cshl.edu/tools/polyadq/polyadq_form.html) [26], and two putative ATTTA instability sequences, a motif possibly involved in rapid message degradation [27]. Blastp analysis indicated that the translated sequence has significant homology to previously characterized PKR-like proteins of eukaryotic origin.

**Analysis of the deduced amino acid sequence of GrPKZ**

The 503 amino acid polypeptide encoded in the amplified gene has a calculated molecular weight of 57,355 Da and isoelectric point of 5.83. Blastp search showed that this gene was most similar to crucian carp PKR-like protein (E-value = 0.0), indicating that it might be a rare minnow homologue of PKR-like (named GrPKZ). GrPKZ has three main structural domains, two Zalpha domains in N-terminal regulatory region and one catalytic domain (S_TKc) in C-terminal (Fig. 1). We selected all the representative PKR homologous proteins from fish and one from human (minimum E-value) to compare their conserved domains and other sequence features (Fig. 2). All the sequences contain a conserved S_TKc domain. Fish PKZs contain two Zalpha domains and the protein sequences consist of 503–513 amino acids (aa). Fish PKRs hold three conserved double-stranded RNA binding motifs (DSRM) instead of Zalpha domains and are composed of 667–682 aa. Human PKR has two DSRM and 551 aa. Other parameters, namely molecular weight and isoelectric point, were also distinguishing. In zebrafish, both PKZ and PKR are identified. To study the molecular evolutionary aspects, all the representative vertebrate PKR homologous protein sequences, including all known fish PKR homologous protein sequences, human PKR standing for mammal and African clawed frog PKR representing amphibian, were used to construct a phylogenetic tree (Fig. 3). These homologue proteins can be divided into two groups, PKZ and PKR.

**Expression analysis of GrPKZ mRNA in different tissues**

RNA was extracted from gill, heart, intestine, kidney, liver, muscle and spleen at 36 h post-injection and the levels of GrPKZ transcripts were assessed using qRT-PCR. The expression of β-actin gene in healthy and infected animals was invariant (Fig. 4A–C). GrPKZ mRNA was detected in all fish tissues tested at low levels, and was significantly up-regulated by viral or bacterial infection (P < 0.05)
To determine the effects of viral or bacterial infection on GrPKZ gene expression, three animals from each group were sacrificed at each time point (0, 12, 24, 36, 48, 72, 96, 120, 144, 168 h post-injection) and qRT-PCR assays were conducted to determine the levels of GrPKZ mRNAs in liver tissue. No significant differences were detected in control and blank groups at any of the time points (P > 0.05) (data not shown). In infected groups, the expressions were significantly up-regulated at 24 h post-injection, reached a peak at 36 h post-injection (P < 0.05), and then gradually decreased. In GCRV infection group, the GrPKZ mRNA expressions were significantly higher than those in the control group from 24 h until the fish became moribund (P < 0.05) (Fig. 5). In the bacterial infection group, the expression of GrPKZ recovered to the original level from 120 h post-injection (P > 0.05) (Fig. 5). The data clearly demonstrated that GrPKZ expression levels were stimulated in infected fish, for both viral and bacterial infection (P < 0.05).

**Discussion**

Recently, cDNAs encoding PKZ have been isolated from crucian carp, zebrafish and Atlantic salmon [17,19,28].
Crucian carp PKZ is detected at a very low level of constitutive expression and can be induced by GCRV, poly(I:C) or interferon in CAB cells [28,29]. Zebrafish PKZ is transcribed constitutively at low levels and is highly induced after injection of poly(I:C). It also inhibits translation of proteins in transfected cells and plays a role in the host response to viruses [17]. Atlantic salmon PKZ expression is up-regulated by interferon in Atlantic salmon TO cells and by poly(I:C) in head kidney. Recombinant PKZ is able to phosphorylate eIF2 and plays a role in host defense against virus infection [19]. These papers studied fish PKZ characterizations from different aspects.

In the present study, GrPKZ was remarkably similar to other fish PKZ (Fig. 2) with two Zalpha domains and an S_TKc domain. The protein sequence was also similar to fish and human PKR proteins with DSRM domains and an S_TKc domain (Fig. 2). Based on the phylogenetic analysis, PKZ is distinct from PKR. Fish PKR firstly cluster with mammal and amphibian PKR, then cluster with fish PKZ (Fig. 3). The kinase domains of fish PKR genes are more closely related to those of fish PKZ than to the PKR kinase domains of other vertebrate species [30]. The similarity in structure inferred the analogy in functions. Both PKZ and PKR are present in zebrafish, and both of them can phosphorylate eIF2α in yeast [30]. In other fishes, either PKZ or PKR is reported. Why fish have both PKZ and PKR is not yet clear, but this may reflect early evolutionary solutions to produce an antiviral protein kinase PKR in innate immunity to viral infection. This feature was first reported in fish PKZ and is similar to mammalian PKR.

PKR’s role as a critical antiviral effector protein leading to inhibition of translation and hence shut-down of infected cells make it a target for viral evasion strategies. The reovirus α3 protein sequesters dsRNA and thus prevents it from activating PKR or other dsRNA receptors [33]. GCRV is a reovirus and whether the α3 related protein in GCRV has a similar function needs further research.

Acknowledgements

The authors thank Wei Hu, Ming Li, Feng Xiong, Jun Dai, Bing Tang, Shangping Chen and other laboratory members for technical assistance and helpful discussion. This work was supported by grants from the National Natural Science Foundation of China (30740009, 30540084), from 973 National Basic Research Program of China (2006CB102100), from the Chinese Academy of Sciences (KSCX2-YW-N-021), from Northwest A & F University in China (01140309, 05ZR096 and 01140508), from China Postdoctoral Science Foundation (20070410298) and from the Institute of Hydrobiology, CAS (2007FB09).

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