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Subcellular localization and inductive expression of the dsRNA-dependent protein kinase PKR from Japanese flounder, *Paralichthys olivaceus*

Rong Zhu [1], Yibing Zhang [1], Qiya Zhang, Jianfang Gui *

State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China

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Abstract

The double-stranded RNA (dsRNA)-dependent protein kinase (PKR) belongs to the eIF2α kinase family and plays a critical role in interferon (IFN)-mediated antiviral response. Recently, in Japanese flounder (*Paralichthys olivaceus*), a PKR gene has been identified. In this study, we showed that *PoPKR* localized to the cytoplasm, and the dsRNA-binding motifs (dsRBMs) played a determinative role in protein localization. In cultured FEC cells, *PoPKR* was detected at a low level of constitutive expression but was highly induced after treatment with UV-inactivated grass carp hemorrhagic virus, active SMRV and Poly I:C although with different expression kinetics. In flounder, *PoPKR* was ubiquitously distributed in all tested tissues, and SMRV infection resulted in significant upregulation at mRNA and protein levels. In order to reveal the role of *PoPKR* in host antiviral response, its expression upon exposure to various inducers was characterized and further compared with that of *PoHRI*, which is another eIF2α kinase of flounder. Interestingly, expression comparison revealed that all inducers stimulated upregulation of *PoHRI* in cultured flounder embryonic cells and fish, with a similar kinetics to *PoPKR* but to a less extent. These results suggest that, during antiviral immune response, both flounder eIF2α kinases might play similar roles and that *PoPKR* is the predominant kinase.

Keywords: Double-stranded RNA (dsRNA)-dependent protein kinase (PKR); Inductive expression; *Paralichthys olivaceus*

1. Introduction

The double-stranded RNA (dsRNA)-dependent protein kinase, PKR, is an essential component of the interferon (IFN)-mediated cellular defense pathway [1,2]. As a serine/threonine kinase, PKR is composed of two functional domains, the N-terminal dsRNA-binding domain comprising two copies of about 65-amino-acid motif homologous to those in other dsRNA-binding proteins, and the C-terminal kinase domain containing 12 conserved kinase subdomains shared by serine/threonine kinases [3]. Through the N terminus, PKR interacts with activator dsRNA, in a sequence independent manner, to carry out the regulation of kinase activity. Low levels of dsRNA activate PKR, while high levels are inhibitory [4]. Both the dsRBMs are necessary for the interaction of PKR with dsRNA, while dsRBM1 exhibits much higher dsRNA affinity than dsRBM2 [5,6], the latter of which is believed to interact with the kinase domain, thus leading to the autoinhibition of PKR in normal cells [7]. Through the C terminus, PKR exerts kinase activity including autophosphorylation and substrate phosphorylation [3].

PKR is present in most mammalian cells at a low constitutive level and in a latent state. During virus infection, PKR is induced by IFN and activated by dsRNA, or single-stranded RNA that possesses secondary structures, which

* Corresponding author. Tel.: +86 27 68780707; fax: +86 27 68780123.
E-mail address: jfgui@ihb.ac.cn (J. Gui).
[1] These authors contributed equally to this work.

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are generated during virus replication [8]. Moreover, PKR may be activated by other immunostimuli such as cytokines, growth factors, oxidative stress, and pro-inflammatory stimuli [9,10]. A number of reports have shown that PKR activation gives rise to an antiviral state during the infection of viruses, such as encephalomyocarditis virus (EMCV), vesicular stomatitis virus (VSV), influenza virus, vaccinia virus, human immunodeficiency virus (HIV), and poliovirus, through phosphorylating the α-subunit of the eukaryotic translation initiation factor 2 (eIF2α) on Ser51 [11,12]. Owing to its antiviral role, many viruses have evolved strategies to circumvent PKR function [1,13]. In addition, PKR has also been implicated in other biological effects such as differentiation, cell growth, apoptosis, tumor suppression, signal transduction, and RNA interference [14–18].

Although four members of eIF2α kinases and their antiviral mechanism underlying phosphorylation of eIF2α have been well studied in mammals, little is known about the fish orthologous genes and their roles in antiviral immune response. Characterization of rainbow trout eIF2α gene revealed *in vitro* phosphorylation of its recombinant proteins by mammalian HRI and PKR and enhanced phosphorylation in rainbow trout cells stimulated with infectious pancreatic necrosis virus (IPNV) or Poly I:C [19]. This result indicates that there seem to be eIF2α kinases involved in fish antiviral response. In crucian carp (*Carassius auratus*), zebrafish (*Danio rerio*) and Atlantic salmon (*Salmo salar*), a gene encoding novel eIF2α kinase PKR-like or PKZ has been identified in cells in the antiviral state [20–22]. This eIF2α kinase most closely resembles PKR, but, unlike PKR, it contains two Z-DNA binding domains in the N-terminus. Recently, a total of 13 PKR genes have been cloned and characterized from eight teleost fish and amphibian species. Phylogenetic analyses revealed that the fish PKR genes are more closely related to PKR-like or PKZ than to non-fish vertebrate PKR [23].

In order to delineate the innate antiviral response in fish, Japanese flounder, *Paralichthys olivaceus,* has been extensively studied for the isolation of immune-related genes. By treatment with UV-inactivated grass carp hemorrhagic virus (GCHV), cultured flounder embryonic cells (FEC) are able to produce IFN-like activity [24], and flounder eIF2α kinase gene *HRI* (heme-regulated initiation factor 2α kinase) has been characterized in virus-infected cells and fish [25]. However, in mammals, HRI is found to be activated by heme deficiency or under conditions of heat shock and oxidative stress, and it just plays an important role in heme-produced cells [26]. Recently, flounder *PKR* gene (*Paralichthys olivaceus PKR, PoPKR*) has been cloned from the same cell system [27]. In order to well understand the roles of fish eIF2α kinases during host antiviral response, this study further investigated the expression profiles of *PoPKR* in response to various stimuli such as UV-inactivated GCHV, *Scophthalmus maximus* rhabdovirus (SMRV), and Poly I:C, verifying that *PoPKR* displayed similar expression kinetics to mammalian orthologues. Subsequently, a comparison between the expression of *PoPKR* and *PoHRI* mRNAs was made, suggesting that *PoPKR* along with *PoHRI* likely participated in flounder defense against SMRV infection but *PoPKR* should be the predominant kinase.

2. Materials and methods

2.1. Cells, virus, and plasmids

Flounder embryonic cells (FEC) were grown at 24 °C and propagated in Dulbecco's modified Eagle's medium (DMEM, Gibco), supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 mg/ml streptomycin. Crucian carp blastulae embryonic cells (CAB), carp leucocyte cells (CLC), and epithelioma papulosum cyprini cells (EPC) were maintained at 28 °C in medium 199 supplemented with the same concentration of FCS and antibiotics. *Scophthalmus maximus* rhabdovirus and grass carp hemorrhagic virus were propagated in CLC cells and CAB cells, respectively. Plasmids wild-type (WT) PKR-pcDNA3.1 (amino acids 1–688), K421R-pcDNA3.1 (amino acids 1–688, Lys421-Arg), ΔN PKR-pcDNA3.1 (amino acids 286–688), and ΔN421R-pcDNA3.1 (amino acids 286–688, Lys421-Arg) have been previously described [27]. For EGFP constructs, the plasmids were cut with NheI and BamHI. The fragments were then inserted into EGFP (Clontech), which yielded PKR-EGFP, K421R-EGFP, ΔN PKR-EGFP, and ΔN 421R-EGFP.

2.2. Transfection

EPC cells were grown on coverslips until 80% confluence and then transfected by using Lipofectamine 2000 (Invitrogen) with 1 μg of the respective EGFP constructs. After 48 h, cells were washed once with PBS, fixed in 4% paraformaldehyde/PBS for 30 min, permeabilized in 0.2% Triton X-100/PBS for 10 min, and stained with Hoechst33258 (Sigma) for 5 min. Cells were then washed three times with PBS, mounted onto glass slides, and visualized under a fluorescence microscope (Leica).

2.3. Challenge of FEC cells and flounders

FEC cells were cultured in 25 cm^2 culture plates till confluence. Cells were washed three times with FCS-free DMEM medium, then treated with 0.5 ml FCS-free DMEM containing UV-inactivated GCHV (1×10^9 $TCID_{50}$/ml exposed to UV irradiation), active SMRV (1×10^4 $TCID_{50}$/ ml), Poly I:C (100 μg/ml), and 0.5 ml FCS-free DMEM alone as a control. After 1 h of incubation, cells were replaced with 5 ml of fresh FCS-free DMEM and then harvested at indicated times.

Prior to challenging, Japanese flounder juveniles (400–500 g weight) were maintained under laboratory conditions for 2 weeks, and no clinical signs were observed during this period. Fish were intraperitoneally injected with 0.5 ml FCS-free medium DMEM containing SMRV (1×10^9 $TCID_{50}$/ml) or with FCS-free medium DMEM

as controls. At 48 h and 72 h after injection, fish were killed for sampling, at least three fish in each group.

2.4. RNA extraction, reverse transcription, and real-time PCR

Total RNA was extracted using Trizol Reagent (Invitrogen), from FEC cells and flounder tissues. For reverse transcription, random primers and the Revert-Aid™ Minus First Strand cDNA Synthesis Kit (Fermentas) were used according to the manufacturer's instructions. Real-time PCR was performed using the DNA Engine Chromo 4 Real-Time System (MJ Research). The PCR primers for *PoPKR* mRNA detection were 5′-TGCGAGACTCAAAGAGAA GAGAAG-3′ (forward) and 5′-GGTTCCTTTGTA CTCCGTTC-3′ (reverse). The PCR was conducted in 20 μl of volumes consisting of 1 μl cDNA, 0.2 μM of each primer, 1 U of *Taq* polymerase (Fermentas), 0.1 μM of each dNTP, 1× buffer for *Taq* polymerase, and 1 μl SYBR Green I Dye. The PCR cycle conditions were denaturation at 95 °C for 4 min, followed by 40 cycles of 95 °C for 20 s, 56 °C for 20 s and 72 °C for 20 s, and a final extension at 72 °C for 10 min. Subsequently a melting curve was acquired with continuous fluorescence acquisition from 65 °C to 95 °C with a rate of 0.5 °C/s. Triplicate reactions were performed for each template cDNA. *β-actin* was used as an endogenous control, and PCR was performed by primers 5′-CAC-TGTGCCCATCTACGAG-3′ (forward) and 5′-CCATCT CCTGCTCGAAGTC-3′ (reverse). Amplification data were analyzed using the relative 2 $(-\Delta\Delta C(T))$ method [28]. Data represent means ± standard errors of triplicate experiments.

2.5. Protein extraction and Western blots

Protein extracts from FEC cells and different tissues were prepared following the methods of the previous report [25]. Briefly, cells seeded in 25 cm^2 culture plates were washed once with PBS and lysed with 500 μl of ice-cold lysis buffer (100 mM glycerophosphate, 20 mM Hepes, 20 mM EGTA, 15 mM $MgCl_2$, pH 7.3, 1 mM DTT and protease inhibitor cocktail). Tissues were harvested and homogenized in the same lysis buffer as above. Lysates were immediately centrifuged at 12,000*g* for 30 min at 4 °C. The supernatants were diluted and boiled for 5 min to detect *PoPKR* and actin with the prepared polyclonal anti-*PoPKR* sera and anti-human actin antibody (Santa Cruz), respectively. Western blots were performed according to the previous report [25].

3. Results

3.1. Subcellular localization of PoPKR

To define the intracellular distribution of *PoPKR*, we expressed EGFP-tagged constructs in EPC cells. As shown in Fig. 1, EGFP alone gave a diffuse signal throughout the cell, especially in the nucleus, while WT PKR was predominantly present in the cytoplasm. However, the isolated kinase domain ΔN PKR showed a nuclear and cytoplasmic localization, which is different from that of WT PKR. This indicates that the N-terminal dsRNA-binding domain is sufficient for the proper localization of PKR. The catalytically inactive mutants, K421R and ΔN-K421R, which were generated from the plasmids WT PKR and ΔN PKR by the replacement of lysine 421 with arginine, exhibited the same distribution as WT PKR and ΔN PKR, respectively. This suggested that the kinase activity of *PoPKR* is not necessary for its localization.

3.2. Induced expression of PoPKR under various inducers

The expression kinetics of *PoPKR* by different stimuli was investigated in FEC cells. As shown in Fig. 2, a basal expression of *PoPKR* in control FEC cells was detected. However, upon exposure to UV-inactivated GCHV, SMRV or Poly I:C, *PoPKR* was significantly induced at the mRNA and protein levels although the kinetics was different.

In response to UV-inactivated GCHV, the *PoPKR* transcript was observed to increase 0.5 h post-treatment, peak 48 h post-treatment followed by a sharp decrease. Real-time PCR showed that the peak expression of *PoPKR* was at least 14-fold over control cells (Fig. 2a, left panel). Compared to induction by UV-inactivated GCHV, the upregulation of *PoPKR* mRNA by SMRV appeared relatively slow along with the time, peaking 96 h post-treatment (about 19-fold) (Fig. 2a, middle panel). However, Poly I:C, a synthetic analog of dsRNA, induced significant increase in *PoPKR* mRNA as early as 0.5 h post-treatment, and a high level of *PoPKR* mRNA still persisted even 96 h post-treatment (about 12-fold) (Fig. 2a, right panel). To investigate whether *PoPKR* translation was upregulated in response to these three stimuli, Western blot analysis was next performed with the prepared polyclonal anti-PKR serum. As shown in Fig. 2(b), a 75-kDa protein was translationally induced and displayed similar increase to those observed in PKR mRNA upon the corresponding inducer.

3.3. Induced expression of PoPKR in flounder tissues

To characterize the spatial distribution of *PoPKR*, expression of *PoPKR* was further investigated in mock-infected and SMRV-infected flounder. As shown in Fig. 3, *PoPKR* was constitutively expressed in all analyzed tissues, including the liver, spleen, anterior kidney, intestine, gill, posterior kidney, brain, heart, and ovary. As compared to the control fish, a significant induction of *PoPKR* was observed in all tissues from flounders infected with SMRV for 72 h. Real-time PCR showed predominant upregulation of *PoPKR* mRNA in the heart (about 19-fold), anterior kidney (about 11-fold), posterior kidney, and spleen (about 4-fold) (Fig. 3a). Western blots also

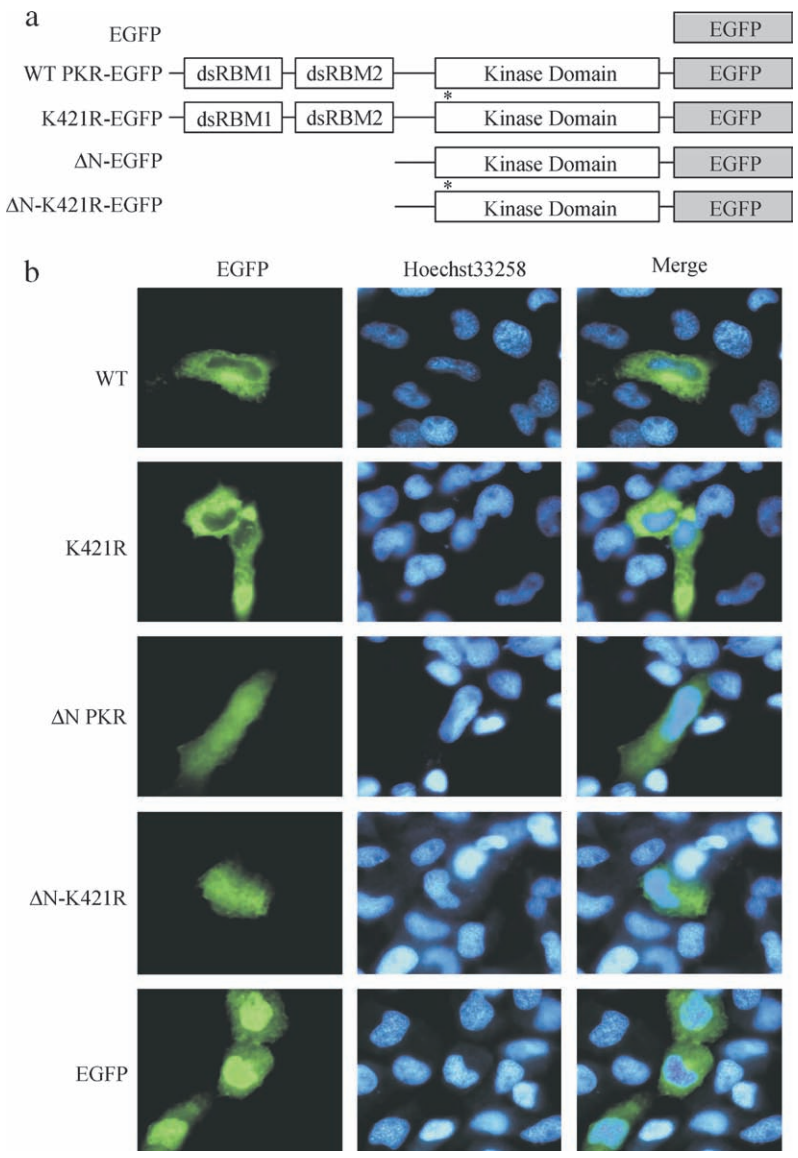

Fig. 1. Subcellular localization of *PoPKR*-EGFP proteins. (a) Schematic constructs of WT and mutant versions of *PoPKR* used in this study. The dsRNA-binding domain and catalytic domain are indicated by the open boxes, and the EGFP domain is indicated by the shaded boxes. Asterisks in the constructs of K421R and ΔN-K421R show the mutated amino acid positions (K421–R421) that abolish kinase activity. (b) EPC cells growing on coverslips were transfected with 1 μg of plasmids expressing the indicated EGFP fusion proteins. Protein localization was visualized by fluorescence microscopy using a lens with 100× magnification. GFP-transfected cells were visualized with a blue filter block (excitation range 450–480 nm), while Hoechst33258-stained cells were visualized with a UV filter block (excitation range 340–380 nm).

detected a consistent upregulation of *PoPKR* protein in the corresponding tissues (Fig. 3b).

3.4. Induced expression comparison between PoPKR and PoHRI

Recent studies have characterized flounder *HRI* (*PoHRI*) as the virus-induced gene [25]. Given that both *PoPKR* and *PoHRI* play important roles during antiviral response, it is of interest to identify which one is most induced. First, the expression patterns of *PoPKR* and *PoHRI* were compared by exposure to the same stresses. As shown in Fig. 4, two eIF2α kinase transcripts were increased in FEC cells after treatment with UV-inactivated GCHV, SMRV and Poly I:C. They both showed almost similar kinetics including a steady increase followed by sharp reduction in inactivated GCHV induction, a delayed upregulation for SMRV induction, and a rapid and persistent upregulation for Poly I:C induction. Despite their parallel kinetics, the *PoPKR* transcript level was obviously observed to increase by a larger percentage than *PoHRI* transcripts in response to the same stimulus. Next, the expression of *PoPKR* and *PoHRI* was compared in five flounder tissues including the liver, spleen, anterior kidney, intestine, and gill. As shown in Fig. 5, although there were different levels of basal expression, SMRV infection led to

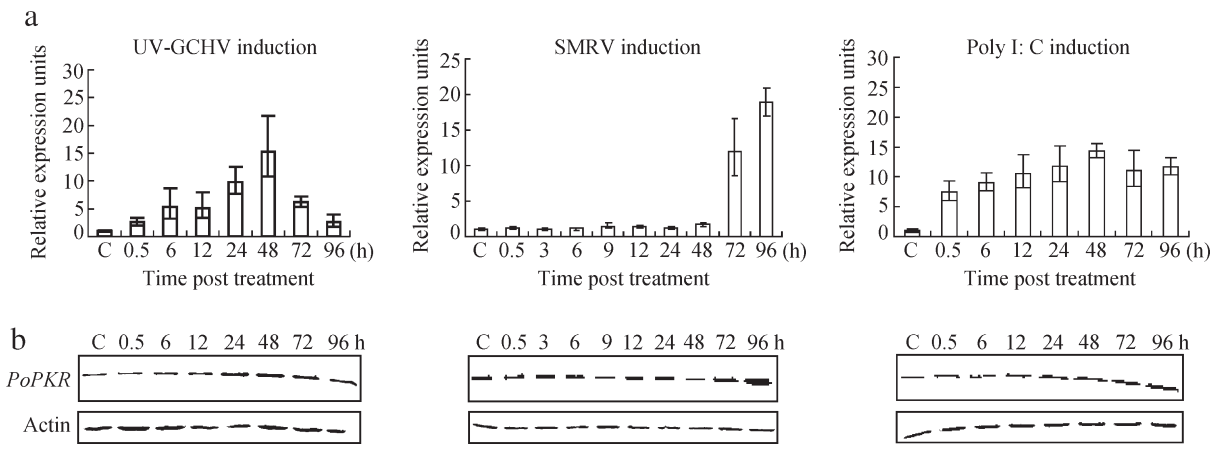

Fig. 2. Induction of *PoPKR* by UV-inactivated GCHV, SMRV, and Poly I:C. Two groups of FEC cells were treated with UV-inactivated GCHV and Poly I:C for 0.5, 6, 12, 24, 48, 72, and 96 h, respectively. The third group was infected with SMRV for 0.5, 3, 6, 9, 12, 24, 48, 72, and 96 h. *PoPKR* mRNA was determined by real-time PCR (a), and *PoPKR* protein was detected by Western blot analyses (b) with polyclonal anti-*PoPKR* serum. For real-time PCR, the ratio of *PoPKR* to *β-actin* in control cells was set to 1, and all treated cells were normalized relative to this value. For Western blotting, actin protein was detected by polyclonal anti-human actin (Santa Cruz) as a loading control. C: mock-treated FEC cells.

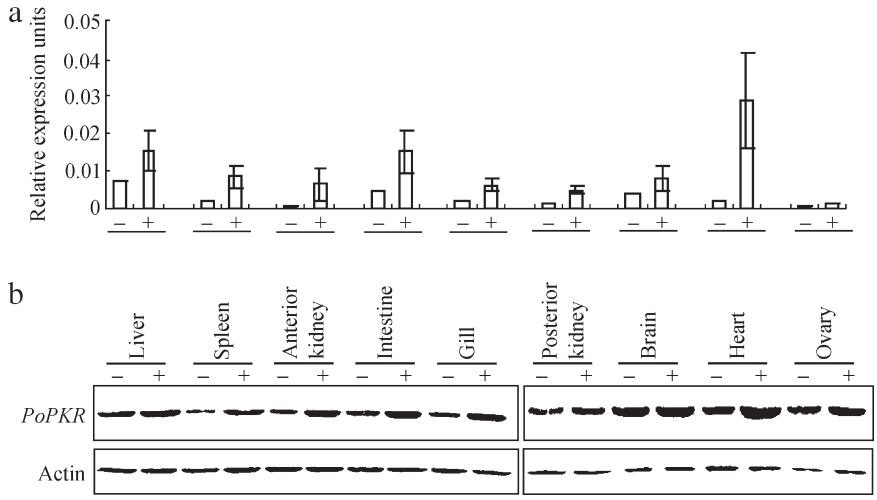

Fig. 3. Induced expression of *PoPKR* in flounder tissues. (a) Real-time PCR detection of *PoPKR* transcripts in flounder tissues infected with (+) or without (−) SMRV for 72 h. The values were expressed as the ratio of *PoPKR* to *β-actin* levels. (b) Western blot detection of *PoPKR* protein in tissues stimulated with (+) or without (−) SMRV. Protein extracts were subjected to 10% SDS–PAGE, transferred to nitrocellulose membranes and probed with polyclonal anti-*PoPKR* serum. Actin protein was detected by polyclonal anti-human actin (Santa Cruz) as a loading control.

a marked increase in the mRNA of both genes, particularly in *PoPKR* mRNA. In mock-infected liver and intestine, a relatively high basal level of *PoPKR* mRNA was observed, whereas upon virus infection, *PoPKR* expression was enhanced more rapidly, resulting in much stronger induction than *PoHRI* (Fig. 5a and d). In the spleen and gill where the constitutive expression was in medium level for both genes (Fig. 5b and e), and in the anterior kidney where a lower level was seen for *PoPKR* (Fig. 5c), SMRV infection still induced a strikingly higher level of *PoPKR* mRNA.

4. Discussion

PKR is well characterized as a major antiviral effector molecule in IFN-mediated antiviral defense. However, little is known about fish eIF2α kinase genes including PKR and their expression. Recently, Japanese flounder *PKR* cDNA has been cloned from UV-inactivated GCHV-infected FEC cells [27]. In agreement with results from mammalian PKR, *PoPKR* is predominantly located in the cytoplasm, and the dsRNA-binding domain determines the subcellular localization of *PoPKR*. In mammals, PKR is bound and activated by dsRNA in the cytoplasm [29]. The dsRNA-binding domain is responsible for localizing PKR to ribosomes, thereby promoting the phosphorylation of its target substrate, ribosome-associated eIF2α that participates in the translation initiation [30,31].

PoPKR was ubiquitously expressed in cultured cells and flounder tissues and was upregulated in response to either virus infection or Poly I:C treatment. These results indicated that *PoPKR* possesses a similar expression profile

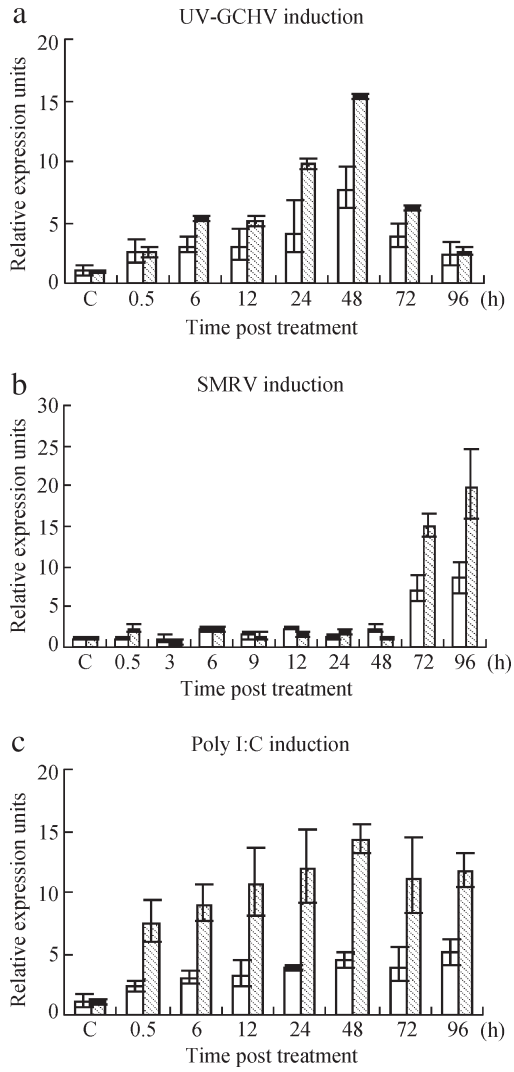

Fig. 4. Expression comparison between *PoPKR* (filled bar) and *PoHRI* (open bar) by UV-inactivated GCHV (a), SMRV (b), and Poly I:C (c). Three groups of FEC cells were treated with UV-inactivated GCHV (1×10^9 $TCID_{50}$/ml exposed to UV irradiation), SMRV (1×10^4 $TCID_{50}$/ml), and Poly I:C (100 μg/ml), respectively. The cells were harvested and extracted for RNA at the indicated times. *PoPKR* and *PoHRI* expression were detected by real-time PCR. C: mock-treated FEC cells.

to that of known mammalian and avian PKR. Numerous reports revealed that PKR is induced by IFN, which is generally produced during virus infection [3,8,11]. To date, flounder IFN has not been characterized, so it is hard to detect whether the induction of *PoPKR* by virus infection or Poly I:C treatment is ascribed to IFN. However, in other fish species, IFN activity and the *IFN* gene have been characterized. In crucian carp (*Carassius auratus*), a set of genes involved in IFN antiviral response have been characterized, revealing that these genes including *PKR-like* can be induced directly by IFN-containing supernatant [32]. IFN-stimulated genes including *IRF1* and *Mx* have been cloned in flounder [33,34]. UV-inactivated GCHV infection of FEC cells also led to production of an IFN activity [24], and *Mx*, the hallmark gene of IFN response in mammals,

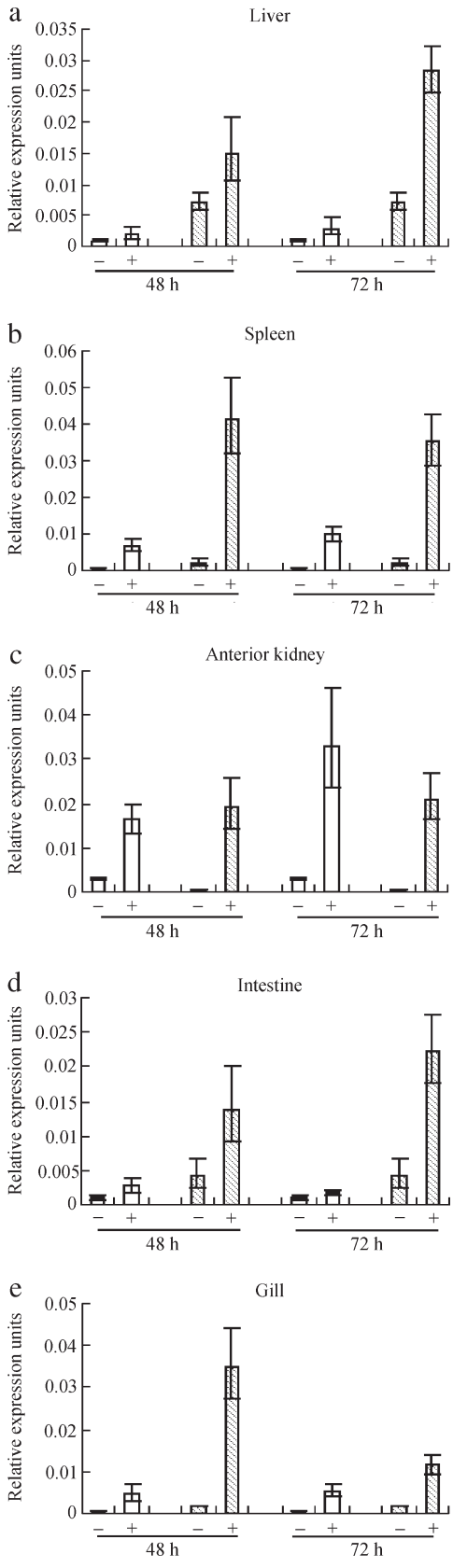

Fig. 5. A comparison between the expression of *PoPKR* (filled bar) and *PoHRI* (open bar) in different tissues. Real-time PCR analyses of *PoPKR* and *PoHRI* mRNA expression in the liver (a), spleen (b), anterior kidney (c), intestine (d), and gill (e) of SMRV-injected flounders for 48 h and 72 h (+) or mock-infected flounders (−).

was induced in FEC cells after treatment with UV-inactivated GCHV, active SMRV and Poly I:C [25]. These results supported the notion that flounder IFN response might be triggered by SMRV infection, which in turn resulted in upregulation of *PoPKR*.

In addition to an antiviral role in blocking translation of general proteins, PKR also affects gene expression by activating diverse transcriptional factors such as IRF1, STAT1, STAT3, p53, ATF3, and NF-κB [35]. During innate antiviral response, PKR is believed to sense intracellular dsRNA and activates the expression of IFN-β [36]. Flounder PKR seemed to function as mammalian orthologues do, and as expected, the antiviral role of *PoPKR* has been confirmed recently by transfection assays with wild-type and the catalytically inactive mutant of *PoPKR* [27]. The results in the present study further revealed that *PoPKR* displayed the expression characteristics similar to that of mammalian orthologues. For example, constitutive expression was observed in cultured cells and flounder tissues. Based on the reports on mammalian PKRs [1,2,13,17,35], the constitutive *PoPKR* is likely in a latent state and prior to activation, it needs to bind its activators, such as dsRNA that is produced during virus replication. Consistent with this hypothesis, a three-step pathway was established to clarify the mechanism underlying PKR-mediated translational shutoff, that is, dsRNA binding triggers dimerization of the kinase, in turn promoting autophosphorylation and subsequent phosphorylation of eIF2α [35]. It is likely that similar to mammalian orthologues, *PoPKR* possesses another function other than translational blockade, since during virus infection, the constitutive *PoPKR* can be rapidly activated and subsequently participate in antiviral response or regulation of related gene expression.

In addition to PKR, another three eIF2α kinases have been well studied in mammals including HRI, PERK and GCN2 [37–39]. In terms of their different regulatory structures, these kinases can respond to different stresses. However, numerous studies have shown that the eIF2α kinases might be interchangeable to some extent. In yeast, either mammalian PKR or HRI can functionally substitute for GCN2 in the general control response [40]. In mouse, knockout of PKR does not increase susceptibility to infection of the influenza virus, vaccinia virus and EMCV, indicating that PKR function might be compensated by other eIF2α kinases [41,42]. This point is further supported by recent findings that PERK is implicated in hepatitis C virus replication and VSV infection [43,44], and GCN2 is involved in Sindbis virus (SV) infection [45]. Interestingly, flounder HRI could be induced by virus infection, suggesting that *PoHRI* might play a crucial role in antiviral immune response [25]. In the present study, further expression comparison confirmed that *PoPKR* and *PoHRI* were upregulated in cultured cells and flounder tissues under virus infection, and the relatively low MOI of the virus might lead to delayed upregulation of *PoPKR* and *PoHRI* by SMRV induction as compared to that by UV-inactivated GVHV induction [25]. It is noted that *PoHRI* displayed similar kinetics to that of *PoPKR*, strongly indicating a similar antiviral role of *PoHRI* to *PoPKR* under virus infection. However, as compared to *PoHRI*, the transcript level of *PoPKR* was increased by a large percentage, suggesting that *PoPKR* should be the predominant eIF2α kinase that inhibits syntheses of virus proteins.

Acknowledgements

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