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RIOK1/2 Negatively Regulates the Antiviral Response by Targeting TBK1 in Yellow Catfish (*Pelteobagrus fulvidraco*)

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Abstract: The yellow catfish (*Pelteobagrus fulvidraco*) industry has expanded to a certain scale and is an important part of aquaculture in China, but frequent diseases have caused huge economic losses. Comprehending the fish's immune mechanisms, particularly the regulation of the interferon (IFN) response, is of paramount importance for future drug development and disease-resistant molecular breeding. Notably, the role of atypical protein kinases, such as the RIO kinase family, in immune regulation is poorly defined. Here, we investigated the roles of yellow catfish RIO kinases, PfRIOK1 and PfRIOK2, in modulating the IFN response through their interaction with PfTBK1, a key player in the RLR signaling pathway. Mechanically, PfRIOK1 and PfRIOK2 negatively regulate the IFN response by interacting with the RIO domains to target and degrade PfTBK1. Our findings reveal that the overexpression of PfRIOK1 and PfRIOK2 led to the decreased expression of IFN-related genes and enhanced viral replication in vitro. Additionally, PfRIOK1 and PfRIOK2 could inhibit PfTBK1-mediated antiviral responses in infected cells. These results suggest that PfRIOK1 and PfRIOK2 act as negative regulators of the IFN response in yellow catfish, providing new insights into the regulatory mechanisms of fish innate immunity and offering target molecules for molecular design breeding in aquaculture.

Keywords: *Pelteobagrus fulvidraco*; RIOK1; RIOK12; TBK1; negative regulation; antiviral response

Key Contribution: The interaction between the core domains of PfRIOK1 and PfRIOK2 and the core domains of PfTBK1 was proven.

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1. Introduction

The innate immune system serves as the initial defense against pathogenic infections, playing a critical role in pathogen recognition and the activation of inflammatory responses to combat microbial damage [1]. The interferon (IFN) signaling pathway is one of the most important mechanisms for eliminating viruses in the host defense against viral infections [2]. Activation of the type I IFN (IFN-I) signaling pathway is the central event in the antiviral innate immune response following the recognition of pathogen-associated molecular patterns (PAMPs) by host pattern recognition receptors (PRRs) [3,4]. Upon activation, a cascade of signaling events initiates, leading to the activation of mitochondrial antiviral signaling (MAVS), which recruits downstream adaptors like tumor necrosis factor receptor-associated

factor protein 3 (TRAF3) and TANK-binding kinase 1 (TBK1) [5,6]. TBK1, in turn, promotes the phosphorylation and nuclear translocation of interferon regulatory factors (IRFs), where they induce the expression of IFNs-I and other immune effectors to clear viruses [7].

In the host, precise regulation of IFN signaling is crucial for maintaining immune homeostasis and effective antiviral responses [3,8]. Excessive or insufficient IFN activity could lead to pathological conditions, such as autoimmune diseases and chronic inflammation, highlighting the importance of fine-tuning this immune pathway [9]. Protein kinases, including atypical kinases like RIOK1 and RIOK2, are critical regulators of immune signaling pathways through post-translational modifications, such as phosphorylation [10,11].

RIOKs are essential for ribosome assembly and function, playing key roles in processing ribosomal RNA and modifying ribosomal proteins, which in turn impacts protein synthesis and cellular responses [12,13]. Moreover, emerging evidence indicates that these kinases also modulate immune responses. Moreover, emerging evidence indicates that these kinases also modulate immune responses. RIOKs, for instance, have been shown to influence antiviral immune responses [14]. RIOK1 functions as an inhibitor in the P38–MAPK pathway in *Caenorhabditis elegans*, which regulates immune responses and maintains immune homeostasis [15]. Furthermore, RIOK1's role in cancer and its involvement in the RAS signaling network suggest that RIOK kinases could be critical in immune modulation during infections and diseases [16]. RIOK2, on the other hand, influences hematopoiesis and immune cell function, which is essential for the innate immune response [17]. The role of RIOK1/2 in the precise modulation of IFN production and interferon-stimulating genes (ISGs) is crucial for effective antiviral defense [18,19]. RIOK1/2 might influence antiviral responses by modulating the expression of ISGs and interacting with molecules in RLR signaling, providing a potential target for therapeutic interventions in viral diseases.

Yellow catfish (*Pelteobagrus fulvidraco*), an economically significant freshwater species in China, is often affected by viral infections, causing substantial economic losses in aquaculture. A deeper understanding of the immune mechanisms, particularly the precise regulation of IFN signaling during viral infections, is critical for improving disease control and developing disease-resistant molecular breeding. In this study, we confirmed that PfRIOK1 and 2 are negative regulators of IFN response and investigated how PfRIOK1 and 2 negatively regulate the antiviral response by targeting TBK1. These findings will provide new insights into the mechanisms of innate immunity and offer potential avenues for molecular design breeding in aquaculture.

2. Materials and Methods

2.1. Experimental Fish, Cell Lines, and Viral Strains

Yellow catfish was purchased from the market, and the liver was rapidly frozen in liquid nitrogen for RNA extraction and cDNA synthesis. Epithelioma papulosum cyprini cells (EPC) and blastulae embryonic cells of crucian carp (CAB) were cultured with M199 medium (Gibco) supplemented with 10% fetal bovine serum. The incubator temperature was maintained at 28 °C. Human embryonic kidney cells (HEK293T) were cultured in DMEM supplemented with 10% fetal bovine serum at 37 °C and 5% CO_2. Spring viremia of carp virus (SVCV) was multiplied in EPC cells, and the virus titer was determined by the TCID 50 method. The specific infection experiment was conducted as described before [19].

2.2. Plasmids

Yellow catfish TBK1 (PfTBK1: XM_047804070.1) sequence was identified from the NCBI website (https://www.ncbi.nlm.nih.gov/nuccore/XM_047804070.1, accessed on 20 April 2022). The open reading frame (ORF) sequence was then amplified. We had already completed the amplification of the ORFs for yellow catfish RIOK1/RIOK2 [19]. The

PfTBK1, PfRIOK1, and PfRIOK2 genes were cloned into expression vectors (pcDNA3.1) and tagged vectors (pcDNA310(+)-HA and pcDNA310(+)-Flag) for the construction of expression vectors PfTBK1-Flag, PfTBK1-HA, PfRIOK1-HA, PfRIOK1-Flag, PfRIOK2-HA, and PfRIOK2-Flag. When constructing the different domain vectors for PfTBK1, the protein was divided into KID, ULD, CCD1, and CCD2 domains, and segmental vectors (PfTBK1, KD, KD+ULD, CCD) were constructed according to the domain classification. Simultaneously, the PfRIOK1/2 domains were divided into the RIO core domain and two flanking segments for the construction of vectors (PfRIOK1/2, R1/2-N1, R1/2-N2, R1/2-C). The primers used in this study are listed in Table 1.

Table 1. Genes and primer sequences.

Primer Name	Sequence (5′–3′)
EPC-*β-actin*-RT-F	CAGATCATGTTTGAGACC
EPC-*β-actin*-RT-R	ATTGCCAATGGTGATGAC
EPC-*mx*-F	GGCTGGAGCAGGTGTTGGTATC
EPC-*mx*-R	TCCACCAGGTCCGGCTTTGTTAA
EPC-*ifn*-F	ATGAAAACTCAAATGTGGACGTA
EPC-*ifn*-R	GATAGTTTCCACCCATTTCCTTAA
EPC-*viperin*-F	AGCGAGGCTTACGACTTCTG
EPC-*viperin*-R	GCACCAACTCTCCCAGAAAA
SVCV-N-F	GGTGCGAGTAGAAGACATCCCCG
SVCV-N-R	GTAATTCCCATCATTGCCCCAGAC
SVCV-L-F	CAAGTTCACAATCGGGAAGACGC
SVCV-L-R	CCAGTTGCTTGTTGGCTTATCCG
SVCV-G-F	CCATTCTGTTCATTTGGAGCCGTA
SVCV-G-R	AATTTCATTCGACAAGACCCCC
Pfriok2-RT-F	GGAAACCAAATGGGTGTCGGC
Pfriok2-RT-R	GTCCACTGGCTTTGGAACAGG
Pfriok1-RT-F	CTAAACGCTACGCTGCGATGC
Pfriok1-RT-R	CCACCGTCGCTCTATCAGAC
PfRIOK1-EcoRV-F	GTGGAATTCTGCAGATATGTCTCAGATTGTCCTGGG
PfRIOK1-EcoRV-R	GCCACTGTGCTGGATTCTTCCTTTCTTCATCTTGGC
PfRIOK2-EcoRV-F	GTGGAATTCTGCAGATATGGGGAAGTTAAACGTCGTT
PfRIOK2-EcoRV-R	GCCACTGTGCTGGATTCCCCAGAACTGGGCTGC
PfTBK1-F	GTGGAATTCTGCAGATATGCAGAGTACGGCCAATTA
PfTBK1-R	CGCCACTGTGCTGGATTCACATCCGCTCCACTGTCC
PfRIOK1-BamHI-F	CTTGTTCTTTTTGCAGATGTCTCAGATTGTCCTGGG
PfRIOK1-BamHI-R	GCGCCACTAGTGGATCCTCTTCCTTTCTTCATCTTGGC
PfRIOK2-BamHI-F	CTTGTTCTTTTTGCAGATGGGGAAGTTAAACGTCGTTG
PfRIOK2-BamHI-R	GCGCCACTAGTGGATCCTCCCCAGAACTGGGCTGCTTC

2.3. Subcellular Localization Assays

The ORFs of PfRIOK1/2 and PfTBK1 were separately inserted into the BamHI site of the 8CmCherry-pcs2+ and pEGFP-N3 vectors to create PfRIOK1-mCherry and PfRIOK2-mCherry constructs. The primers used are listed in Table 1. HEK293T cells were transfected in six-well plates with either TBK1-GFP and 8CmCherry-pcs2+ or co-transfected with PfRIOK1-mCherry or PfRIOK2-mCherry along with PfTBK1-GFP (1.0 μg each). The transfected cells were incubated overnight on microscope slides. After 24 h, the cells were washed three times with PBS and fixed at room temperature for 30 min with 4% (*v*/*v*) paraformaldehyde. After additional washing with PBS three times, the cells were incubated with 0.2% Triton X-100 for 15 min, followed by DAPI (500 μg/mL) staining for 10 min, and finally inspected under a confocal microscope (ZEISS, Oberkochen, Germany). [Objective: ×40; Analysis Software: ZEN 2.3 (blue edition); Scale bar: 10 μm].

2.4. Transfection, Luciferase Activity Assay, and RT-PCR

Transfections were performed according to the manufacturer's instructions and laboratory protocols using Polyethylenimine Linear reagent (MW 25000, Aldrich, St. Louis, MO, USA). For the luciferase activity assay, EPC cells seeded in 48-well plates were co-transfected with the reporter plasmid (promoter-driven luciferase construct IFNφ1/CaIFNpro-luc) (100 ng) and increasing amounts of PfRIOK1 and PfRIOK2 at doses of 0, 2.5, 5, 10, 25, 50, 100, and 150 ng, along with pRL-TK (10 ng) as an internal control. Then, 12 h post initial transfection, the cells were infected with SVCV (10^3 TCID 50/mL). An empty expression vector was added for the control group. After 24 h post infection, cells were harvested and lysed according to the Dual-Luciferase Reporter Assay System (Promega, Wisconsin Madison, WI, USA). The luciferase activity was measured using a Junior LB9509 luminometer (Berthold, Welford, Germany).

The above experiment was repeated for overexpression and viral infection, relevant cells were harvested, and then total RNA extraction, cDNA synthesis, and quantitative real-time PCR (qPCR) were performed as described previously [19]. For qPCR, the specificity of amplification for each reaction was analyzed by dissociation curves. All samples were analyzed in triplicate, and the abundance of mRNA expression values was normalized to β-actin or showed as fold induction relative to the expression level in control cells, which was set to 1 using the $2^{-\Delta\Delta Ct}$ program. Primer availability was confirmed by qPCR testing, and the sequences were also confirmed by sequencing. qPCR was performed on a Bio-Rad PCR system by CFX96 Optics Module with chamq universal SYBR qPCR Master Mix (Vazyme, Nanjing, China) as described previously [19]. Primers used for qPCR analysis are listed in Table 1.

2.5. Antiviral Activity Analysis

Antiviral experiments were performed as previously described [19,20]. EPC cells plated in 24-well plates and incubated overnight were transfected with empty vector (EV) or PfRIOK1 and PfRIOK2 (each at 0.5 μg), followed by infection with SVCV (10^3 TCID 50/mL) after 12 h post-transfection. Approximately 48 h later, the cells were treated with 0.1% crystal violet solution for 3 h, and the supernatants were harvested for TCID 50 determination to assess viral titers.

2.6. Co-Immunoprecipitation (Co-IP) and Western Blot (WB)

Co-IP and WB were performed according to previously established protocols [21,22]. HEK293T cells cultured in 10 cm^2 plates were transfected with the indicated plasmids, EGFP-HA (1.0 μg), PfTBK1-HA, KD-HA, KD-ULD-HA, and CCD-H (5.0 μg) in combination with either PfRIOK1-Flag (5.0 μg) or PfRIOK2-Flag (5.0 μg). Additional combinations included EGFP-HA (1.0 μg), PfRIOK1/2-HA, R1/2-N1-HA, R1/2-N2-HA, R1/2-C-HA (5.0 μg each) with PfTBK1-Flag (5.0 μg). For the protein degrading, PfTBK1-Flag (1.0 μg) was co-transfected with PfRIOK1/2-HA in differential doses (0, 0.2, 0.5, 0.8, 1.0 μg each) supplemented by empty vector (EV) (1.0 μg in total).

Thirty hours post-transfection, cells were collected and lysed with NP-40 lysis buffer. The lysis supernatants were mixed with 40 μL of Anti-tag antibody and incubated overnight at 4 °C. The agarose beads were washed five times with wash buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM DTT, 1% NP-40], dissolved in 1× loading buffer, boiled for 10 min, and then subjected to WB using tag-specific antibodies. Flag and HA tag-specific antibodies were obtained from Cell Signaling Technology (CST, Danvers, MA, USA).

2.7. Data Collection and Statistical Analysis

Data are shown as mean ± SD. The figures were drawn using GraphPad Prism 6 software. All significance analyses were conducted in the SPSS software (version v.29), and significant differences were assessed by the *t*-test analysis. Statistical significance is represented by asterisks (* $p < 0.05$).

3. Results and Analysis

3.1. PfRIOK1/2 Negatively Regulates the IFN Response by Targeting the RLR Pathway

Viral infection can mediate the IFN response in fish via the RLR pathway [23]. Our previous studies indicated that PfRIOK1 and PfRIOK2 act as negative regulators of the IFN response. As shown in Figure 1A,B, we further validated that both PfRIOK1 and PfRIOK2 significantly inhibit the activity of the CaIFN promoter induced by various RLR signaling molecules, except for IRF3 in fish cell lines (Figure 1A,B). This finding is consistent with our earlier results, leading us to focus on the node molecule TBK1.

In vitro experiments confirmed that PfRIOK1/2 can weaken the IFN response by binding to and degrading zebrafish TBK1. Subsequently, we wonder whether PfRIOK1/2 also inhibits the endogenous yellow catfish TBK1 protein (PfTBK1). As demonstrated in Figure 1C, PfTBK1 can strongly induce the activity of the zebrafish IFNφ1 promoter in EPC cells. Moreover, the level of PfTBK1 transfection, up to 50 μg, resulted in a dose-dependent increase in promoter activity, which was significantly diminished upon the addition of PfRIOK1. We then replicated these experiments in CAB cells, observing that PfRIOK1 also significantly inhibited PfTBK1-induced IFN promoter activity (Figure 1E). Simultaneously, we replaced PfRIOK1 with PfRIOK2 and repeated the same experiments (Figure 1D,F).

To further validate the regulatory effects of PfRIOK1/2 on PfTBK1, we transfected varying doses of PfRIOK1/2 plasmids to assess their impact on PfTBK1-induced IFN activity. As illustrated, both PfRIOK1 and PfRIOK2 remarkably diminished PfTBK1-induced IFN activity, and this inhibitory effect was more pronounced with increasing concentrations of PfRIOK1/2 (Figure 1G,H). These results showed that PfRIOK1/2 could significantly suppress the activity of the IFNφ1 promoter (EPC) and CaIFN (CAB) induced by PfTBK1.

3.2. Co-Localization of PfRIOK1/2 with PfTBK1

Next, we continued to explore the regulatory relationship between PfRIOK1/2 and PfTBK1 through in vitro experiments. We employed subcellular localization techniques to assess the co-localization of PfRIOK1/2 and PfTBK1 proteins. As illustrated, upon co-transfection of the PfTBK1-GFP vector fused with green fluorescent protein and an empty red fluorescent vector (8CmCherry-pcs2+), confocal microscopy analysis revealed that the green signal of PfTBK1-GFP was predominantly distributed in the cytoplasm rather than the nucleus. The red signal from the empty Cherry vector was localized within the nucleus, with no overlap observed with the green signal (Figure 2A).

To further investigate the subcellular localization of PfTBK1 and PfRIOKs, we co-transfected EPC cells with PfTBK1-GFP and a red fluorescent PfRIOK1-mCherry vector. The results demonstrated that PfRIOK1 co-localized with PfTBK1 in the cytoplasm (Figure 2B). Repeating the experiment with PfRIOK2-mCherry in place of PfRIOK1-mCherry yielded similar results, indicating that PfRIOK2 also co-localizes with PfTBK1 in the cytoplasm (Figure 2C). Collectively, these results demonstrate that PfRIOK1 and PfRIOK2 co-localize with PfTBK1 in the cytoplasm.

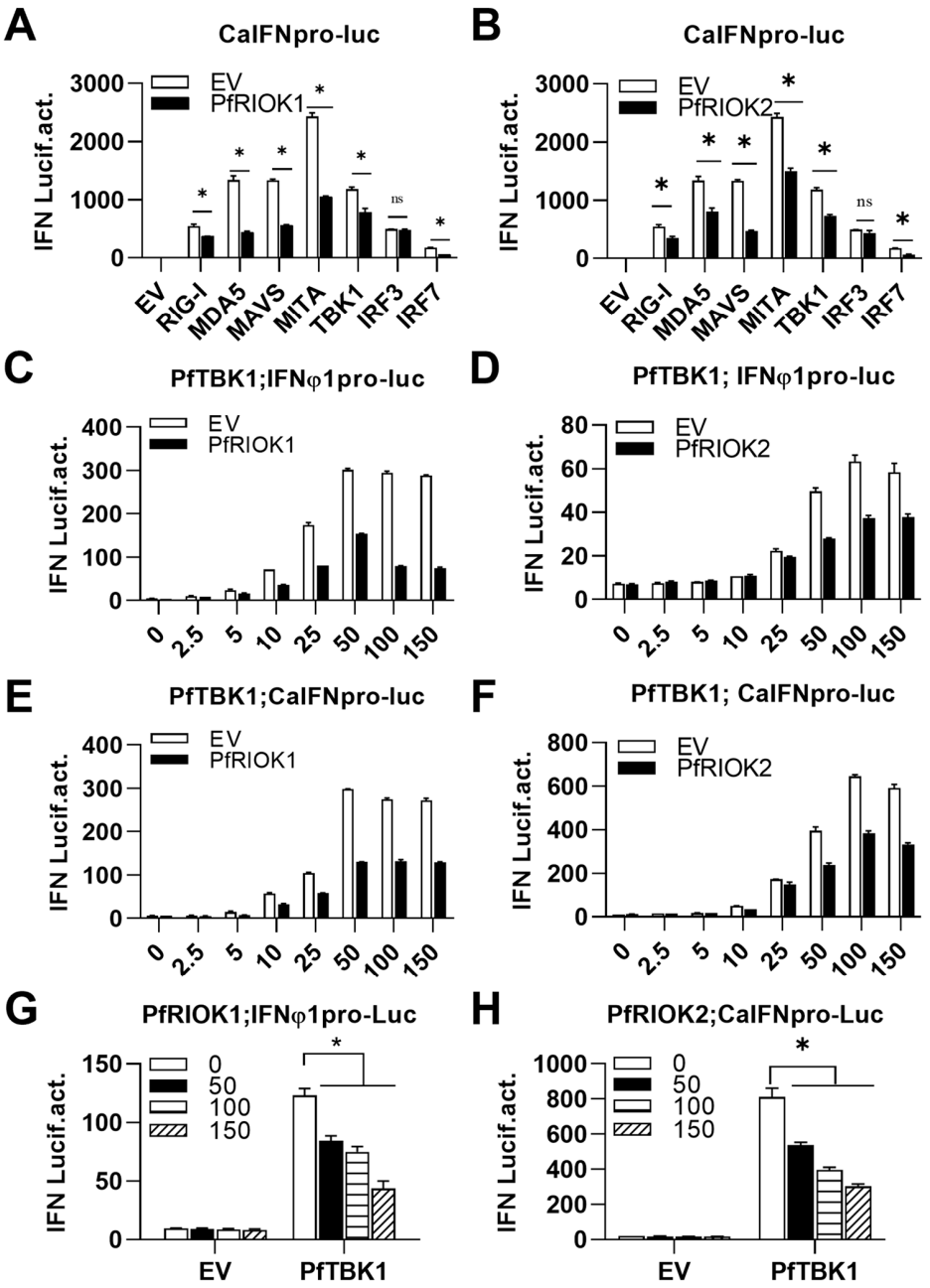

Figure 1. PfRIOK1/2 negatively regulates the IFN response. (**A**,**B**) PfRIOK1 and PfRIOK2 block the activation of CaIFNpro-luc induced by key components of the RLR pathway except for IRF3. EPC cells seeded overnight in a 48-well plate were co-transfected with the key components of RLR signaling (RIG-I, MDA5, MAVS, MITA, TBK1, IRF3) (100 ng each) and CaIFNpro-luc (100 ng). They were co-transfected with empty vectors (EVs) or PfRIOK1 or PfRIOK2 plasmids (100 ng each). Transfected pRL-TK (10 ng) was an internal control, and 24 h after transfection, cells were collected for luciferase assay. (**C**–**F**) PfRIOK1 and PfRIOK2 inhibit the activity of PFTBK1-induced IFNφ1 promoter (EPC) and CaIFN (CAB). EPC cells were seeded in a 48-well plate, PfTBK1 with 0, 2.5, 5, 10, 25, 50, 100, 150 ng, and empty plasmid or PfRIOK1, or PfRIOK2 with IFNφ1pro-luc (100 ng) or CaIFNpro-lu and pRL-TK (10 ng), respectively, were transfected. Cells were collected 24 h after transfection for luciferase assay. (**G**,**H**) PfRIOK1 and PfRIOK2 significantly reduced PFTBK1-induced IFN activity in a dose-dependent manner. EPC or CAB cells inoculated overnight in 48-well plates were transfected with PfRIOK1 and PfRIOK2 (100 ng), respectively. The cells were transfected with empty plasmid or PfTBK1 (0, 50, 100, 150 ng) and IFNφ1pro-luc (100 ng) or CaIFNpro-lu and pRL-TK (10 ng). Cells were collected for luciferase assay 24 h after transfection. Statistical significance is represented by asterisks (* $p < 0.05$); ns stands for no significant difference.

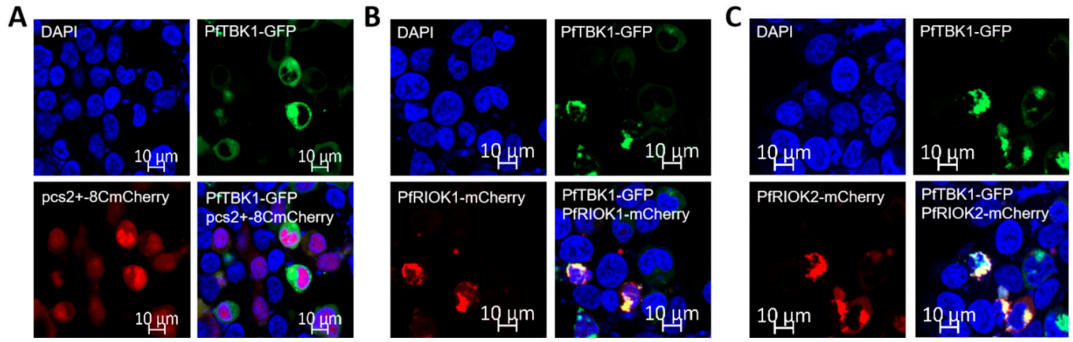

Figure 2. Co-localization of PfRIOK1/2 and PfTBK1 protein. (**A**–**C**) HEK293T cells were transfected with 8CmCherry-pcs2+ PfRIOK1-mCherry or PfRIOK2-mCherry and PfTBK1-GFP 1.0 μg each on a glass cover slide in a 6-well plate overnight. The cells were immobilized and examined using confocal microscopy. DAPI staining shows the nucleus. Scale, 10 μm.

3.3. Interaction of PfRIOK1/2 with PfTBK1

In previous studies, we confirmed that PfRIOKs interact with TBK1 extracellularly [19]. To further investigate the interaction between PfRIOKs and the endogenous TBK1 of yellow catfish, we analyzed the functional domains of PfTBK1, PfRIOK1, and PfRIOK2 using SMART software (https://smart.embl.de/, accessed on 20 April 2022). Based on this analysis, we constructed three different functional domain plasmids for PfTBK1 (KD-HA, KD+ULD-HA, and CCD-HA) and six different functional domain plasmids for PfRIOK1/2 (R1/2-N1-HA, R1/2-N2-HA, and R1-C-HA) (Figure 3A,B).

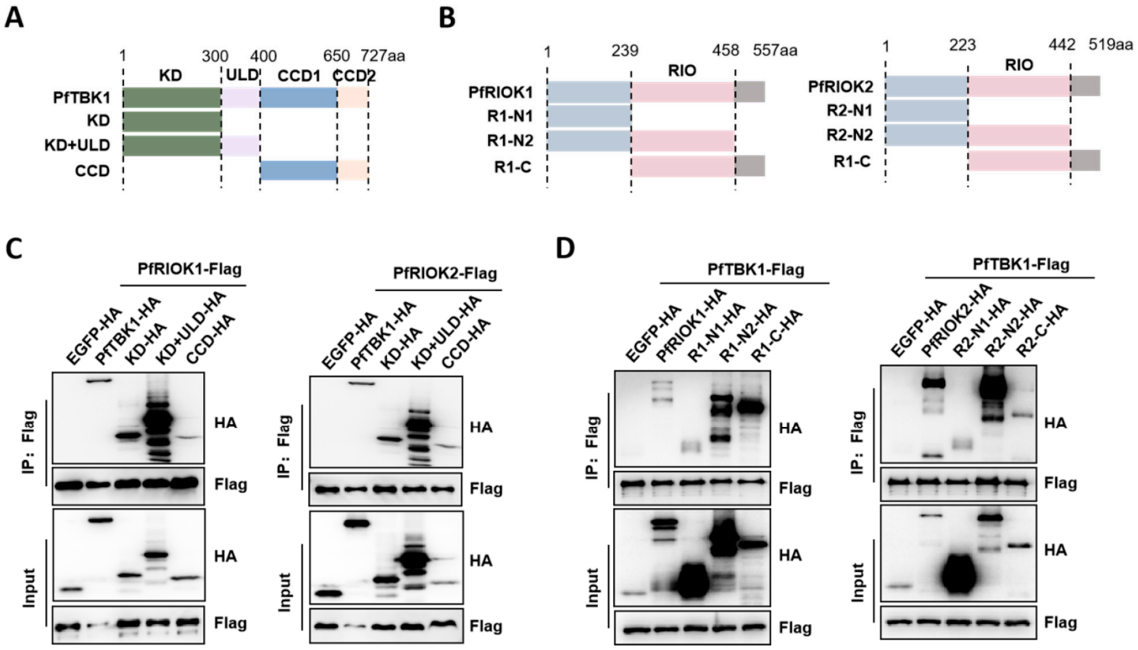

Figure 3. PfRIOK1/2 interact with PfTBK1. (**A**,**B**) Structure diagrams of PfTBK1 (KD-HA, KD+ ld-ha and CCD-HA) (**A**) and PfRIOK1/2 (R1/2-N1-HA, R1/2-N2-HA and R1/2-C-HA). (**B**,**C**) PfRIOK1/2 interacts with different domains of PfTBK1, EPC cells were seeded into a 10 cm^2 dish and co-transfected with EGFP-HA, PfTBK1-HA, KD-HA, KD+ lD-HA, CCD-HA with PfRIOK1-Flag (**Left**) or PfRIOK2-Flag (**Right**), respectively (each 5 μg). (**D**) PfTBK1 interacts with different domains of PfRIOK1/2. In the same way, EPC cells were co-transfected with PfTBK1-Flag and EGFP-HA, PfRIOK1-HA, R1-N1-HA, R1-N2-HA, R1-C-HA (**Left**) or EGFP-HA, PfRIOK2-HA, R2-N1-HA, R2-N2-HA, R2-C-HA (**Right**) (each 5 μg). After transfection for 30 h, the cell lysates were immunoprecipitated (IP) with anti-FLAG affinity gel. IP samples and cell lysates were then analyzed by Western blotting with the specified Abs.

We employed Co-IP to examine the interactions between the different structural domains of PfRIOK1/2 and PfTBK1. As shown in Figure 3C, PfRIOK1/2 did not interact with EGFP but exhibited a significant interaction with PfTBK1. Furthermore, PfRIOK1/2 interacted with the KD, KD+ULD, and CCD domains of PfTBK1, with the interaction being most pronounced with the KD+ULD domain and observable to a certain extent with the CCD domain. We speculate that PfRIOK1/2 primarily exerts regulatory effects through interactions with the KD domain of PfTBK1.

Next, we continued to investigate the binding relationship between PfTBK1 and the different domains of RIOKs. As depicted in Figure 3D, PfTBK1 did not interact with EGFP, but there was a clear interaction with PfRIOK1/2. Additionally, there were interactions among PfTBK1 and the R1/2-N1, R1/2-N2, and R1-C domains of PfRIOK1/2, with the most significant interactions noted with R1/2-N2 and R1/2-C, while R1/2-N1 exhibited a weaker binding signal. These findings suggest that PfTBK1 primarily exerts its regulatory functions through interactions with the RIO domains of PfRIOK1/2.

3.4. PfRIOK1/2 Degrade PfTBK1 Protein

To further delve into the regulatory relationship between PfRIOK1/2 and PfTBK1, we co-transfected Pf-TBK1-Flag plasmid (1 μg) with various concentrations of PfRIOK1-HA plasmid (0, 0.2 μg, 0.5 μg, 0.8 μg, and 1 μg) in EPC fish cell lines. The results indicated that the overexpression of PfRIOK1 led to a significant reduction in PfTBK1 protein levels, demonstrating a dose-dependent decrease in protein levels with increasing concentrations of PfRIOK1 (Figure 4A). Similarly, when we replaced PfRIOK1-HA with PfRIOK2-HA, the results showed that PfRIOK2 also significantly degraded PfTBK1 protein, with a more pronounced degradation effect observed at higher concentrations of PfRIOK2 (Figure 4B). Collectively, these experimental results confirm that PfRIOK1/2 can degrade PfTBK1.

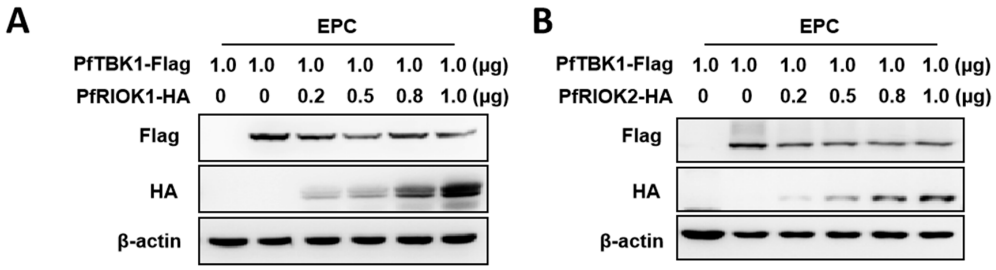

Figure 4. PfRIOK1/2 degrade PfTBK1 protein. (**A**,**B**) EPC cells cultured in 3.5 cm^2 plates were transfected with plasmids PfTBK1-Flag (1.0 μg) and PfRIOK1-HA (**A**) or PfRIOK2-HA (**B**) at specified doses (0, 0.2, 0.4, 0.8, 1.0 μg) for 30 h. The cells were then collected for Western blot using the corresponding label Abs.

3.5. PfRIOK1/2 Inhibits PFTBK1-Mediated Antiviral Response

To determine the effects of PfRIOK1 and PfRIOK2 on viral replication, we conducted transfection experiments with PfRIOK1/2 in EPC cells, followed by infection with SVCV. Crystal violet staining results showed more pronounced cytopathic effects (CPE) in cell plates transfected with PfRIOK1/2 compared to those transfected with an empty vector (Figure 5A).

As previously established, PfRIOK1/2 primarily negatively regulate the IFN response by targeting the TBK1 molecule in the RLR pathway. Given that TBK1 is also an antiviral molecule, we hypothesized whether PfRIOK1/2 could suppress PfTBK1's antiviral response. We transfected equal amounts of plasmid-empty vector, PfTBK1, and PfRIOK1+PfTBK1 into EPC cells, respectively, followed by SVCV infection, and then measured the viral titers in the cell supernatants of the three groups. The results showed that consistent with previous findings, the viral titers in the supernatant of the cells transfected

with PfRIOK1 and PfRIOK2 were significantly higher than those in the control group (Figure 5B).

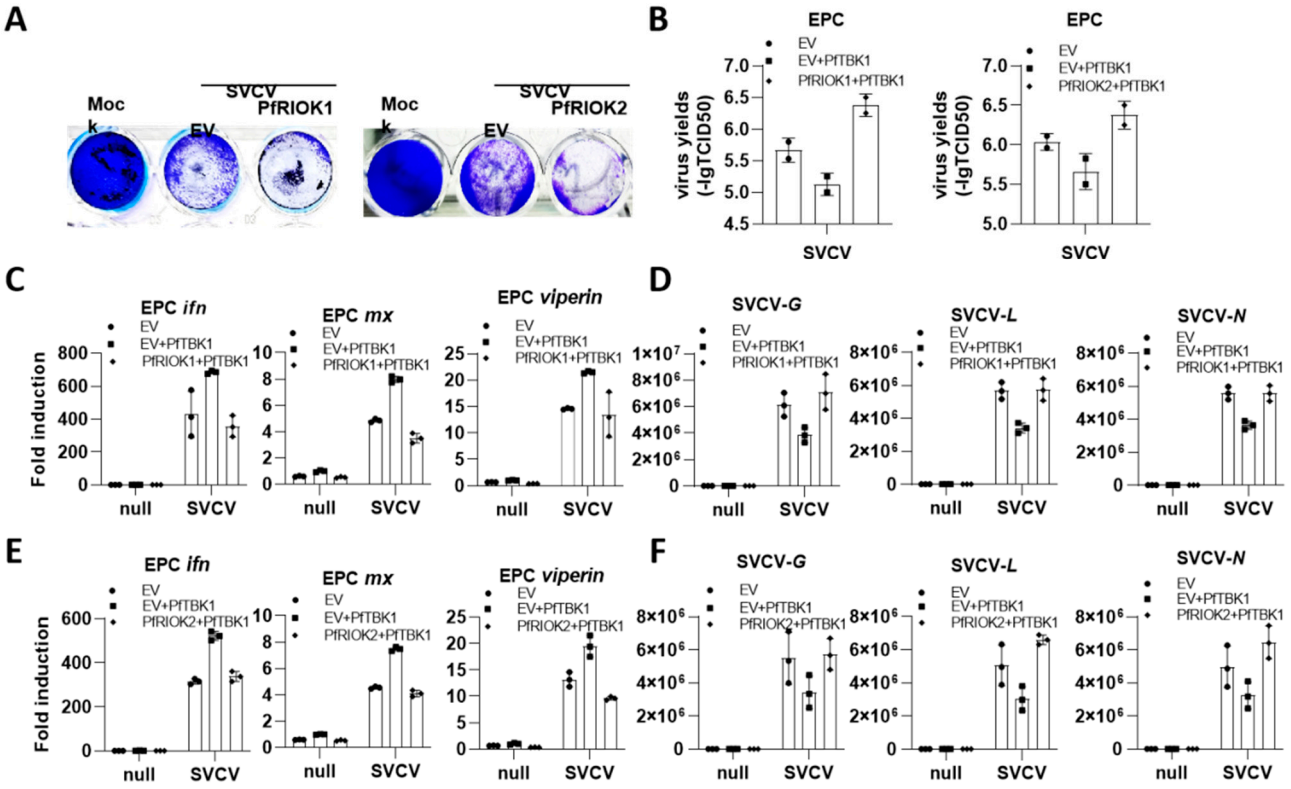

Figure 5. PfRIOK1/2 inhibits PFTBK1-mediated antiviral response. (**A**) PfRIOK1 and PfRIOK2 inhibit the cellular cytopathic effects. EPC cells cultured in 24-well plates were transfected with empty vectors (EV), PfRIOK1, or PfRIOK2 (0.5 μg each). The cells were treated with SVCV (10^3 TCID 50/mL) 12 h later and observed with 0.1% crystal violet after 48 h. (**B**–**F**) RIOK1/2 negatively regulates the TBK1-induced cellular antiviral response. EPC cells cultured in 24-well plates were transfected with empty vector (EV) (1 μg), PfTBK1 (1 μg), PfTBK1, and PfRIOK1/2 (1 μg). After 12 h, the cells were treated with SVCV (10^3 TCID 50/mL), and after another 30 h, RT-PCR was performed for *ifn*, *mx*, *viperin*, and SVCV genome genes (N, L, and G). The supernatant was taken, and the virus titer of the supernatant was detected.

Furthermore, qPCR results confirmed that the overexpression of PfTBK1 significantly reduced the transcription levels of three SVCV genes: nucleoprotein (N), glycoprotein (G), and RNA polymerase (L) in EPC cells. At the molecular level, the mRNA levels of three ISG genes, *ifn*, *mx*, and *viperin*, significantly increase upon PfTBK1 overexpression, suggesting enhanced intracellular antiviral responses, which aligns with previous studies [24]. However, the suppressed expressions of SVCV-N, SVCV-G, and SVCV-L by PfTBK1 were significantly counteracted after overexpressing PfRIOK1/2. Moreover, the mRNA levels of *ifn*, *mx*, and *viperin* induced by PfTBK1 were also markedly inhibited following the overexpression of PfRIOK1/2 (Figure 5C,D). In summary, PfRIOK1/2 downregulates the IFN response to promote viral replication and effectively inhibits the antiviral responses enhanced by TBK1.

4. Discussion

In mammals, TBK1 plays a pivotal role in antiviral immunity, activated by PRRs, such as toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I), and melanoma differentiation-associated protein 5 (MDA5) [25]. Upon activation, TBK1 phosphorylates and facilitates the nuclear translocation of interferon regulatory factors IRF3 and IRF7,

thereby inducing the expression of IFNs-I. Our previous study has demonstrated that TBK1 plays an equally vital role in yellow catfish, where its expression is remarkably upregulated in response to viral stimuli, underscoring its essential role in the antiviral immune response in fish [26].

However, the precise regulation of IFN signaling is fundamental to maintaining an effective antiviral response while preventing immune dysregulation [27]. RIO kinases have emerged as key modulators of various cellular functions, including cell cycle progression, ribosome biogenesis, and immune signaling [24]. These findings highlight the interconnected roles of TBK1 and RIO kinases in orchestrating immune responses, offering insights into the complex regulatory networks underlying antiviral immunity.

RIOK1 and RIOK2 have also been identified as important regulators of innate immunity, with RIOK1 inhibiting the p38 Mitogen-Activated Protein Kinase (MAPK) pathway in *Caenorhabditis elegans* and regulating immune responses in macrophages [15]. Our findings confirm that PfRIOK1/2 serve as negative regulators of the RLR-mediated IFN response, offering novel insights into the molecular mechanisms involved in viral immune responses in aquatic species. Specifically, we demonstrate that PfRIOK1/2 negatively regulate PfTBK1, thereby modulating the IFN response and influencing viral replication. RIOK1 and RIOK2 exert their negative regulatory effects on the IFN response by interacting with TBK1, thereby inhibiting the activation of transcription factors IRF3 and IRF7. This suppression of the IFN response may facilitate viral replication by reducing the expression of interferon-stimulating genes (ISGs). These findings highlight the role of RIOK1/2 in negatively regulating antiviral immune responses, but the precise molecular mechanisms of their interaction with TBK1 and their broader impacts on antiviral immunity require further investigation.

Understanding how RIOK1 and 2 modulate the IFN response in yellow catfish offers insights into the broader mechanisms of antiviral immune regulation and provides potential therapeutic targets for controlling viral diseases in aquaculture. In vitro systems allow for the precise manipulation of target genes or proteins, such as overexpression or silencing, and enable rapid testing of pharmacological modulators, such as agonists or inhibitors, to assess the specific function of target genes in various biological processes. For instance, Liu et applied a series of in vitro experiments, such as luciferase reporter assays and Co-IP, to investigate how SIRT3 regulates the activation of IFN-I signaling pathways and the physical interaction between SIRT3 and MAVS [28]. In this study, we also utilized Co-IP and WB to confirm that RIOK1/2 interact with and degrade TBK1 to modulate the IFN response. Despite the advantages of in vitro experiments, in vivo studies remain the gold standard for validating gene function. Therefore, further in vivo experiments, such as gene editing, are encouraged to better elucidate the immune functions and the physiological function of RIOKs.

Interestingly, the overexpression of RIOK1 and RIOK2 promoted SVCV replication, and co-transfection with TBK1 led to the downregulation of antiviral genes, providing strong evidence that RIOK1/2 serve as negative regulators of the antiviral response. Further investigations revealed that PfRIOK1/2 and their core domains directly interact with the core structure of PfTBK1, effectively targeting TBK1 to suppress the IFN response. This suppression primarily facilitates SVCV replication by inhibiting the expression of interferon-stimulated genes (ISGs). These findings underscore the potential of RIOK1/2 as targets for enhancing antiviral immunity in yellow catfish, paving the way for innovative strategies in disease management. Moreover, the functional parallels between RIOK1/2 and other kinases involved in immune regulation suggest that these proteins may play broader roles in immune system modulation, potentially contributing to viral persistence and immune evasion. Further studies are needed to fully elucidate their functions and mechanisms.

The role of RIO kinases in various biological processes is well-established, particularly in cell cycle regulation, ribosome biogenesis, and immune response modulation. In yeast, RIOK1 has been shown to play a pivotal role in maintaining cell cycle progression [29]. The absence of RIO1 in yeast results in cell cycle arrest at either the G1 phase or mitosis, underscoring its critical function in cell division [30]. Furthermore, RIO1 and RIO2 are involved in the cell viability and cell migration [31]. In yellow catfish, the regulatory roles of RIOK1/2 in antiviral immune responses have been increasingly elucidated; however, their contributions to other biological functions remain to be fully explored. A more comprehensive understanding of RIO kinases' multifaceted roles is essential to comprehensively assess their biological significance in aquaculture, with potential implications for enhancing disease resistance and advancing antiviral therapeutic strategies. Moreover, the introduction of RIOK-specific agonists or inhibitors in future experimental studies could provide valuable insights into their precise functions, thereby establishing a robust foundation for their application in aquaculture practices.

In conclusion, this work highlights the importance of yellow catfish RIOK1 and RIOK2 in regulating the antiviral immune response. By targeting PfTBK1, PfRIOK1 and 2 modulate the IFN response. Our findings provide valuable insights into the molecular mechanisms underlying antiviral immunity in fish and suggest that RIOK1/2 could be potential targets for therapeutic intervention to control viral infections and target molecules for further molecular design breeding in aquaculture.

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