Fish & Shellfish Immunology 31 (2011) 1173–1178

Contents lists available at SciVerse ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Cloning and functional analysis of PKZ (PKR-like) from grass carp (*Ctenopharyngodon idellus*)

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ARTICLE INFO

Article history:
Received 29 July 2011
Received in revised form
14 September 2011
Accepted 6 October 2011
Available online 14 October 2011

Keywords:
Z-DNA
PKR
PKZ
Z-DNA binding protein
Teleost fish

ABSTRACT

The new teleost fish PKZ (PKR-like) full-length cDNA (GU299765) had been cloned and identified from grass carp (*Ctenopharyngodon idellus*). The cDNA of grass carp *PKZ* (*CiPKZ*) has 2185 bp in length with a largest open reading frame (ORF) encoding 513aa. CiPKZ possesses a conserved C-terminal catalytic domain of eIF2α kinase family. Within its N-terminal there are two binding domain (Zα) named Zα1 (1–67aa) and Zα2 (81–152aa). BLAST homologous search reveals that CiPKZ has a high-level homology with other fish PKZs and PKRs. Like other fish PKZs and PKRs, *CiPKZ* is a ubiquitous tissue expression gene that had a very low level of constitutive expression but up-regulated in response to Poly I:C or hot stress (34 °C). For the purpose of searching for the potential function of CiPKZ, we obtained CiPKZ polypeptide via *Escherichia coli* Rosetta prokaryotic expression and purified with Ni-NTA His-Bind Resin affinity chromatography. CiPKZ polypeptide was used for the test of phosphorylating eIF2α *in vitro*. The results demonstrated that CiPKZ could be activated by Z-DNA but not by Poly I:C, and with subsequent could phosphorylate eIF2α. Meanwhile, four pcDNA3.1/PKZ recombinant plasmids, including pcDNA3.1/PKZ-wet, pcDNA3.1/PKZ-wet-K198R, pcDNA3.1/PKZ-wet-C, pcDNA3.1/PKZ-wet-C-K198R had been constructed, respectively. Mouse Myeloma cells (Sp2/0) and Human Umbilical Vein Endothelial Cells (HUVEC) were transiently cotransfected with pcDNA3.1/PKZ recombinant plasmid and PGL-3-promoter plasmid. The results revealed that CiPKZ could greatly decrease luciferase level in these cells. Zα and the K198 amino acid residue may play a key role in its function.

1. Introduction

In recent years, the protein kinase PKR-like (PKZ) genes had been reported successively in some teleost fish, such as *CaPKZ* in Goldfish (*Carassius auratus*) [1], *DrPKZ* in Zebrafish (*Danio rerio*) [2], *SsPKZ* in Atlantic salmon (*Salmo salar*) [3] and *GrPKZ* in Rare minnow (*Gobiocypris rarus*) [4]. Because the proteins encoded by these genes contain two Zα domains in N-terminal and can bind Z-DNA, so they were designated as "PKZ" (protein kinase containing Z-DNA binding domain) [2]. It is interested that the C-terminus of PKZ also contains 11 conserved subdomains which are more closely to fish PKR (dsRNA-activated protein kinase) [5], so Rothenburg et al. (2008) considered *PKZ* as replication of fish *PKR* and stopped at tetrapod [6]. Hence, PKZ is a special protein kinase, not only is a kind of Z-DNA binding protein but also belongs to an eIF2α protein kinase family. The research on PKZ has a good beginning. The preliminary conclusion is that PKZ is extremely similar to PKR, so it is likely related to the anti-viral effects in cell [1,2]. For the past few years, there are some articles about the affinity between PKZ Zα and Z-DNA [7,8]. Moreover, like the others member of eIF2α kinase, DrPKZ and SsPKZ could inhibit protein synthesis [2,3]. In spite of that, there are many unknown remained about the function of fish PKZ in cell.

Grass carp (*Ctenopharyngodon idella*) is one of the major fresh fish in china. Because it is very sensitive to many stresses, so the PKZ from grass carp may be beneficial for us to comprehend the function of fish PKZ. In the paper, we had cloned and identified grass carp *PKZ* (*CiPKZ*, GU299765) by taking advantage of the homologous *CaPKZ* (AY293929). The cDNA of *CiPKZ* has 2185 bp in length with a largest open reading frame (ORF) encoding 513aa that has a high-level homology with other PKZ. CiPKZ possesses a conserved C-terminal catalytic domain of eIF2α kinase family. Within its N-terminal there are two Z-DNA binding domains (Zα). *CiPKZ* is

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1050-4648/$ – see front matter © 2011 Published by Elsevier Ltd.
doi:10.1016/j.fsi.2011.10.012

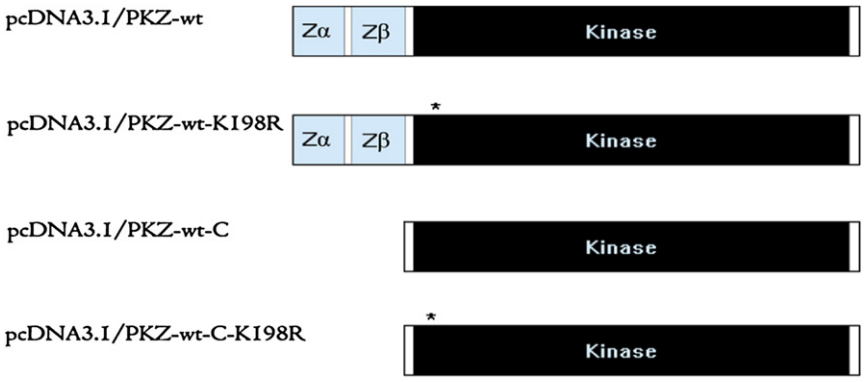

Fig. 1. Construction of pcDNA3.1/PKZ recombinant plasmids. An asterisk denoted the position of K198R mutation.

a ubiquitous tissue expression gene that had a very low level of constitutive expression but was up-regulated in response to Poly I:C challenge or hot stress (34 °C). CiPKZ could be activated by Z-DNA but not by Poly I:C, and with subsequent could phosphorylate eIF2α. After pcDNA3.1/PKZ recombinant plasmids and PGL-3-promoter were transiently cotransfected into Mouse Myeloma Sp2/0 cells and Human Umbilical Vein Endothelial Cells (HUVEC), respectively, the results revealed that CiPKZ could restrain the level of luciferase in these cells. Zα and the K198 amino acid residue may play a key role in its function.

2. Materials and methods

2.1. Cloning and sequence analysis of grass carp PKZ cDNA

Grass carp, came from Jiangxi Provincial Fisheries Research Institute, were injected 10 μg/g bodyweight Poly I:C (Pharmacia Biotech). 3d later, total RNA was prepared using SV Total RNA Isolation System (Premega). SMART cDNA was prepared using SuperScript II reverse polymerase (Invitrogen). The ORF of *CiPKZ* was cloned with primers ORF-F, ORF-R (designed in accordance with the CaPKZ cDNA sequence). PCR system: 10× LA Buffer (plus Mg2+) 5 μl, SMART cDNA 2.0 μl, primers-F (10 mmol/L) 1 μl, primers-R (10 mmol/L) 1 μl, dNTP (10 mmol/L) 2 μl, Ex Taq (TAKARA) 2.5 U, add DDH_2O to 50 μl. *CiPKZ* cDNA was cloned into pMD18-T vector (Takara) and transform DH5α. After *CiPKZ* had been sequenced, we forecasted polypeptide by ORF finder, SMART etc online-software. Based on the ORF of *CiPKZ*, the 3′ end was obtained by RACE-PCR with primer 3′-F (GGC CCT CCG GAA ACC TGG ACC CAT TA) and SMART-R. The full length cDNA of *CiPKZ* was got by PCR with primers GSP-F and GSP-R (Table 1).

2.2. Tissues expression of CiPKZ

Grass carp were treated with 34 °C 2 h and injected 0.5 mg/ml Poly I:C challenge 3d, respectively. The control group fish were injected with PBS. Liver (30 mg), kidney (20 mg), muscle (30 mg), spleen (15 mg), heart (60 mg), brain (60 mg) from every group were harvested. Total RNA was extracted by using the kit (Promega). cDNA was reverse transcribed using reverse polymerase M-MLV (Takara). cDNA was diluted to suitable concentration referenced to *β-actin* (β-actin-F and β-actin-R). According to RT-PCR assay, tissues expressed character of *CiPKZ* was analyzed with primers ND597-F6′ and ND597-R7′ (Table 1).

2.3. CiPKZ phosphorylated eIF2α

CiPKZ ORF cDNA was inserted into expression vector pET32a (Novagen), and then recombinant vector pET32a/PKZ was transformed into *E. coli* Rosetta (DE3) (Clontech). DE3 was grown in LB at 37 °C to an A600 of 0.6–0.8, then induced with 1 mM isopropyl thio-β-D-galactoside (IPTG) for 4 h. The cells were collected and suspended in binding buffer (20 mM Tris–HCl, 50 mM NaCl, 5 mM imidazole, pH7.9), and broken by sonication and centrifuged for 30 min (4 °C, 14 000 g). The supernatant was collected and purified with Ni-NTA His-Bind Resin affinity chromatography (QIAGEN)

Table 1
Primers used in this paper.

Name	Sequence (5′ to 3′)	Application
GSP-F	AGACCGACCAGAGAAGTTGCAG	*CiPKZ* full
GSP-R	CCAGCCAGCACAACATTTCTTC	length cDNA
ORF-F	GAATTCATATGTCTGCCGAAACTCAAA	*CiPKZ* ORF
ORF-R	GCTCGAGTCAAATCGTTTTCTGGCTTA	
ND597-F6′	CACCGTGAACAGACATTTG	*CiPKZ* expression
ND597-R7′	TCCCTTACGTGTTTCTCTTC	
β-actin-F	CACTGTGCCCATCTACGAG	*β-actin* expression
β-actin-R	CCATCTCCTGCTCGAAGTC	
PKZ-K198R-F	TCTACGCTGTAAGGAAAGTCGTCTTAACTG	pcDNA3.1/PKZ-wt-K198R
PKZ-K198R-R	AAGACGACTTTCCTTACAGCGTAGATCTTG	
PKZ-C-K198R-F	TCTACGCTGTAAGGAAAGTCGTCTTAACTG	pcDNA3.1/PKZ-wt-C-K198R
PKZ-C-K198R	AAGACGACTTTCCTTACAGCGTAGATCTTG	
PKZ-C-F	CGGAATTCATGGGGTTGTCTCAAAGTTTTGATGT	pcDNA3.1/PKZ-wt-C
PKZ-C-R	CGCTCGAGCTGGCTTAACAGATCCTCATCC	
PKZ-WT-F	CGGAATTCTGATGGCCGAAACTCAAATGGAGAG	pcDNA3.1/PKZ-wt
PKZ-WT-R	CGCTCGAGCTACTGGCTTAACAGATCCTCATCC	