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Identification and function analysis of the three dsRBMs in the N terminal dsRBD of grass carp (*Ctenopharyngodon idella*) PKR

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ABSTRACT

The protein kinase R (PKR) can inhibit protein translation and lead to apoptosis under the circumstances of virus invasion and multiple other stress conditions. PKR is a dsRNA binding protein with a dsRBD and a kinase domain (KD). dsRBD is mostly composed of two (in mammal PKR) or three (in some fish PKR) dsRNA binding motifs (dsRBMs). Multiple sequences alignment and Phylogenetic analysis indicate that the three dsRBMs of fish PKR share analogous structure but show to be divergence origination. In this study, we have identified and analyzed the three dsRBMs from grass carp (*Ctenopharyngodon idellus*) PKR (*Ci*PKR), which was cloned previously in our laboratory. dsRBMs of *Ci*PKR have two or three conserved regions involved in dsRNA binding. Among the three dsRBMs, dsRBM1 was peculiar to some fish PKRs, while dsRBM2 and dsRBM3 were closely related to the dsRBM1 and dsRBM2 of mammal PKRs respectively. Dimerization assay indicated that dsRBM1 and dsRBM2 formed not only homo-dimer but also homo-multimer; whereas dsRBM3 formed merely homo-dimer. Meanwhile, dsRBM1-2, dsRBM2-3 and dsRBM1-2-3 could homo-dimerize and homo-multimerize also. Poly I:C pull-down assay showed that the binding of dsRBM to Poly I:C needed two or three dsRBMs to cooperate *in vitro*, meaning one dsRBM from *Ci*PKR could not bind to dsRNA efficiently. To further investigate the effect of dsRBM on the function of *Ci*PKR, we constructed pcDNA3.1/*Ci*PKR-wt and a series of *Ci*PKR mutants recombined plasmids including pcDNA3.1/*Ci*PKR-ΔdsRBM2-3, pcDNA3.1/*Ci*PKR-ΔdsRBM1,3, pcDNA3.1/*Ci*PKR-ΔdsRBM1-2, pcDNA3.1/*Ci*PKR-ΔdsRBM3, pcDNA3.1/*Ci*PKR-ΔdsRBM1. The recombined plasmids respectively were co-transfected with plasmid PGL3 promoter into CIK cells. In comparison with the control group, the luciferase translation inhibitions were 78.7%, 15%, 0, 0.5%, 61.8%, 67.3% respectively. The results indicated that the protein translation inhibition caused by *Ci*PKR mutants with only one dsRBM were very weak, while those with two or three dsRBMs inhibited the protein translation powerfully. Cell viability were 34.2%, 98.2%, 112%, 108%, 50.3%, 47.5% respectively after transfected with pcDNA3.1/*Ci*PKR-wt, pcDNA3.1/*Ci*PKR-ΔdsRBM2-3, pcDNA3.1/*Ci*PKR-ΔdsRBM1,3, pcDNA3.1/*Ci*PKR-ΔdsRBM1-2, pcDNA3.1/*Ci*PKR-ΔdsRBM3, pcDNA3.1/*Ci*PKR-ΔdsRBM1 in order into CIK cells for 48 h. The results from cell counting also indicated that transfection of *Ci*PKR-wt and the mutants *Ci*PKR-ΔdsRBM3, *Ci*PKR-ΔdsRBM1 could inhibit the protein translation and facilitated the decrease of CIK cells number. In conclusion, our observations suggested that two dsRBMs ranking in tandem at N terminal were essential for the function of *Ci*PKR, and the presence of the extra dsRBM1 enhanced its function.

1. Introduction

Protein kinase R (PKR), a dsRNA-activated protein kinase, is linked to the two different protein families due to its structure characteristics [1–4]. One is the eIF2α protein kinase family for this reason of its C terminal kinase domain (KD), the other one is the dsRNA binding protein family because of dsRNA binding domain (dsRBD) containing two dsRNA binding motifs (dsRBM) in N terminal [2,5]. dsRBD and KD are linked with a linker in tandem.

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Compared with KD, dsRBD is more multifarious in the structure [1,2]. dsRBM is classified into type A and type B according to its structure and function. In a general way, type A dsRBM is supposed to have three conserved regions and performs the binding of dsRNA, whereas type B has not all of the three conserved regions, it generally has one or two conserved regions [6,7]. Particularly, the first and the second region in N terminal of dsRBMs are of diversity [6]. The diversity leads PKR to adapt well under different circumstances, such as virus invasion, bacterium infection, some of the physiological and pathological intracellular RNAs and cytokines [3,4]. Therefore, PKR is involved in multiple cell functions [1,8].

dsRBMs can sense the endogenous and exogenous dsRNAs and bind to it. Through binding, dsRBMs brings two PKR monomers to close proximity and to form a homo-dimer; subsequent leads the allosteric PKR auto-phosphorylation and activation [3,4]. Finally, the activated PKR phosphorylates its substrate eIF2α and shuts down the global protein translation, results in apoptosis [1,9]. Hence, dsRBM is pivotal in the function of PKR.

In the last decade, many fish PKR genes have been cloned and identified [10–14]. Different from mammalian counterparts, it is interesting that the number of dsRBM in fish PKR varies from 1 to 3 [10]. There are no data regarding the functional differentiation and implication of the alterable dsRBMs in fish PKR. It is proposed that more dsRBMs might be responsible for the binding of the varying size of RNA and efficiency of the combination [10,12,13].

Grass carp (*Ctenopharyngodon idellus*) PKR (*Ci*PKR) has three dsRBMs in series in its N terminus [14]. In this study, phylogenetic analysis illustrated that its dsRBM1 was peculiar to fish PKR; dsRBM2 and dsRBM3 were more closely related to dsRBM1 and dsRBM2 of mammalian PKR respectively. The prokaryotic expressed isolated fusion protein of dsRBM1, dsRBM2, dsRBM3, dsRBM1-2, dsRBM2-3 and dsRBM1-2-3 can dimerize respectively *in vitro*. Poly I:C pull-down assays indicated that the mutational *Ci*PKR with one-dsRBM could not bind to dsRNA obviously, whereas the proteins with two-dsRBM or three-dsRBM bound to dsRNA distinctly. Likewise, the result of co-transfection indicated that two dsRBMs ranking in tandem in N terminal dsRBD were crucial for the function of *Ci*PKR. CCK-8 and cell counting assays further confirmed the results above.

2. Materials and methods

2.1. Phylogenetic analysis and multiple sequences alignment of dsRBM

The amino acid sequences of PKR dsRBMs were extracted and predicted by SMART (Table 1). Multiple alignments of dsRBMs from some reported PKR were performed by using Clustal ×2, BioEdit 7.0.9 and modified manually.

Evolutionary analysis and phylogenetic tree were conducted with MEGA version 4.0 by the Neighbour-Joining method and optimized manually. The bootstrap confidence values were based on 1000 bootstrap replications.

2.2. Vectors, strains and cells

The plasmids of pcDNA3.1, pRL-TK and pGL3-promoter were purchased from Transgen Biotech (China), TaKaRa (Japan) and Promega (USA) respectively. *Escherichia Coli* DH5α and BL21 (DE3) pLysS were bought from Promega (USA). CIK cells that derived from *C. idellus* kidney tissue were kindly provided by professor Pin Nie, Institute of Hydrobiology, Chinese Academy of Sciences and kept in our lab. CIK cells were maintained in M199 containing 10% fetal bovine serum (Gbico, Australia) at 28 °C.

2.3. Construction of CiPKR mutants

The construction of the eukaryotic expressed mutants and the prokaryotic expressed proteins were based on the retrieving results of the open database SMART. The results from other databases were listed as reference, such as CATH, CCD (Table 2, Fig. 4A).

*Ci*PKR-ORF was used for constructing the mutants. Over-lapping PCR was applied to construct the series of *Ci*PKR mutants with varying number of dsRBM. The following mutants and control only need one round of PCR procedure to construct, including *Ci*PKR-ORF, *Ci*PKR-ΔdsRBM1 and *Ci*PKR-ΔdsRBM1-2. PCR was performed in a volume of 50 μl mixtures containing 35.5 μl of ddH_2O, 4 μl of LaTaq buffer, 5 μl of dNTP, 0.5 μl LaTaq, 1 μl of each primer, and 3 μl of template. The thermal cycling parameters of PCR were as

Table 2
Recognition of the dsRBMs of CiPKR and its boundary by various databases.

Name of database	aa boundary of dsRBMs	E-value
SMART	(dsRBM1) 8-74	1.83e-22
	(dsRBM2) 100-169	0.0105
	(dsRBM3) 215-281	7.89e−18
CATH	(dsRBM1) 6-77	6.1e-61
	(dsRBM2) 99-171	7.2e-30
	(dsRBM3) 214-278	6.9e−124
CDD	(dsRBM1) 7-71	1.19e−18
	(dsRBM2) 99-168	2.67e−05
	(dsRBM3) 214-280	7.79e−19

Table 1
The member of PKR family used for phylogenetic analysis in this study.

Name of protein	Species	Database accession number
*Ci*PKR	*Ctenopharyngodon idellus*	GenBank JX511974.1
*Dr*PKR	*Danio rerio*	GenBank AM421526.1
*Ca*PKR	*Carassius auratus*	GenBank JN091442.1
*Po*PKR	*Paralichthys olivaceus*	GenBank EU118259.1
*Tn*PKR1	*Tetraodon nigroviridis*	GenBank AM421523.1
*Tn*PKR2	*Tetraodon nigroviridis*	GenBank AM421524.1
*Tn*PKR3	*Tetraodon nigroviridis*	GenBank AM421525.1
*Hs*PKR	*Homo sapiens*	GenBank M35663.1
*Ss*PKR	*Sus scrofa*	GenBank AB104654.1
*Tr*PKR1	*Takifugu rubripes*	GenBank JN103425.1
*Tr*PKR2	*Takifugu rubripes*	GenBank JN103426.1
*Ga*PKR	*Gasterosteus aculeatus*	GenBank AM850085.1
*Om*PKR	*Oncorhynchus mykiss*	GenBank EF523423.1
*Bt*PKR	*Bos Taurus*	GenBank AB104655.1
*Mm*PKR	*Mus musculus*	GenBank BC016422.1
*Rn*PKR	*Rattus norvegicus*	NCBI NM_019335.1 (NCBI Reference Sequence)