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# The Zα domain of PKZ from *Carassius auratus* can bind to d(GC)$_n$ in negative supercoils

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ABSTRACT

PKZ was the most recently discovered member of eIF2α kinase family in fish. *Ca*PKZ, the first identified fish PKZ, possessed a conserved eIF2α kinase catalytic domain in C-terminal and two Z-DNA binding domains (Zα) in N-terminal. The Zα of *Ca*PKZ closely resembled that of other Z-DNA binding proteins: ADAR1, DLM-1, and E3L. In order to understand more about the function of *Ca*PKZ, we expressed and purified three constructed peptides of *Ca*PKZ ($P_{Z\alpha}$): $P_{Z\alpha1Z\alpha2}$, $P_{Z\alpha1\underline{Z\alpha1}}$ and $P_{\underline{Z\alpha2}Z\alpha2}$. Moreover, most of the plasmids containing d(GC)$_n$ inserts were maintained in the Z-conformation, as confirmed by using inhibition of methylation experiments and anti-Z-DNA antibody. Gel mobility shift assays were then used to examine the affinity of these $P_{Z\alpha}$ to the recombinant plasmids. Meanwhile, a competition experiment using $P_{Z\alpha1Z\alpha2}$ and anti-Z-DNA antibody was performed. The results revealed that $P_{Z\alpha1Z\alpha2}$ and $P_{Z\alpha1\underline{Z\alpha1}}$ were able to bind to the recombinant plasmids with high affinity, whereas $P_{\underline{Z\alpha2}Z\alpha2}$ could not bind to it. In addition, dimerization of $P_{Z\alpha1Z\alpha2}$ indicated the function unit of Zα of *Ca*PKZ would be a dimer.



## 1. Introduction

*CaPKR-like* gene (AY293929), which encoded a 513aa protein with a conserved eIF2α kinase domain in its C-terminal and two Z-DNA binding domains (Zα) in its N-terminal regulatory domain, was first identified from IFN-producing CAB cells after treatment with UV-inactivated GCHV at 2004 [1]. Later, some other *PKR-like* homologous cDNA were cloned from Zebrafish (*Danio rerio*) (NM_001040376) [2], Atlantic salmon (*Salmo salar*) (DQ182560) [3] and Rare minnow (*Gobiocypris rarus*) (EF661570) [4]. As all the proteins encoded by these homologous genes containing two Zα domains (Z-DNA binding domain), they were designated "PKZ" (protein kinase containing Z-DNA binding domain) [2]. Moreover, it was likely that PKZ was a unique and recently discovered member of eIF2α kinase family in fish [5].

The highly conserved Z-DNA binding domain was identified from human double-stranded RNA adenosine deaminase (ADAR1) [6], Z-DNA binding protein 1 (DLM-1/ZBP1) [7], vaccinia virus E3L protein [8], and fish PKZ. Primarily, the background of the structure and function of Zα came from the analysis of ADAR1 [9–11]. However, the biological role of Zα from PKZ remains unclear. In order to investigate the function of *Ca*PKZ Zα, we expressed and purified three constructed peptides of *Ca*PKZ ($P_{Z\alpha}$): $P_{Z\alpha1Z\alpha2}$, $P_{Z\alpha1\underline{Z\alpha1}}$ and $P_{\underline{Z\alpha2}Z\alpha2}$. In addition, recombinant plasmids containing d(GC)$_n$ inserts ($n = 6, 8, 10, 13$) were constructed. Gel mobility shift assays were used to examine the binding activity of $P_{Z\alpha}$ to the recombinant plasmids which could be in a potential Z-DNA. Our results showed that $P_{Z\alpha1Z\alpha2}$ and $P_{Z\alpha1\underline{Z\alpha1}}$ were able to bind with high affinity to Z-DNA.

## 2. Materials and methods

### 2.1. DNA

Synthetic Oligonucleotides d(GC)$_n$ sequences (Table 1) were purchased from Shanghai Sangon Ltd. After annealing, duplex oligomers were inserted into the pMD18-T vector (TaKaRa).

### 2.2. Conformation of pMD18-T/d(GC)n analysis

A total of 500 ng of pMD18-T/d(GC)$_n$ was incubated in 10 μl with 10× NEB 2 buffer (New England Biolab) containing 1 unit of *Sss* I methylase and 6.4 mM of *S*-adenosylmethionine. Reaction mixtures were incubated for an hour at 37 °C and stopped by phenol extraction and ethanol precipitation. DNA was dissolved in 15 μl *Hha* I reaction buffer that contained 10 units of *Hha* I restriction endonuclease, then digested overnight at 37 °C. Products were analyzed by 1% agarose gel electrophoresis.

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**Table 1**
Different length of d(GC)n sequences used for cloning.

| Name | Sequence (5′ to 3′) |
|---|---|
| $d(GC)_6$ | CTGATACTACATTGAATTCGCGCGCGCGCGCGAATTCAATGTAGTATCAGA |
| $d(GC)_8$ | CTGATACTACATTGAATTCGCGCGCGCGCGCGCGCGAATTCAATGTAGTATCAGA |
| $d(GC)_{10}$ | CTGATACTACATTGAATTCGCGCGCGCGCGCGCGCGCGCGAATTCAATGTAGTATCA |
| $d(GC)_{13}$ | CTGATACTACATTGAATTCGCGCGCGCGCGCGCGCGCGCGCGCGAATTCAATGTAGTATCAGA |

pMD18-T/$d(GC)_n$ (280 ng) and the sheep polyclonal antibody to Z-DNA (Abcam, UK) (350 ng) were mixed together in a final volume of 15 μl, complexes were analyzed by 1% agarose gel.

### 2.3. Construction of expression plasmids and proteins preparation

With pMD18-T/*CaPKZ* (the full-length cDNA of *CaPKZ*) as the template (AY293929), different portions of the cloned cDNA coding for Zα of *Ca*PKZ were amplified by PCR and inserted into the *Nde* I/*Xho* I cleaved expression vector pET-22b(+) (Novagen). Three recombinant expression vectors were pET-22b(+)/Zα1Zα2, pET-22b(+)/Zα1Zα1 and pET-22b(+)/Zα2Zα2, which yielded the C-terminal His-tagged (LEHHHHHH) proteins. These DNA were confirmed by PCR and sequencing. In Zα1Zα1, the Zα2 motif was replaced with a second copy of Zα1, while a Zα2 substituted Zα1 in Zα2Zα2. To construct Zα1Zα1, primers (Table 2) SF1 and SR1 were used to amplify DNA coding for Zα1 and the linker region, then ligated to the *Nde* I and *Bam*H I cleaved pET-22b(+). At the same time, primers SF2 and SR2 were used to amplify Zα1. Cleaved with *Bam*H I and *Xho* I, Zα1 was ligated to the former pET-22b(+)/Zα1 with the same enzyme cut, resulting in pET-22b(+)/Zα1Zα1. Similarly, two pairs of primers: SF3 and SR3, SF4 and SR4, were used to construct pET-22b(+)/Zα2Zα2.

The recombinant expression vectors were transformed into *E. coli* BL21 (DE3) plysS (Novagen). Bacteria were grown at 37 °C in Luria–Bertani medium to $A_{600}$ of 0.6–0.8 and then induced with 1 mM isopropyl thio-β-D-galactoside (IPTG) for 4 h. The cells were harvested and suspended in binding buffer (20 mM Tris–HCl, 500 mM NaCl, 5 mM imidazole, pH 7.9), 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). Pooled fractions containing $P_{Z\alpha}$ were dialyzed overnight against dialysis buffer (20% glycerol, 150 mM NaCl, 1 mM DTT, 0.5 mM EDTA, 20 mM HEPES, pH 7.5), and then analyzed by 12% SDS-PAGE. The protein concentration was measured by Bradford assay. Samples were stored at −80 °C.

**Table 2**
Primers used in the study.

| Primer name | Sequence (5′ to 3′) | Purpose used |
|---|---|---|
| WF | CGCCATATGTCTGCCGAAACTCAAATGGAGAGGAAGATCATTG | Zα1Zα2 |
| WR | ACGCTCGAGACTTTCATTGCTCTCCTCGCCTTCCAGAAGCCA | |
| SF1 | CGCCATATGGAGAGGAAGATCATTGATTTC | Zα1 and |
| SR1 | AGCGGATCCTTGCTCTGGTGTTTTGAC | the linker region |
| SF2 | AGCGGATCCGAGAGGAAGATCATTGAT | Zα1 |
| SR2 | ACGCTCGAGCTTCTCCATTAGATCCCA | |
| SF3 | CGCCATATGACGACGACAGCAGAAACATGTG | Zα2 |
| SR3 | ACGGGATCCCTCCTCGCCTTCCAGAAG | |
| SF4 | CGCGGATCCGGGATCAAACAAACAGTC | the linker |
| SR4 | ACGCTCGAGGCCTTCCAGAAGCCACAAGC | region and Zα2 |

### 2.4. Dimerization assay

For each reaction, $P_{Z\alpha}$ (0.6 μg/μl) was incubated on ice in the presence or absence of pMD18-T/$d(GC)_6$ (50 μg/ml) in a volume of 10 μl. After an hour, the reaction was stopped by adding 6 × gel loading buffer (0.25% bromophenol blue, 40% sucrose solution). Samples were run on a 12% native polyacrylamide gel and analyzed by Coomassie brilliant blue staining. Meanwhile, reactions in the presence of 2% SDS or 100 mM 2-mercaptoethanol (2-ME) were used as the controls.

### 2.5. Gel mobility shift assays

DNA and $P_{Z\alpha}$ were mixed together in a final volume of 15 μl, with 280 ng of plasmid and about 350 ng of protein. Meanwhile, 10 μg of pMD18-T/$d(GC)_6$ was incubated with 20 units of DNA topoisomerase I (TaKaRa) in the buffer for 2 h at 37 °C. DNA was phenol extracted and ethanol precipitated, then mixed with $P_{Z\alpha1Z\alpha2}$ or $P_{Z\alpha1Z\alpha1}$ the same as above. In addition, pMD18-T/$d(GC)_6$ was mixed with the same amount of $P_{Z\alpha1Z\alpha2}$ and anti-Z-DNA antibody, which a competition experiment was performed.

Complexes were incubated on ice for an hour and resolved on a 1% agarose gel with 1 × TAE buffer at 5 V/cm. Gels were stained with ethidium bromide (0.5 μg/ml) and photographed.

## 3. Results

### 3.1. The conformation of pMD18-T/d(GC)n

Inhibition of methylation at *Hha* I sites in $d(GC)_n$ was restrained by Z-conformation that was stabilized by negative supercoiling. In addition to the insert sequence of $d(GC)_n$, there were seventeen GC sites in the parental plasmid of pMD18-T. As shown in lanes 3, 6, 9, 12 and 15 of Fig. 1A, the plasmids were cleaved in lots of fragments by *Hha* I in the absence of *Sss* I methylase. When pre-incubated with *Sss* I, the recombinant plasmids were mostly cleaved in linear by *Hha* I (Fig. 1A, lanes 5, 8, 11 and 14). As the control, some parental plasmids remained intact because GC sites were completely methylated (Fig. 1A, lane 2). Moreover, the linear bands were brighter as the length of $d(GC)_n$ increased. There were still some fragments below the linear DNA because of the incomplete methylation of these sites.

The gel shift assay was applied to assess the binding activity of the anti-Z-DNA antibody to pMD18-T/$(GC)_n$. As indicated by the arrow in Fig. 1B, the antibody could produce bandshifts that migrated with more slower mobility (Fig. 1B, lanes 4, 6, 8 and 10). As for the parental plasmid of pMD18-T (Fig. 1B, lane 2), there was no shifting band.

### 3.2. Construction, expression and purification of $P_{Z\alpha}$

These recombinant vectors were transformed into *E. coli* BL21 (DE3) plysS and induced with IPTG. Three kinds of $P_{Z\alpha}$ with His tag in its C-terminal were produced. After affinity chromatography with His–Bind Resin, the purified $P_{Z\alpha}$ were obtained.