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Original Article

The Zα domain of fish PKZ converts DNA hairpin with d(GC)$_n$ inserts to Z-conformation

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PKZ, protein kinase containing Z-DNA domains, is a novel member of the vertebrate eIF2α kinase family. Containing a catalytic domain in C-terminus and two Z-DNA binding domains (Zα1 and Zα2) in N-terminus, PKZ can be activated through the binding of Zα to Z-DNA. However, the regulatory function of PKZ Zα remains to be established. Here, to understand the impact of PKZ Zα on DNA conformational transition, wild-type Zα1Zα2 and 11 mutant proteins were expressed and purified. At the same time, several different lengths of DNA hairpins—d(GC)$_n$T$_4$(GC)$_n$ (n = 2–6) and an RNA hairpin—r(GC)$_6$T$_4$(GC)$_6$ were synthesized. The effects of Zα1Zα2 and mutant proteins on the conformation of these synthetic DNA or RNA hairpins were investigated by using circular dichroism spectrum and gel mobility shift assays. The results showed that DNA hairpins retained a conventional B-DNA conformation in the absence of Zα1Zα2, while some of the DNA hairpins ($n \geq 3$) were converted to Z-conformation under Zα1Zα2 induction. The tendency was proportionally associated with the increasing amount of GC repeat. In comparison with Zα1Zα2, Zα1Zα1 rather than Zα2Zα2 displayed a higher ability in converting d(GC)$_6$T$_4$(GC)$_6$ from B- to Z-DNA. These results demonstrated that Zα1 sub-domain played a more essential role in the process of B–Z conformational transition than Zα2 sub-domain did. Mutant proteins (K34A, N38A, R39A, Y42A, P57A, P58A, and W60A) could not convert d(GC)$_6$T$_4$(GC)$_6$ into Z-DNA, whereas S35A or K56A retained some partial activities. Interestingly, Zα1Zα2 was also able to induce r(GC)$_6$T$_4$(GC)$_6$ RNA from A-conformation to Z-conformation under appropriate conditions.

Keywords PKZ; Zα; Z-DNA; hairpin; Z-DNA binding protein

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Introduction

PKR-like gene was initially identified from crucian carp (*Carassius auratus* L.) blastula embryonic cells after treatment with UV-inactivated grass carp hemorrhage virus in 2004 [1]. Then, some other PKR-like orthologs were cloned and characterized from zebrafish (*Danio rerio*) [2], Atlantic salmon (*Salmo salar*) [3], and rare minnow (*Gobiocypris rarus*) [4]. PKR-like is referred to as 'PKZ' (protein kinase containing Z-DNA binding domain), because it contains two Z-DNA binding domains (Zα1 and Zα2) in the N-terminus [2]. The kinase domain with 11 conserved sub-domains in its C-terminal is closely related to mammalian PKR [5,6], so PKZ is a novel member of vertebrate eIF2α kinase family [7,8]. Rothenburg *et al*. [9] considered *PKZ* as a duplication of *PKR* after the divergence of the tetrapod lineage. Like mammalian *PKR*, *PKZ* is strongly up-regulated after immunostimulation [6,10].

The highly conserved Zα domain had been identified from human double-stranded RNA (dsRNA) adenosine deaminase (ADAR1) [11], Z-DNA binding protein 1 (DLM-1/ZBP1) [12], vaccinia virus E3L protein [13], and fish PKZ. The unique Zα domain (named regulatory domain) in N-terminal is able to specifically recognize and bind to Z-DNA with high affinity. The Zα of PKZ ($Zα_{PKZ}$) also recognizes and binds to Z-DNA [2] as well as recombinant plasmids with d(GC)$_n$ (n = 6, 8, 10, 13) inserts [14]. In addition, $Zα_{PKZ}$ facilitates the conversion of oligonucleotides with d(GC)$_n$ inserts from B- to Z-form [15]. Although recent studies have provided a more accurate view of fish PKZ [6,10], the regulatory function of PKZ Zα remains unclear.

Z-DNA, a left-handed double helical DNA, was first described in the late 1970s [16]. It is named Z-DNA because of the zigzag arrangement of its sugar-phosphate backbone [17]. *In vivo*, Z-DNA was found to be stabilized by negative

supercoiling that is generated by a moving RNA polymerase as it plows through the DNA double helix [17]. Although the roles of Z-DNA in biological processes are not fully elucidated, accumulating evidence has revealed that Z-DNA exists widely within genomes and may be involved in diverse biological functions *in vivo* such as antiviral defense, homologous chromosomal recombination, and regulation of gene expression, etc. [18–21]. It is well established that alternating purine-pyrimidine sequences, especially poly d(GC), are usually transformed into the left-handed structure under high-salt concentration. This transition can be stabilized by negative supercoiling or methylation of cytosine [22–27]. Since the stem region of d(CGCGCGTTTTCGCG CG) or d(CGCGCG) DNA adopts the left-handed Z-conformation under appropriate conditions [28,29], hairpins $d(GC)_nT_n(GC)_n$ and poly d(GC) are widely used for mimicking Z-DNA *in vitro*.

The Zα domain of ADAR1 ($Z\alpha_{ADAR1}$) is able to flip poly d(GC), hairpin $d(CG)_3T_4(CG)_3$, or $d(CG)_6T_3(CG)_6$ from B- to Z-conformation [30–32]. To better understand the biological functions of $Z\alpha_{PKZ}$ from goldfish (*C. auratus*) (*Ca*PKZ), we expressed and purified 12 recombinant mutant proteins: Zα1Zα2, Zα1Zα1, Zα2Zα2 and 9 point-mutated proteins (K34A, S35A, N38A, R39A, Y42A, K56A, P57A, P58A, and W60A). Different lengths of DNA or RNA hairpin were designed and synthesized. Circular dichroism spectrum (CD) and non-typical gel mobility shift assays were employed to detect the interaction of proteins with DNA or RNA hairpins.

In vivo, RNA often forms double-stranded structure in some region, especially in the region containing $r(GC)_n$ sequence [32]. As $Z\alpha_{ADAR1}$ facilitates the transition of RNA from A to Z, ADAR1 is considered as an important mechanism against RNA virus infection in living cells [17]. Interestingly, the A–Z transition of RNA hairpin $r(GC)_6T_4(GC)_6$ was also induced by $Z\alpha_{PKZ}$.

Materials and Methods

Protein preparation

The recombinant proteins including Zα1α2, Zα1Zα1, and Zα2Zα2 were expressed and purified as described previously [14]. Based on the sequence alignment of *C. auratus* $Z\alpha_{PKZ}$ and *Homo sapiens* $Z\alpha_{ADAR1}$, nine mutation vectors, pET-32a (+)/(K34A, S35A, N38A, R39A, Y42A, K56A, P57A, P58A, or W60A) were constructed and expressed by polymerase chain reaction site-directed mutagenesis method. All of the recombinant plasmids were sequenced by Sangon Biotech (Shanghai, China) before use. All of the samples were stored at −20°C.

Preparation of DNA and RNA hairpins

DNA hairpins $d(CG)_nT_4(CG)_n$ ($n = 2-6$) and 6-R (GAC TGGTTAGCATTTTTGCTAACCAGTC, as a control) were purchased from Sangon Biotech. After dissolution in buffer solution (pH 7.4, 50 mM Tris, 25 mM NaCl, 0.1 mM ethylenediaminetetraacetic acid), all DNA samples were incubated at 95°C for 5 min, then cooled to room temperature. RNA hairpin $r(CG)_6T_4(CG)_6$ was purchased from TaKaRa (Dalian, China). All of the DNA and RNA hairpins were stored at −20°C.

CD spectroscopy

CD spectra were recorded on a JASCO J-715 spectropolarimeter (Jasco Inc., Easton, USA) as described previously [15]. DNA hairpins were mixed with the recombinant proteins at molar ratios of 24 : 1, 12 : 1, 6 : 1, 3 : 1, respectively. RNA hairpin $r(CG)_6T_4(CG)_6$ was just mixed with Zα1Zα2 at molar ratio of 3 : 1. Each mixture was incubated at 25°C for 30 min before measurement. All spectra were corrected by subtracting the buffer baseline. The data were converted to jws (Java Web Service) and txt file formats for final analysis.

Gel mobility shift assays

The recombinant protein (Zα1Zα2, Zα1Zα1, Zα2Zα2, K34A, or S35A, respectively) was mixed with DNA hairpins $d(CG)_nT_4(CG)_n$ ($n = 3$ or 6) for gel mobility shift assays. After centrifugation at 13,400 *g* for 10 min and incubation at 95°C for 5 min, DNA hairpins (290 ng) were mixed with each fusion protein (4 μg) in a final volume of 10 μl. In addition, $d(CG)_6T_4(CG)_6$ was mixed with the same amount of proteins (Zα1Zα2, Zα1Zα1, or S35A, respectively) and 4 μg anti-Z-DNA antibody (ab2079; Abcam, Cambridge, UK) used for the competitive experiment under the same condition. After incubation at 30°C for 30 min, the reaction products were subject to 2% agarose gel electrophoresis. Gels were then stained with ethidium bromide (0.5 mg/ml) and photographed by using Gel Doc XR system (Bio-Rad, Hercules, USA).

Results

Effect of Zα1Zα2 on the conformational transition of DNA or RNA hairpins

To investigate the molecular mechanism responsible for B–Z transition of PKZ Zα, we performed the titration analysis of Zα1Zα2 to DNA or RNA hairpin. $d(CG)_nT_4(CG)_n$ ($n = 2, 3, 4, 5, 6$) or hairpin 6-R was titrated with Zα1Zα2 protein and then CD spectra were obtained at 25°C (**Fig. 1**). It was shown that $d(CG)_2T_4(CG)_2$ adopted a conventional B-DNA conformation at low ionic strength and was not shifted to Z-DNA even in the presence of Zα1Zα2 at a DNA

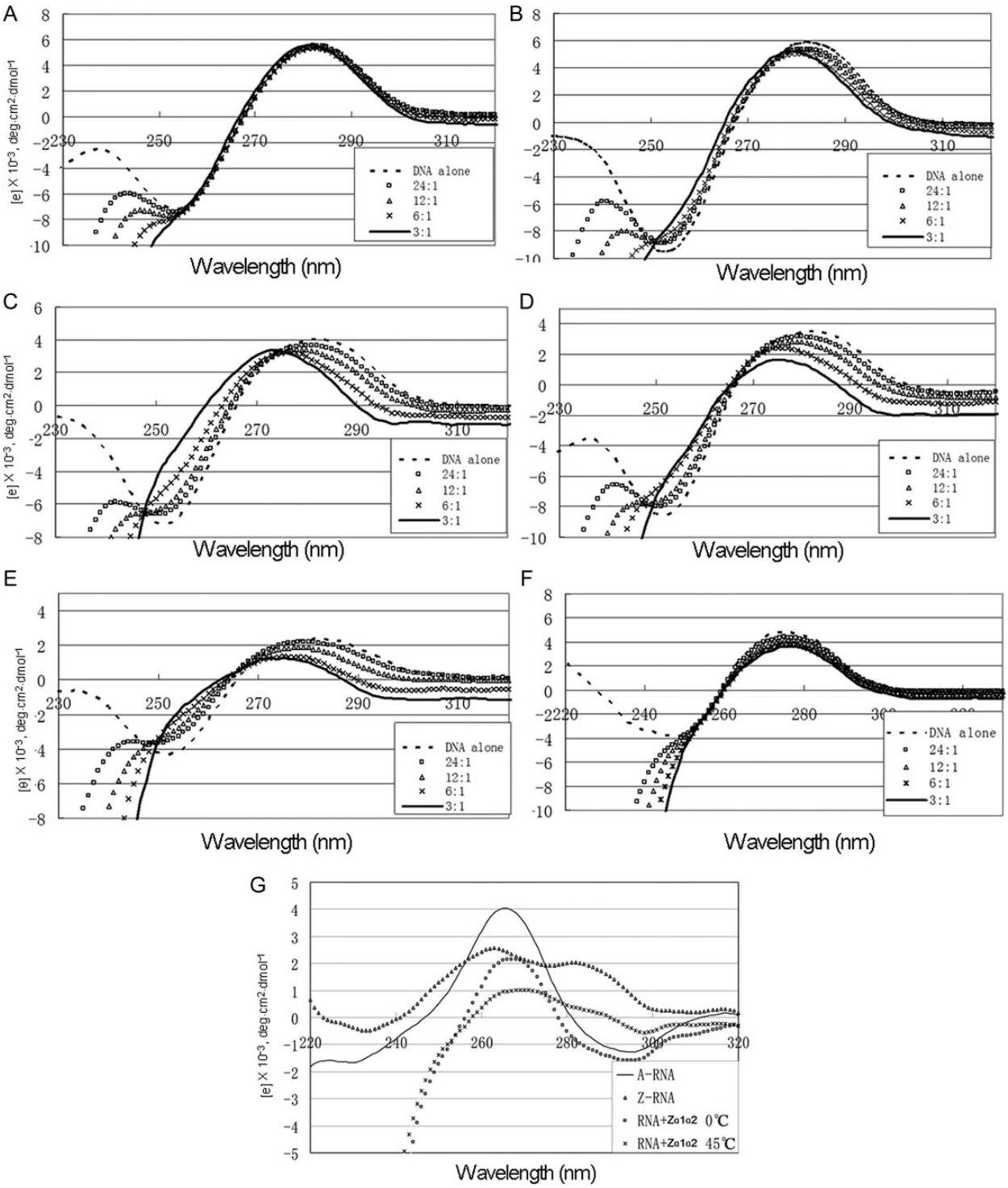

Figure 1 DNA hairpins and RNA hairpin $r(CG)_6T_4(CG)_6$ were titrated with Zα1Zα2 DNA hairpins were titrated with Zα1Zα2. CD spectra of hairpin/Zα1Zα2 complex were obtained in buffer at 25°C. The stochiometric ratio of base pair-to-peptide was 24 : 1, 12 : 1, 6 : 1, and 3 : 1, respectively, and spectra were measured after a 10 min equilibration period. No correction was applied to the spectrum of mixture. (A) $d(CG)_2T_4(CG)_2$ adopted a conventional B-DNA conformation at low ionic strength and could not be converted to Z-conformation in the presence of Zα1Zα2. (B－E) $d(CG)_3T_4(CG)_3$, $d(CG)_4T_4(CG)_4$, $d(CG)_5T_4(CG)_5$, or $d(CG)_6T_4(CG)_6$ was mixed with Zα1Zα2, respectively. (F) DNA hairpin 6-R kept B-conformation in the presence of Zα1Zα2. 'Em dash' represents a typical A-RNA at different salt solution; 'Empty triangle' represents a Z-RNA at different salt solution. 'Empty circle' or 'Multiplication sign' denoted that Zα1Zα2 was titrating RNA hairpin $r(CG)_6T_4(CG)_6$ at 0 or 45°C, respectively. (G) At 45°C, the spectrum feature was just like Z-RNA conformation.

base pair-to-peptide ratio of 3 : 1. In contrast, CD curves of $d(CG)_3T_4(CG)_3$ induced by Zα1Zα2 began to change. The positive peak slightly shifted from 285 nm (low-salt form) to 295 nm [**Fig. 1(B)**]. With the increasing d(CG) portion, the change of CD curves became more obvious [**Fig. 1(C–E)**]; however, as a control, the CD curve of hairpin 6-R/Zα1Zα2 changed little under the same condition [**Fig. 1(F)**].

$r(CG)_6T_4(CG)_6$ could be converted to Z-RNA by Zα1Zα2 and the trend became more obvious with increasing experimental temperature [**Fig. 1(G)**]. Under Zα1Zα2 induction, the CD spectrum of $r(CG)_6T_4(CG)_6$ was similar to that of typical A-RNA at 0°C, while it tended towards Z-RNA to a certain degree at 45°C.

Zα1 sub-domain plays a major role in the binding of Zα to Z-DNA

Under Zα1Zα1 induction, the CD curves of $d(CG)_6T_4(CG)_6$ became more pronounced in comparison with that under Zα1Zα2 induction [**Fig. 2(A)**]. With the increasing amounts of Zα1Zα1 titer, the molar ellipticities from 253 to 294 nm were altered dramatically. This implied that Zα1 sub-domain promoted the transition of B- to Z-DNA conformation. On the contrary, no matter how much Zα2Zα2 titer it was, the DNA hairpin retained a conventional B-DNA conformation [**Fig. 2(B)**].

The conserved residues within Zα are very important in Z-DNA binding

To investigate whether the conserved residues within Zα are very important in Z-DNA binding, we performed a series of mutant protein-$d(CG)_6T_4(CG)_6$ titration analyses. The CD spectra were recorded from 230 to 330 nm at 25°C. Since Zα1Zα2 itself did not show any significant CD signals above 250 nm, no baseline correction was applied. According to titration results (**Fig. 3**), these nine mutant proteins were divided into two groups. Group 1 was comprised of two non-conserved mutant proteins, S35A and K56A. Group 2 was comprised of seven conserved mutant proteins, K34A, N38A, R39A, Y42A, P57A, P58A, and W60A.

DNA hairpin $d(CG)_6T_4(CG)_6$ was titrated with S35A or K56A mutant protein. Under S35A [**Fig. 3(A)**] or K56A (data not shown) induction, the CD curves of $d(CG)_6T_4(CG)_6$ were very similar. The molar ellipticities around 254 and 280 nm were altered with increasing amounts of mutant protein S35A or K56A. S35A (K56A) mutant protein retained ~78.01% (56.36%) ability of wild-type Zα1Zα2. These results indicated that non-conserved amino acid residues such as S35 and K56 were not very important for the activity of Zα.

In contrast, when DNA hairpin $d(CG)_6T_4(CG)_6$ was titrated with K34A [**Fig. 3(B)**] or N38A, R39A, Y42A, P57A, P58A, W60A (data not shown), the CD spectra of hairpin was unchanged even at a DNA base pair-to-peptide ratio of 3 : 1. Obviously, these seven mutations were unable to trigger the B- to Z-transition of $d(CG)_6T_4(CG)_6$. These results indicated that conserved residues were very important for Zα.

Binding of protein to $d(CG)_6T_4(CG)_6$ revealed by gel mobility shift assays

In accordance with CD results above, the results of gel mobility shift assays revealed that Zα1Zα1, Zα1Zα2, or S35A could bind to $d(CG)_6T_4(CG)_6$ and converted it from B-form into Z-form [**Fig. 4(A)**] to some extent. As control, when 4 μg of anti-Z-DNA antibody was added to the mixture of protein/DNA, the antibody would compete with protein in Z-DNA binding. The band shift patterns of $d(CG)_6T_4(CG)_6$/Zα2Zα2 and $d(CG)_6T_4(CG)_6$/K34A were very weak when compared with those of $d(CG)_6T_4(CG)_6$/Zα1Zα1, $d(CG)_6T_4(CG)_6$/Zα1Zα2, or $d(CG)_6T_4(CG)_6$/S35A. Although the CD spectra of proteins/DNA hairpins were similar, the band shift pattern of hairpin $d(CG)_3T_4d(CG)_3$ was generally weaker than that of hairpin $d(CG)_6T_4(CG)_6$ in the presence of proteins [**Fig. 4(B)**].

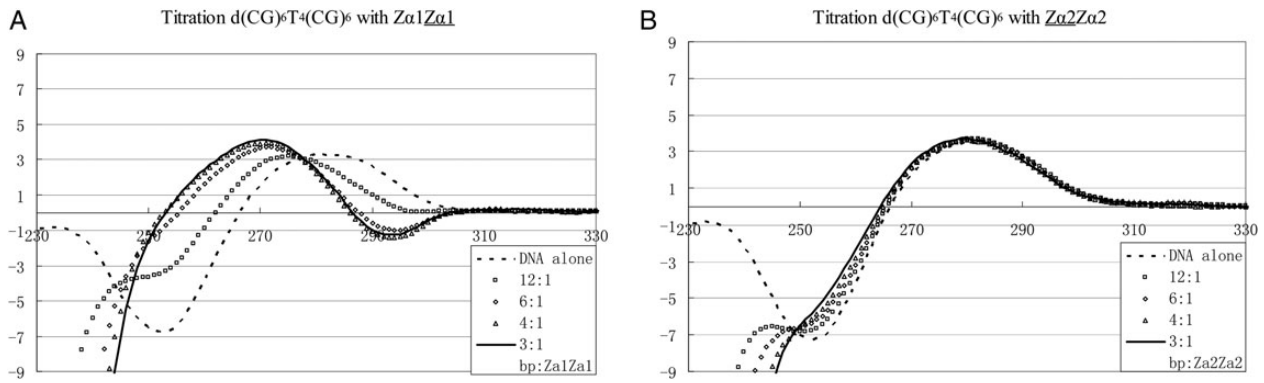

Figure 2 Hairpin $d(CG)_6T_4(CG)_6$ was titrated with Zα1Zα1 (A) or Zα2Zα2 (B) When the amounts of Zα1Zα1 increased, the pronounced alteration of molar ellipticity from 270 to 295 nm was 1.25-fold higher than that of Zα1Zα2.

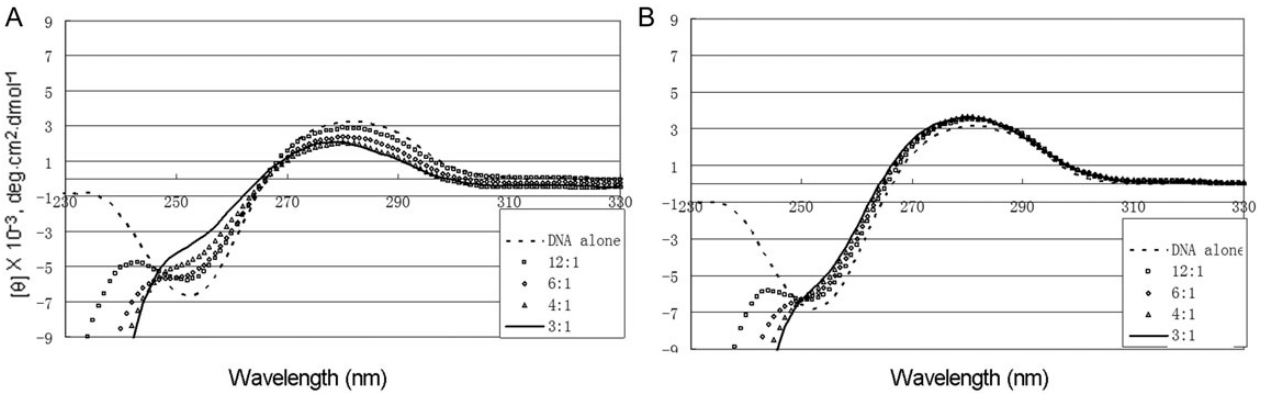

Figure 3 Hairpin $d(CG)_6T_4(CG)_6$ was titrated with S35A or K34A Mutants were used at a stochiometric base pair-to-peptide ratio of 24 : 1, 12 : 1, 6 : 1, and 3 : 1, respectively. Spectra were measured after a 10 min equilibration period. The spectrum from 240 to 320 nm showed the transition of DNA conformation. No correction was applied to the spectrum of mixture. (A) S35A maintained part of the activity. (B) K34A lost the activity.

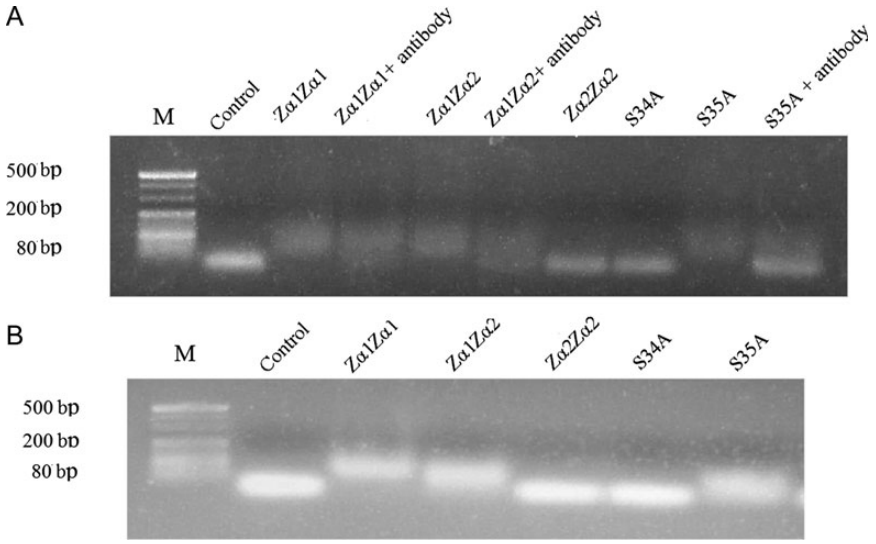

Figure 4 Binding of Zα to DNA hairpin $d(CG)_3T_4(CG)_3$ or $d(CG)_6T_4(CG)_6$ analyzed by gel mobility shift assays (A) Zα1Zα1, Zα1Zα2, Zα2Zα2, S34A, or S35A protein was mixed with hairpin $d(CG)_6T_4(CG)_6$. Anti-Z-DNA antibody was used to compete binding. (B) Zα1Zα1, Zα1Zα2, Zα2Zα2, K34A, or S35A protein was mixed with hairpin $d(CG)_3T_4(CG)_3$. DNA alone was used as control.

Discussion

CD spectroscopy is an ideal method for tracing the conformational transitions between discrete B-form and Z-form arrangements [33]. The conformational transition from B- to Z-DNA at high-salt concentrations is accompanied by a near inversion of its CD spectrum [34]. NaCl and cobalt hexamine $[Co(NH_4)_6]$ prompt the conformational transition of poly d(GC) from B- to Z-form [35,36]. Similarly, we found that $d(CG)_6T_4(CG)_6$ formed the conventional Z-form DNA at high-salt solution of 4.5 M NaCl, while $d(CG)_3T_4d(CG)_3$ was converted to Z-form partially under same condition (data not shown). These results indicated that these DNA hairpins could be converted from B- to Z-DNA in high-salt solution and the length of DNA segment might play some critical roles in the transition. Consequently, the B–Z transition of poly d(GC) might emerge in a length-dependent manner. Due to lack of enough base-stacking energy, it was very difficult for shorter DNA fragments such as $d(CG)_nT_4(CG)_n$ ($n < 3$) to form a typical Z-DNA structure [**Fig. 1(A,B)**].

$d(CG)_3T_4(CG)_3$, a double-stranded $d(CG)_3$ stem connected covalently with a T4 loop, is used as a defined substrate to determine the binding constant and the stoichiometry of Zα/Z-DNA in solution. $Z\alpha_{ADAR1}$ was able to bind to $d(CG)_3T_4(CG)_3$ with high affinity and converts it into Z-DNA almost completely, which resembled to that of poly d(CG) [30]. In the present study, the effect of $Z\alpha_{PKZ}$ on the conformation of hairpins was investigated by using CD spectroscopy. DNA hairpins were converted to Z-conformation in a d(GC) length-dependent manner. That was similar to the oligonucleotides with $d(GC)_n$ inserts [15], although $d(CG)_nT_4(CG)_n$ formed Z-DNA without negative supercoiling.

There are several conserved residues in Zα that play important roles in Z-DNA binding. For example, residue Y145 in $Z\alpha_{DLM-1}$ is critical for Z-DNA recognition [37]. An insight into the crystal structure of $Z\alpha_{ADAR1}$/Z-DNA complex revealed that nine residues of $Z\alpha_{ADAR1}$ are essential for Z-DNA recognition and binding, and these residues are highly conserved in Zα [2,11,12,36,38,39]. Among them, residues K169, K170, N173, R174, Y177, and T191 are involved in the formation of helix α3 and bind to DNA directly. Residue W195 helps Y177 to interact with DNA. P192 and P193 form another important van der Waals interaction with DNA in the C-terminal of β-hairpin [38]. Sequence alignment of *Ca*PKZ Zα and *Hs*ADAR1 Zα revealed that PKZ Zα1 sub-domain also contains nine residues corresponding to those of $Z\alpha_{ADAR1}$. Residues K34, N38, R39, Y42, P57, P58, and W60 are identical to those of $Z\alpha_{ADAR1}$, while residues S35 and K56 share no homology with that of $Z\alpha_{ADAR1}$ (data not shown). Here, we found that these seven conserved residues in $Z\alpha_{PKZ}$ were very important in the B−Z transition of DNA (**Fig. 3**).

Zα (Zα1) sub-domain rather than Zβ (Zα2) displays a high affinity with Z-DNA. Zβ sub-domain might be responsible for dimerization [39]. However, Kim *et al*. [40] found that Zβ of human ZBP1 (DLM-1) binds to Z-DNA via a novel B−Z transition pathway. So the binding mechanism of Zα to Z-DNA is much more complicated. Our results were in accordance with a previous report [14]. Zα1Zα1 protein was more effective in the B−Z transition of DNA hairpins than Zα1Zα2, while Zα2Zα2 almost lost the function (**Fig. 2**).

In addition, $Z\alpha_{ADAR1}$ binds to left-handed Z-RNA as well as to Z-DNA [17]. The observation of Zα binding to Z-RNA revealed a well conserved pathway of the interaction between Zα and the nucleic acid backbone of Z-DNA or Z-RNA [41]. $Z\alpha_{PKZ}$ could also convert A-RNA to Z-RNA [**Fig. 1(G)**].

Temperature is also an important factor that affects the binding constant between riboses in nucleic acid. The higher the temperature, the easier the conformational transition for nucleic acid. Ribose of dsRNA generally adopts the 3′-endo conformation, while deoxyribose of double-stranded (ds DNA) adopts the 2′-endo conformation [41]. Ribose has a 2′-hydroxyl group that stabilizes its pucker (pseudorotation), and more energy is required for altering the pucker in dsRNA than in dsDNA consequently. The transformation constant of A- to Z-RNA at 45°C is approximately the same as that of B- to Z-DNA at 20°C [17]. In the present study, it was found that the degree of A- to Z-RNA transition at 45°C was more prominent than that at 0°C [**Fig. 1(G)**] or 30°C (data not shown).

Taken together, $Z\alpha_{PKZ}$ was able to recognize and convert DNA or RNA hairpin to Z-conformation, indicating that it might serve as a 'flippase' as $Z\alpha_{ADAR1}$ [42]. Because PKZ is mainly located in the cytoplasm [3], it could be speculated that $Z\alpha_{PKZ}$ would actively induce the B- to Z-DNA transition or A- to Z-RNA transition by advantage of a series of short DNA or RNA sequence. It is now well-known that PKZ could protect cell against DNA or RNA virus [2]. PKZ could be activated by binding to Z-DNA or Z-RNA, followed by the activation of eIF-2α, and finally resulting in the shut-down of the cellular protein synthesis [10].

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