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Novel zinc finger nuclease created by combining the Cys_2His_2- and His_4-type zinc finger domains

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ABSTRACT

To improve the DNA hydrolytic activity of the zinc finger nuclease, we have created a new artificial zinc finger nuclease (ZWH4) by connecting two distinct zinc finger domains possessing different types of Zn(II) binding sites (Cys_2His_2- and His_4-types). The overall fold of ZWH4 is similar to that of the wild-type Sp1 zinc finger (Sp1(zf123)) as revealed by circular dichroism spectroscopy. The gel mobility shift assay demonstrated that ZWH4 binds to the GC box DNA, although the DNA-binding affinity is lower than that of Sp1(zf123). Evidently, ZWH4 hydrolyzes the covalently closed circular plasmid DNA (form I) containing the GC box (pBSGC) to the linear duplex DNA (form III) in the presence of a higher concentration (50 times) of the protein than DNA for a 24-h reaction. Of special interest is the fact that the novel mixed zinc finger protein containing the Cys_2His_2- and His_4-type domains was first created. The present results provide the useful information for the redesign strategy of an artificial nuclease based on the zinc finger motif.

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Introduction. The design of functional proteins is one of the most attractive issues in the post-genomic era. Especially, the artificial nucleases that promote efficient cleavage of DNA are valuable as biotechnological and gene therapeutic reagents. A zinc finger protein provides the ideal frameworks for creating the artificial nuclease for the following reasons;[1,2] (i) zinc finger domains fold into a compact ββα structure with the aid of Zn(II), (ii) zinc finger domains possess two important molecular recognition abilities, that is, metal- and DNA-binding abilities, (iii) the DNA recognition mode can be easily controlled by manipulating the key DNA contacting amino acids on the α-helix[3–7] and (iv) the zinc finger domain has a tandemly connected structure with the linker parts.

The most widely used strategy for creating an artificial zinc finger nuclease is to fuse a DNA-cleaving domain to a zinc finger protein. Chandrasegaran's group reported the artificial zinc finger nuclease comprised of zinc finger domain and the DNA cleavage domain of a *Fok*I endonuclease.[8] This chimeric zinc finger nuclease could cleave the DNA sequence at a position close to the zinc finger binding site. Urnov et al. also succeeded in the endogenous human gene correction using the *Fok*I-type zinc finger nuclease.[9] Recently, it is widely recognized that engineering zinc finger nucleases is the most promising tool for manipulating and editing the target genomic DNA in human cells.[10,11]

We previously reported the construction of artificial zinc finger proteins with hydrolytic ability in a metal dependent manner by mutating the Cys_2His_2-type to the His_4-type in the second domain (Sp1(zf2H4)) of the transcription factor Sp1 zinc finger protein (Sp1(zf123)) without adding the *Fok*I DNA cleavage domain.[12,13] The designed peptides successfully acquired both an ester bond and DNA hydrolytic ability, while retaining the proper zinc-finger folding and DNA targeting ability. However, the low DNA cleavage activity of the His_4-type zinc finger nuclease is still an important problem to be solved. In order to improve the low catalytic activity, we designed a new type of zinc finger nuclease (ZWH4) possessing four zinc finger domains in this study (Fig. 1). ZWH4 was created by combining the Cys_2His_2 and His_4 zinc finger domains without the use of any native DNA cleaving enzymes. This chimera protein is expected to function as a novel artificial nuclease with a high sequence specificity for DNA duplexes.

Results and discussion. Construction of ZWH4: Sp1(zf123) and Sp1(zf2H4) are coded on the plasmids, pEVSp1 (530–623) and pEVSp1(zf2H4) as previously described, respectively.[14–16] The fragments of Sp1(zf123) and Sp1(zf2H4) were amplified and combined together with the obtained fragments using the standard PCR technique. The resultant fragment encoding ZWH4 was digested by restriction enzymes (Nde I and Eco RI) and inserted into pEV3b. The sequence was confirmed by a 3130 Genetic Analyzer (Amersham Biosciences). ZWH4 was overexpressed as a soluble form in the *Escherichia coli* strain BL21(DE3)pLysS at 20 °C and purified according to the following procedure at 4 °C. The *E. coli* cell was

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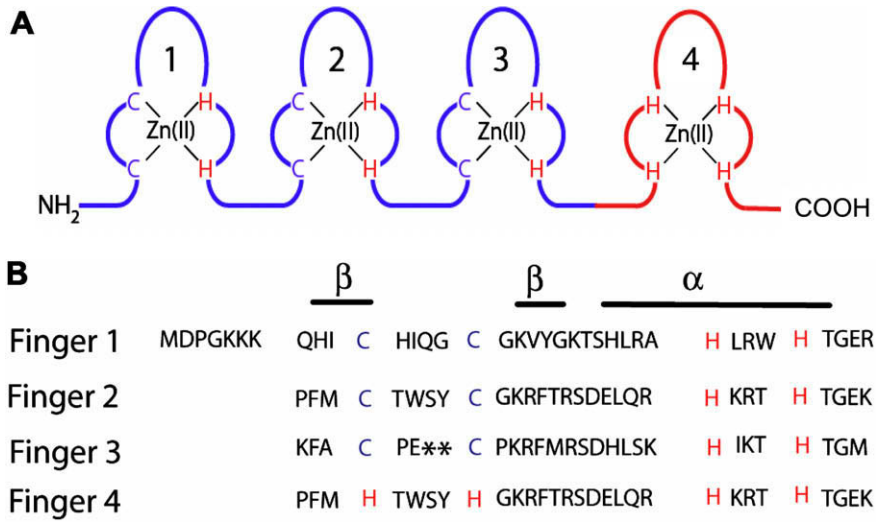

Figure 1. (A) Schematic representation of ZWH4 and (B) the amino acid sequence of ZWH4.

resuspended and lysed in PBS buffer. After centrifugation, the supernatant containing the soluble form of ZWH4 was purified by cation-exchange chromatography (BioRad). After desalting, final purification was performed by HPLC on a Cosmosil $5C_{18}$-ARII (10 mm × 250 mm) column (Nakalai Tesque). The fidelity of the purified protein was confirmed by SDS–PAGE (data not shown) and matrix-associated laser desorption ionization time-of-flight mass spectroscopy (MALDI-TOF-MS) using a Voyager-DE STR system (Applied Biosystems): [MH+] calcd 14860.2 and observed 14860.9.

Secondary structure of ZWH4: Circular dichroism (CD) spectroscopy is an effective tool to determine the secondary structure content of peptides and proteins in solution.[17] The relative amounts of the α-helical, β-sheet and random coil conformations can be estimated on the basis of their characteristic ellipticities. The folding properties of ZWH4 were investigated by monitoring the CD spectra.[18] Figure 2 shows the CD spectra of ZWH4 in the absence and presence of Zn(II). In the absence of Zn(II), the peptide gives a negative band near 200 nm, suggesting that the peptide possesses largely random coil features without Zn(II). Upon the addition of Zn(II), a large change in the CD spectrum was observed; the intensity significantly increases in the helix diagnostic negative molar ellipticity at 222 and 205 nm, and the random coil signature band near 200 nm undergoes a dramatic reduction in the negative molar ellipticity. As compared to the CD feature of Sp1(zf123),[14,15] ZWH4 has a secondary structure similar to Sp1(zf123), indicating that ZWH4 is folded into the zinc finger typical of the ββα conformation.

DNA-binding ability of ZWH4: The Sp1 zinc finger protein specifically recognizes and binds to the GC box, 5′-GGG GCG GGGCC-3′.[16,19] DNA binding of ZWH4 for the cognate DNA was evaluated by a gel mobility shift assay (Fig. 3).[20] Based on this result, ZWH4 clearly showed band shifts during protein binding depending on the protein concentration. In order to determine the dissociation constant (K_d), the quantity of shifted bands was estimated as described previously.[16] The K_d value of ZWH4 was approximately 58 nM, indicating that ZWH4 has a sufficiently high DNA-binding ability for the GC-box, though the affinity was lower compared to that of Sp1(zf123) (K_d = ca. 3 nM). Presumably, the reduction of the DNA-binding affinity is caused by changing the protein conformation. Although ZWH4 exhibits secondary structure similar to Sp1(zf123) based on the CD measurement, the ternary structure of ZWH4 would be slightly changed by the fusion of Sp1(zf2H4) to the C-terminus of Sp1(zf123). These data indicate that our designed zinc finger seems to have an adequate DNA-binding affinity for the target DNA.

Evaluation of site-specific DNA cleavage of ZWH4: We tried to examine the ability of ZWH4 to cleave the targeting DNA due to the sufficient DNA-binding affinity for the C box DNA as shown by the gel mobility shift assay. In order to test the sequence selectivity of the ZWH4, supercoiled plasmid DNAs, pBS and pBSGC were used as the substrates; pBSGC contains a GC box, and the pBS is free from a GC box. The cleavage of the supercoiled plasmid DNA (form I) was followed by its conversion to the nicked-circular

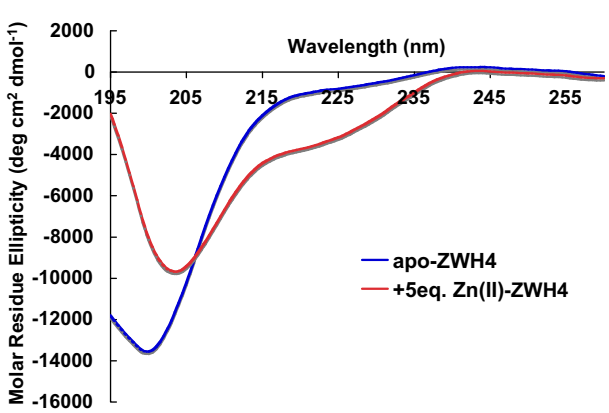

Figure 2. CD spectra of ZWH4 in the absence (blue line) and presence of 5 equiv of $ZnCl_2$ (red line).

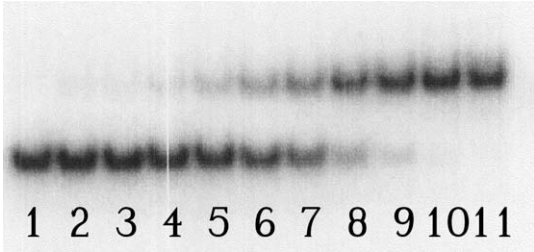

Figure 3. Gel mobility shift assay of ZWH4 for binding to the target DNA. The lanes represent the following protein concentrations: 0, 1.8, 3.7, 7.5, 15, 31, 62, 125, 250, 500 and 1000 nM, respectively.

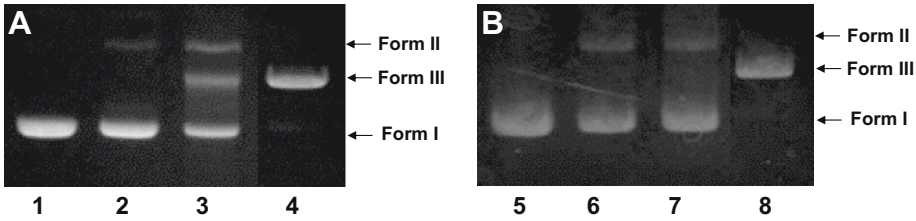

Figure 4. Cleavage of the pBS plasmid DNA by ZWH4. The reactions were carried out in 5 mM HEPES buffer (pH 7.5) at 37 °C for 24 h. (A) pBSGC; [pBSGC] = 24 nM; Lanes 1–3 represent 0, 25 and 50 times of ZWH4 for DNA. Lane 4 shows the DNA treated by the HindIII-treated DNA. (B) pBS; [pBS] = 24 nM; Lanes 5–7 represent 0, 25 and 50 times of ZWH4 for DNA. Lane 8 shows the DNA treated by the HindIII-treated DNA.

form (form II) and then to the linear form (form III).[21,22] The apo ZWH4 alone without Zn(II) exhibited no hydrolytic activity for the pBS plasmid under the present conditions (data not shown). This result agrees with our previous data that the zinc ion coordinated by the His_4-type zinc finger domain plays a critical role in the catalytic reaction.[12,13]

Figure 4 shows the results of the plasmid DNA cleavage experiment. In the case of pBSGC (Fig. 4A), ZWH4 had a clear DNA cleavage activity depending on the concentration of proteins under the experimental conditions in 24 h. In particular, at the 50 equiv concentration (lane 3), ZWH4 converted pBSGC to the form II and even to form III. The new zinc finger protein ZWH4 induced a strong cleavage for pBSGC DNA. On the other hand, ZWH4 showed a very small hydrolytic activity for pBS even at the higher protein concentrations (Fig. 4B). We have already reported that a H4 type Sp1zf(123) (H4Sp1), where two of the Cys residues in each finger domain were replaced by His residues, also has a high DNA hydrolytic activity.[23] However, the hydrolysis reaction of H4Sp1 requires longer reaction time compared to that of the present ZWH4.[12] The difference in the DNA hydrolytic activity between H4Sp1 and ZWH4 might be due to the difference in the geometry of the His_4-type zinc finger domain against the phosphate back bone, because the H4Sp1 showed an affinity to the GC box similar to that of ZWH4 to GC box.[14] These results indicate that the high hydrolytic activity for the pBSGC by ZWH4 was achieved through specific DNA binding for GC box by fusing Sp1(zf123) (DNA binding domain) to Sp1(zf2H4) (catalytic domain).

In summary, we successfully demonstrated the selective hydrolysis of the plasmid DNA containing the GC box by a new artificial zinc finger nuclease (ZWH4) that was created by mixing two kinds of zinc finger domains having different types of Zn(II) binding sites (Cys_2His_2- and His_4-types). Interestingly, ZWH4 could convert the plasmid DNA up to the form III under mild conditions. This design strategy of the combination of the different kinds of zinc finger domains would provide a new and valuable approach for novel artificial nucleases based on the zinc finger proteins.

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