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Site-specific DNA cleavage by artificial zinc finger-type nuclease with cerium-binding peptide ☆,☆☆

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Abstract

The addition of a new function to native proteins is one of the most attractive protein-based designs. In this study, we have converted a C_2H_2-type zinc finger as a DNA-binding motif into a novel zinc finger-type nuclease by connecting two distinct zinc finger proteins (Sp1 and GLI) with a functional linker possessing DNA cleavage activity. As a DNA cleavage domain, we chose an analogue of the metal-binding loop (12 amino acid residues), peptide P1, which has been reported to exhibit a strong binding affinity for a lanthanide ion and DNA cleavage ability in the presence of Ce(IV). Our newly designed nucleases, Sp1(P1)GLI and Sp1(P1G)GLI, can strongly bind to a lanthanide ion and show a unique DNA cleavage pattern, in which certain positions between the two DNA-binding sites are specifically cleaved. The present result provides useful information for expanding the design strategy for artificial nucleases.

Keywords: Zinc finger protein; Lanthanide ion; Calcium-binding loop; DNA binding; DNA cleavage; Artificial nuclease

The complete analysis of the human genome reveals close relationships between DNA sequences and diseases. Therefore, the application of this knowledge to gene therapy has become of great importance. In order to desire high selectivity for a target DNA sequence, the construction of an artificial nuclease has been investigated widely as a promising tool [1–12]. One of the most versatile strategies is the concept that a metal-chelating ligand or a catalytic domain of a restriction enzyme is connected to DNA-binding proteins [1–10] or motifs [11,12]. In these approaches, the catalytic domains have been designed to locate at one side of the DNA-binding domain, and thus the DNA cleavage site was observed in the vicinity of the DNA-binding site, together with fluctuation in the DNA cleavage. To achieve a highly site-specific DNA cleavage inside the DNA recognition site, we first designed novel nucleases in which the catalytic domain is held by two DNA-binding domains.

A C_2H_2-type zinc finger motif is one of the most promising molecules for a DNA-binding domain of a nuclease for the following reasons [13–15]: (i) artificial zinc finger motifs for any DNA sequences are easily designed, because each domain strongly recognizes three-base-pairs with amino acid residues at specific positions in the α-helix, and (ii) this motif has a tandemly connected structure with the linker region. In our opinion,

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☆☆ *Abbreviations:* Tris, tris(hydroxymethyl)aminomethane; TN, Tris–NaCl; CD, circular dichroism; zf, zinc finger; NMR, nuclear magnetic resonance.
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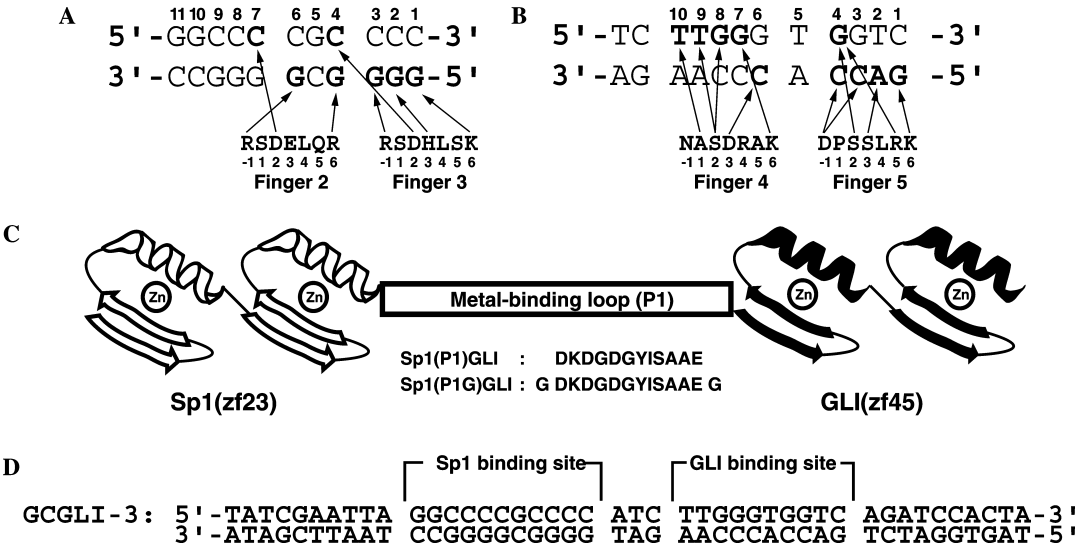

Fig. 1. (A,B) Base recognition mode of Sp1(zf23) and GLI(zf45) determined from NMR or X-ray analysis. Amino acid residues at the N-terminus of the α-helix in each finger are depicted by their one-letter codes with the number of helical positions below. Solid arrows show the base recognition, and the bases recognized by amino acids are written in bold characters. (C) Schematic representation of designed zinc finger-type nucleases. The designation of each zinc finger is shown by the original name. The amino acid residues of each peptide are indicated by their one-letter codes. (D) Substrate DNAs used in this study.

the conversion of the linker region into a functional linker with DNA cleavage ability should result in site-specific DNA cleavage. Considering steric hindrance, a short peptide is suitable for the DNA cleavage domain. The analogue (P1) of a 12-residue calcium-binding loop (sequence: DKDGDGYISAAE) strongly and specifically binds to a lanthanide ion [16–19]. On the other hand, chimeric molecules composed of DNA-binding domains and a Ce(IV)-based catalytic domain have been designed, because Ce(IV)-chelates have been reported to catalyze DNA hydrolysis with high efficiency [7–12]. In this research, we inserted the P1-Ce(IV) complex as a linker to connect two different zinc finger motifs. We previously reported that a peptide segment corresponding to fingers 2 and 3 of the Sp1 zinc finger (Sp1(zf23)) or to fingers 4 and 5 of the GLI zinc finger (GLI(zf45)) has sufficient DNA-binding affinity for their binding sites (Figs. 1A and B) [20–23]. In this report, based on these findings, we newly created the unique zinc finger-type nucleases, Sp1(P1)GLI and Sp1(P1G)GLI (Fig. 1C). In Sp1(P1)GLI, P1 was placed between Sp1(zf23) and GLI(zf45). In Sp1(P1G)GLI, glycine residues were inserted at both ends of peptide P1 of Sp1(P1)GLI to achieve subtle flexibility. This study describes the characteristics of their unique DNA cleavage properties and the potential of our approach in designing sophisticated metallonucleases.

Materials and methods

Chemicals. Fmoc-amino acid derivatives and peptide resin were purchased from Novabiochem and Applied Biosystems. The T4 polynucleotide kinase and restriction enzymes were obtained from New England Biolabs. The *Taq* DNA polymerase and synthesized oligonucleotides for cloning each peptide were acquired from Qiagen and Sigma Genosys, respectively. The labeled [γ-^{32}P]ATP compound was supplied by DuPont. The plasmid pBS-Sp1-fl was kindly provided by Dr. R. Tjian. All other chemicals were of commercial reagent grade.

Peptide synthesis. The peptide P1 (DKDGDGYISAAE-amide) was prepared by Fmoc-solid-phase synthesis on a TGS-RAM resin (Shimadzu). The peptide chain was constructed using a Shimadzu PSSM-8 synthesizer with a standard protocol including a benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP)/1-hydroxybenzotriazole (HOBt)/4-methylmorpholine (NMM) coupling system. The peptide segment was removed from the resin and deprotected by a treatment with trifluoroacetic acid/1,2-ethanedithiol (95:5) at room temperature for 2 h, followed by HPLC-purification on a Cosmosil 5C_{18}-ARII (10 mm × 250 mm) column (Nakalai Tesque). The fidelity of the product was confirmed by matrix-associated laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) using a Voyager-DE STR system (Applied Biosystems): P1 [$M + H^+$] calcd. 1240.3, observed 1240.2.

Construction of zinc finger-type nucleases and substrate DNA fragments. Sp1(zf23) and GLI(zf45) are coded on the plasmids pEVSp1(566–623) and pEVGLI(99–160), respectively, as previously described [21,22]. The fragments of Sp1(zf23) and GLI(zf45) were amplified and combined with the fragment coded P1 sequence using the standard PCR technique. The resultant fragments were digested by restriction enzymes (*Nde*I and *Eco*RI) and inserted into pEV3b. Their sequences were confirmed by a GeneRapid DNA sequencer (Amersham Biosciences). These peptides were 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. *E. coli* cells were resuspended and lysed in PBS buffer. After centrifugation, the supernatant containing the soluble form of the zinc finger peptides was purified by cation-exchange chromatography using a 0.05–2.0 M NaCl gradient (Mono S HR 5/5; Amersham Biosciences). Final purification was achieved by a gel filtration technique (Superdex 75; Amersham Biosciences) using TN buffer [10 mM Tris–HCl (pH 8.0), 50 mM NaCl, and 1 mM dithiothreitol]. To prepare the substrate DNA, oligonucleotides containing the DNA-binding sites of Sp1(zf23) and