Enhanced Cleavage of Double-Stranded DNA by Artificial Zinc-Finger Nuclease Sandwiched between Two Zinc-Finger Proteins

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ABSTRACT: To enhance DNA cleavage by zinc-finger nucleases (ZFNs), we sandwiched a DNA cleavage enzyme with two artificial zinc-finger proteins (AZPs). Because the DNA between the two AZP-binding sites is cleaved, the AZP-sandwiched nuclease is expected to bind preferentially to a DNA substrate rather than to cleavage products and thereby cleave it with multiple turnovers. To demonstrate the concept, we sandwiched a staphylococcal nuclease (SNase), which cleaves DNA as a monomer, between two three-finger AZPs. The AZP-sandwiched SNase cleaved large amounts of dsDNA site-specifically. Such multiple-turnover cleavage was not observed with nucleases that possess a single AZP. Thus, AZP-sandwiched nucleases will further refine ZFN technology.

Restriction endonucleases recognize double-stranded DNA (dsDNA)[1] substrates sequence-specifically. After cleaving, restriction endonucleases detach from the DNA products due to the DNA structural change and bind to substrates again, resulting in multiple-turnover cleavage of dsDNA substrates. While efforts to isolate homing endonucleases such as I-SceI continue, the number of homing endonucleases available for genome manipulation is limited at this stage. Therefore, development of artificial endonucleases that recognize long genomic DNA sequences is desired.

The most popular strategy for generating artificial endonucleases is to fuse a DNA-binding protein to a DNA-cleaving enzyme. Such hybrid enzymes include a λ repressor−staphylococcal nuclease hybrid (*1*) and a chimeric nuclease comprising a zinc-finger protein (ZFP) and the cleavage domain of a FokI endonuclease (*2*). Among them, the zinc-finger nucleases (ZFNs) seem most useful; ZFNs efficiently cleave their genomic DNA target in human cells, thereby enhancing rates of homologous recombination (*3*) or mutagenesis (*4*). While efforts to improve ZFN technology (e.g., reduction of off-target cleavage) (*5, 6*) continue, a study focused on enhancement of dsDNA cleavage rates has not been reported. Because current ZFNs bind to both dsDNA substrates and cleavage products equally, they are not effective for cleaving large amounts of DNA.

We reasoned that if a DNA-cleaving enzyme (or domain) were placed between two zinc-finger proteins, the resulting artificial endonuclease could distinguish substrate DNA and cleavage products and thereby cleave more dsDNA with multiple turnovers. In this study, we split a six-finger artificial zinc-finger protein (AZP) (*7*) used for recognition of a genomic DNA target into two three-finger AZPs and sandwiched the staphylococcal nuclease (SNase), which cleaves DNA as a monomer, with these two AZPs. We examined whether the resulting artificial zinc-finger nuclease cleaved a dsDNA target with multiple turnovers in vitro.

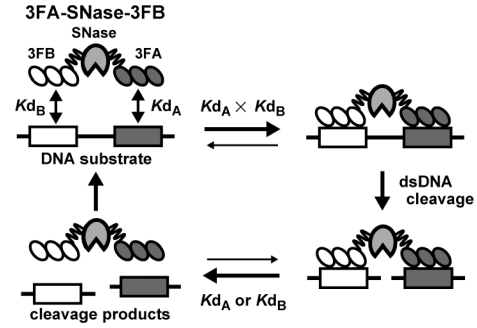

FIGURE 1: Multiple-turnover DNA cleavage by an AZP-sandwiched nuclease, 3FA−SNase−3FB. The scheme presents a nuclease sandwiched between two AZPs, 3FA−SNase−3FB, that binds to its DNA substrate and cleaves it with multiple turnovers. Because the cleavage site resides between two AZP-binding sites, 3FA−SNase−3FB binds to the DNA substrate much more strongly than to the resulting cleaved DNA products. In contrast, a nuclease fused to a continuously joined six-finger AZP should not show such multiple-turnover cleavage because the nuclease has the same affinity for the cleavage product and for the DNA substrate. The dark gray and open ellipses represent zinc-finger domains 3FA and 3FB, respectively. The light gray Pacman shape represents a SNase cleavage domain. The wavy lines represent linker peptides between SNase and 3FA or 3FB. The dark gray and open rectangles represent 3FA- and 3FB-binding sites, respectively.

The reason the AZP-sandwiched SNase is expected to cleave dsDNA with multiple turnovers is illustrated in Figure 1. When the dissociation constants (K_ds) of three-finger AZPs, 3FA and 3FB, for target DNA are defined as K_{dA} and K_{dB}, respectively, the K_d of the AZP-sandwiched 3FA−SNase−3FB for its DNA substrate is ideally expressed as $K_{dA} \times K_{dB}$. Because the region between the two AZP-binding sites should be cleaved by 3FA−SNase−3FB, the K_d of 3FA−SNase−3FB for the DNA cleavage products is significantly increased to K_{dA} or K_{dB}. Therefore, 3FA−SNase−3FB is expected to revisit and cleave the DNA substrate again, leading to multiple-turnover DNA cleavage.

To demonstrate the concept, we constructed a 3FA−SNase−3FB sandwich. The 3FA (AZP-10 designated in ref *7*) and 3FB (a three-finger moiety of AZP_{HPV}-2 designated

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[1] Abbreviations: dsDNA, double-stranded DNA; ZFP, zinc-finger protein; ZFN, zinc-finger nuclease; AZP, artificial zinc-finger protein; SNase, staphylococcal nuclease; K_d, dissociation constant.

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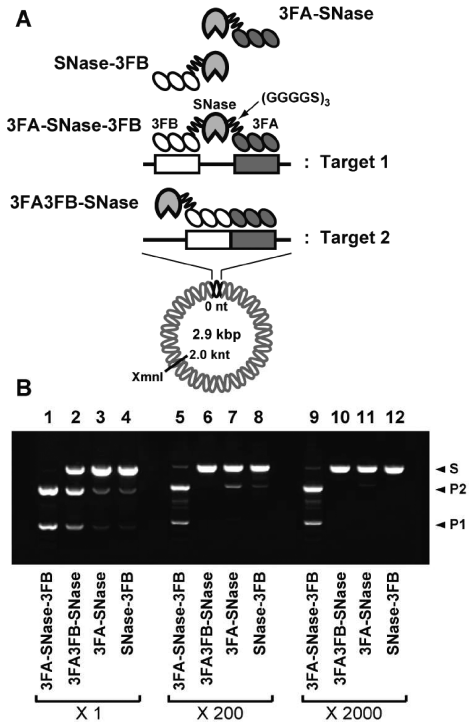

FIGURE 2: AZP-sandwiched nuclease, 3FA−SNase−3FB, cleaved dsDNA more efficiently. (A) Schematic representation of structures of 3FA−SNase−3FB and the controls, 3FA3FB−SNase, 3FA−SNase, and SNase−3FB, together with target plasmids, Target 1 and Target 2. (B) Comparison of cleavage rates of SNase variants. The SNase variant used for each cleavage is indicated below the figure. Target 1 was used for cleavage by 3FA−SNase−3FB, 3FA−SNase, and SNase−3FB. Target 2 was used for cleavage by 3FA3FB−SNase. A target plasmid was incubated with a SNase variant at 37 °C for 1 (lanes 1−4), 30 (lanes 5−8), or 300 min (lanes 9−12) and then digested with XmnI. Amounts of DNA used are shown relative to SNase variants (final concentration of 5 nM). S, a 2.9 kilobase DNA substrate; P1, a 0.9 kilobase cleavage product; P2, a 2.0 kilobase cleavage product.

in ref *8*) were previously generated to recognize the 10 bp DNAs 5′-GTTGCGGGAT-3′ and 5′-GGTCGGGACC-3′, respectively (amino acid sequences of 3FA and 3FB are shown in Figure S1 of the Supporting Information). The apparent K_ds of 3FA and 3FB were 25 and 4 nM, respectively. As controls, we also produced a six-finger nuclease (designated 3FA3FB−SNase) and two three-finger nucleases (designated 3FA−SNase and SNase−3FB) possessing only one three-finger AZP of 3FA−SNase−3FB (Figure 2A). Each of these nucleases was expressed in *Escherichia coli* and purified as previously described (*7*). However, when we tried to determine the apparent K_ds of 3FA−SNase−3FB and 3FA3FB−SNase to compare their affinities directly, we failed because the SNase moiety cleaved the ^{32}P-labeled DNA probe even at 4 °C in the absence of Ca^{2+} during DNA binding reactions (data not shown). We could not add EDTA to the binding buffer to remove Ca^{2+} because EDTA also inhibits chelation of Zn^{2+} by AZP.

To evaluate the cleaving efficiency of 3FA−SNase−3FB, we performed in vitro DNA digestion experiments. A target DNA plasmid was digested by each artificial nuclease under the conditions described in the legend of Figure 2. Target 1 (Figure 2A) contained a 40 bp sequence (5′-GGTCGGGACCATATGTATACATATGTATACGTTGCGGGAT-3′) harboring 3FA- and 3FB-binding sites (underlined) with a 20 bp spacer, which is recognized by 3FA−SNase−3FB. Target 2 (Figure 2A) contained the 19 bp sequence 5′-GGTCGGGACGTTGCGGGAT-3′, which is recognized by 3FA3FB−SNase. The resulting products were digested by XmnI to distinguish the DNA substrate and cleavage products more clearly, and the final products were analyzed on a 0.8% agarose gel. First, DNA substrates (0.1 μg) were cleaved by SNase variants in equimolar concentrations. Under the single-turnover conditions, all artificial nucleases cleaved their target DNA site-specifically as shown in Figure 2B (lanes 1−4). Among these nucleases, 3FA−SNase−3FB demonstrated the highest cleaving activity: 3FA−SNase−3FB digested the target DNA completely. In contrast, 3FA3FB−SNase, a nuclease variant comprising a continuously joined six-finger AZP, which was expected to have roughly the same (or similar) affinity as 3FA−SNase−3FB, cleaved approximately 50% of a DNA substrate. The three-finger nucleases, 3FA−SNase and SNase−3FB, which were expected to have lower affinities for their targets than six-finger nucleases (*7*), cleaved a target DNA plasmid much less effectively. It is known that SNase alone cleaves DNA randomly (*1*).

Next, DNA cleavage by SNase variants was investigated under multiple-turnover conditions. When a 200-fold excess of a target plasmid (20 μg) was used, 3FA−SNase−3FB again demonstrated the highest cleaving activity as shown in Figure 2B (lanes 5−8). Under these conditions, DNA cleavage by 3FA3FB−SNase was not observed (lane 6), showing lower activity than 3FA−SNase and SNase−3FB (lanes 7 and 8, respectively). 3FA3FB−SNase was expected to have a much greater affinity for its DNA target than three-finger nucleases (*7*), and in fact, 3FA3FB−SNase seemed to detach from DNA products and bind to a DNA substrate again less frequently than 3FA−SNase and SNase−3FB. Otherwise, 3FA3FB−SNase should have cleaved more efficiently than the three-finger SNases under multiple-turnover conditions as well as under single-turnover conditions.

3FA−SNase−3FB cleaved the 2000-fold excess of target DNA (200 μg) (Figure 2B, lane 9). Under the same conditions, no control nuclease (lanes 10−12) showed detectable DNA cleavage. These experiments showed that our AZP-sandwiched nuclease distinguished DNA substrates and products and cleaved its DNA target with multiple turnovers. If the multiple-turnover cleavage was due to enhanced substrate binding rather than enhanced product dissociation, 3FA3FB−SNase should have demonstrated multiple-turnover cleavage as well. However, as shown in Figure 2B (lanes 6 and 10), 3FA3FB−SNase did not demonstrate it because 3FA3FB−SNase bound to both DNA substrate and cleavage products equally.

Finally, we identified the cleavage sites of 3FA−SNase−3FB. The 5′-end-labeled 200 bp DNA probes containing the target site recognized by 3FA−SNase−3FB were digested by 3FA−SNase−3FB (Figure 3A), and then these products were separated on a 6% denaturing gel. Comparison with a DNA (C + T) marker generated by the Maxam−Gilbert method (*9*) demonstrated that 3FA−SNase−3FB cleaved the target DNA at specific sites of both strands, producing two-base sticky ends. As designed, 3FA−SNase−3FB cleaved the DNA region between the binding sites of 3FA and 3FB.

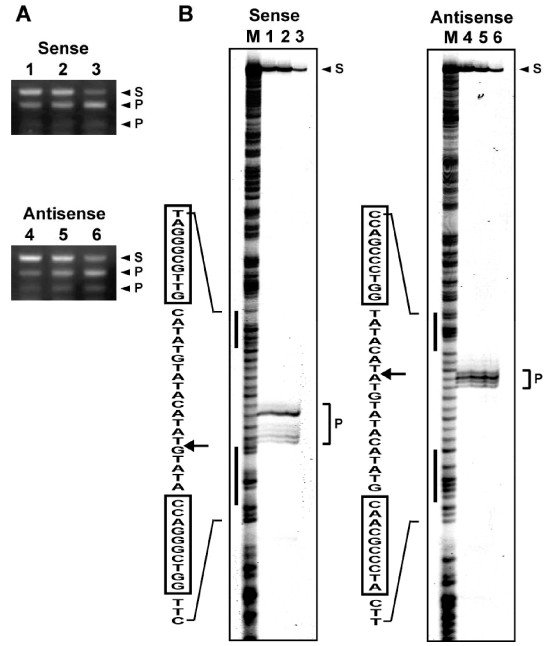

FIGURE 3: 3FA−SNase−3FB cleaved dsDNA at specific sites. (A) Cleavage of 5′-end-labeled 200 bp dsDNA targets by 3FA−SNase−3FB. After reaction with 3FA−SNase−3FB for 4 (lanes 1 and 4), 6 (lanes 2 and 5), and 8 min (lanes 3 and 6), the reaction mixtures were analyzed on a 2% agarose gel. (B) Determination of sites cleaved by 3FA−SNase−3FB. The reaction mixtures shown in panel A were separated on a 6% denaturing gel. Black bars next to the C + T marker lane (M) indicate the binding sites of the two AZPs used. Boxes indicate DNA sequences of the binding sites of these AZPs, and the main cleavage sites are denoted with black arrows. S, DNA substrate; P, DNA cleavage products.

While we fused SNase, which cleaves DNA nonspecifically (*1*), to 3FA and 3FB with a flexible linker, $(GGGGS)_3$, mainly one site was cleaved. By adjusting the length and amino acid content of the linker moieties, we may be able to cleave target DNA at the desired positions between two AZP-binding sites.

In this study, the catalytic domain of SNase was used as a model enzyme to demonstrate our concept because the nuclease cleaves DNA as a monomer. Because SNase produces 5′-hydroxyl and 3′-phosphate termini after DNA cleavage (*10*), AZP-sandwiched SNase cannot be used directly for cloning. After DNA products are cleaved, the phosphate at the 3′-ends must be eliminated with alkaline phosphatase, and then the 5′-ends must be phosphorylated with T4 polynucleotide kinase before DNA ligation. Therefore, single-chain restriction endonucleases such as PvuII (*11*) and FokI (*12*; T. Mino, Y. Aoyama, and T. Sera, manuscript submitted for publication) may be more useful as a cleaving domain of AZP-sandwiched nucleases. In particular, an AZP-sandwiched "single-chain FokI" will solve the problem of off-target cleavage (*13*) caused by homodimerization of conventional ZFP−FokI through the catalytic FokI domain. Conversely, AZP-sandwiched SNase is expected to have an advantage. The phosphate at the 3′-ends may reduce the chance of self-religation after cleavage, thereby enhancing homologous recombination, mutagenesis, or inhibition of DNA virus replication.

We note that Sugiura's group first reported an artificial nuclease in which a cerium-binding peptide as a DNA-cleaving domain was placed between two two-finger ZFPs (*14*). However, they showed only nicking, but not dsDNA cleavage. It is likely that the cleaving peptide in their molecule reached only one proximal strand because the short cleaving peptide was directly connected to two ZFPs with no peptide linker. Moreover, there were no data on or description of multiple-turnover cleavage. In our nuclease, the SNase domain was linked to AZPs via a flexible peptide linker, $(GGGGS)_3$, thereby enabling the SNase domain to contact both DNA strands. We also demonstrated the multiple-turnover dsDNA cleavage experimentally.

In summary, we demonstrated that AZP-sandwiched nucleases enhanced cleavage of dsDNA by distinguishing the DNA substrate and products. Because AZP-sandwiched nucleases are able to cleave large amounts of DNA, they will be a useful molecular tool for in vitro as well as in vivo genome manipulations such as homologous recombination and mutagenesis. AZP-sandwiched nucleases will be used for synthesis of artificial genomes or genome reconstruction (*15*). Furthermore, AZP-sandwiched nucleases will be useful for digestion of viral genomic DNA in vivo, which is amplified rapidly after infection, leading to the development of new antiviral therapies.

SUPPORTING INFORMATION AVAILABLE

Experimental procedures and Figure S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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