Biochemical and Biophysical Research Communications 453 (2014) 289–295

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Characterization of functional, noncovalently assembled zinc finger nucleases

So-Young Park, Xu Zheng, Yang-Gyun Kim *

Department of Chemistry, Sungkyunkwan University, Suwon 440-746, Republic of Korea

ARTICLE INFO

Article history:
Received 2 September 2014
Available online 7 October 2014

Keywords:
Noncovalent assembly
Zinc finger nuclease
Coiled-coil interaction
DNA cleavage
Protein design

ABSTRACT

Zinc finger nuclease (ZFN) is a chimeric restriction enzyme made of a C_2H_2-type zinc finger protein (ZFP) and the *Fok*I nuclease domain (F_N). ZFN technology has been considered as a powerful tool for genome editing. Here, we report a new type of ZFN system based on the coiled-coil interaction used as a noncovalent assembler. Like conventional ZFNs, noncovalently assembled ZFNs (ncZFNs) structurally have two domains, a ZFP and a F_N. Each domain carries one of antiparallel heterodimeric leucine zippers, respectively, to form an ncZFN through leucine zipper assembly. The characterization of ncZFNs revealed that they behave as fully functional sequence-specific endonucleases, comparable to those of conventional ZFNs. Interestingly, some ncZFNs displayed augmented off-target cleavage, possibly by degenerate DNA binding of the ZFP domain of ncZFNs based on our data. We postulate that DNA cleavage of ncZFN(s) seems to be more sensitive to the ZFP binding to lesser-optimal sites. Facile design of ncZFNs through the mix-and-assemble approach could be applicable to other DNA binding proteins for evaluation of sequence-specificity. In addition, our work establishes that the coiled-coil interaction could be used as the peptide-based noncovalent assembler for the formation of a noncovalently-linked functional multi-domain protein.

1. Introduction

*Fok*I restriction enzyme is composed of two separable domains: a sequence-specific DNA recognition domain (F_R) at the N terminus, and a DNA cleavage domain (F_N) at the C terminus (Fig. S1A) [1]. This modular structure of *Fok*I has made it possible to create a chimeric nuclease with novel sequence specificity by replacing the F_R domain of *Fok*I with other DNA binding domain (DBD) (Fig. S1B) [2]. This approach has successfully created novel sequence-specific endonucleases with various DBDs [3–8].

ZFN is largely based on the modular structure of *Fok*I. ZFN is composed of a C_2H_2-type zinc finger protein (ZFP) domain and the F_N domain of *Fok*I, and it has been utilized as a tailor-made, rare-cutter restriction enzyme [9]. In the advance of genome editing technology, zinc finger nuclease (ZFN) has been one of the leading and most investigated gene targeting methods [9]. The sequence-specific gene targeting using ZFNs was first described by Bibikova et al. [10]. Since then, a number of works have been reported, notably the gene targeting against X-linked severe combined immune deficiency (SCID) by Urnov et al. [11]. There have been several new designing approaches such as the single-chain F_N domain dimer [12–14] and the photocaged F_N domain [15]. In addition, the heterodimerization of the mutant F_N domains has been successfully used for avoiding the homodimerization of ZFNs to reduce off-target cleavage [16–18].

The coiled-coil is a versatile structural motif consisting of two or more α-helices [19]. In nature, it is used for a vast variety of biologically important interactions. The coiled-coil has been also utilized as a building block to produce polypeptide polyhedral [20,21], new biomaterials such as hydrogels [22,23], and assembling fragmented enzyme [24,25]. As the simplest way to make a dimer complex, the coiled-coil dimer-typically, leucine zippers-is particularly an attractive model system for the study of protein assembly and design [26,27]. It can be used to effectively guide polypeptide chains for assembly in preconceived ways.

In this study, we reasoned that the coiled-coil interaction can be devised as a 'noncovalent assembler'. We designed a new type of ZFN system by noncovalently associating a ZFP and a F_N through the leucine zipper assembly (Fig. 1). Noncovalently assembled ZFNs (ncZFNs) were successfully created through noncovalently combining two separately expressed, folded protein domains. Characterization of ncZFNs showed high sequence-specific DNA cleavages, comparable to those by conventional ZFN counterparts.

* Corresponding author. Fax: +82 31 299 4575.
E-mail address: ygkimmit@skku.edu (Y.-G. Kim).

http://dx.doi.org/10.1016/j.bbrc.2014.09.140
0006-291X/

2. Materials and methods

2.1. DNA construction

Expression vectors, pZFP-F_N, pZFP-CZ and pNZ-F_N (Fig. S2), were constructed with the pET28a vector (Novagen). The genes of ZFPs and the antiparallel heterodimeric leucine zipper pair (NZ and CZ) were synthesized by assembling oligonucleotides. To construct a vector for expression of ZFP-CZ fusion protein, pZFP-CZ, a ZFP gene was cloned into the modified pET28a at *Nde*I/*Age*I sites, and the CZ gene was then cloned at *Age*I/*Xho*I sites. pNZ-F_N vector was constructed by cloning the NZ gene and the PCR-amplified F_N gene into the modified pET28a at *Nde*I/*Spe*I and *Age*I/*Xho*I sites, respectively. To construct pZFP-F_N that expresses a conventional ZFN, we first constructed pZif268-F_N made by inserting the *Nco*I-*Xho*I DNA fragment of the Zif268-ZFN gene from the Zif268-ZFN expression vector (gift from Professor Jin Soo Kim at Seoul National University) into pET28a. For other conventional ZFP-ZFNs, an appropriate ZFP gene was inserted into pZif268-F_N vector to replace the Zif268 gene at *BamH*I/*Age*I sites. For the construction of mammalian expression vectors, PCR-amplified DNAs of natural or synthetic Zif268-ZFPs from pZFP-F_N vectors were digested with *Nhe*I/*Mlu*I. The *Nhe*I-*Mlu*I DNA fragment was then cloned into pIRES vector at *Nhe*I/*Mlu*I sites.

Construction of substrate DNAs for DNA cleavage assay was performed by inserting an annealed oligonucleotides (Bioneer Corporation) into pUC19 plasmid at *BamH*I/*Hind*III sites. DNA substrates with different spacer lengths were cloned by the same manner.

2.2. In vitro protein synthesis

Expression of all genes in vector constructs based on the pET28a was controlled by T7 promoter. For *in vitro* protein expression, $T_NT^{®}$T7 Quick Coupled Transcription/Translation System (Promega), T_NT in short, was used. All plasmid DNAs were purified using the plasmid cleanup kit (Qiagen) to remove RNases before the T_NT reaction. Proteins were produced by following the manufacturer's manual (Promega).

2.3. DNA cleavage assay

For sequence-specific DNA cleavage assay, proteins produced from pZFP-CZ and pNZ-F_N by the T_NT reaction were pre-incubated for 1 h at 22 °C for facilitating self-assembly of antiparallel heterodimeric leucine zippers. The ratio of reaction products from pZFP-CZ and pNZ-F_N was optimized to 1:4 for reproducible and efficient reaction condition after titration (Fig. 2). Typically, a reaction volume was 20 μl containing 2 μl of the 10× reaction buffer (50 mM potassium acetate, 20 mM Tris–acetate, pH7.9, 10 mM magnesium acetate, 1 mM DTT), 500 ng of a *Sca*I-linearized plasmid DNA substrate, and 5 μl of the preincubated ncZFN mix (the T_NT reactions of pZFP-CZ and pNZ-F_N) as 1:4 ratio. The reaction was incubated for 16 h at 22 °C. Similarly, sequence-specific DNA cleavage of conventional ZFNs was also performed by using 2 μl of the T_NT reaction and incubated for 16 h at 22 °C. After completing the DNA cleavage reaction, 10 μg of RNase A was added into reactions and incubated for 30 min at 22 °C. Then, 10 μg of protease K was added

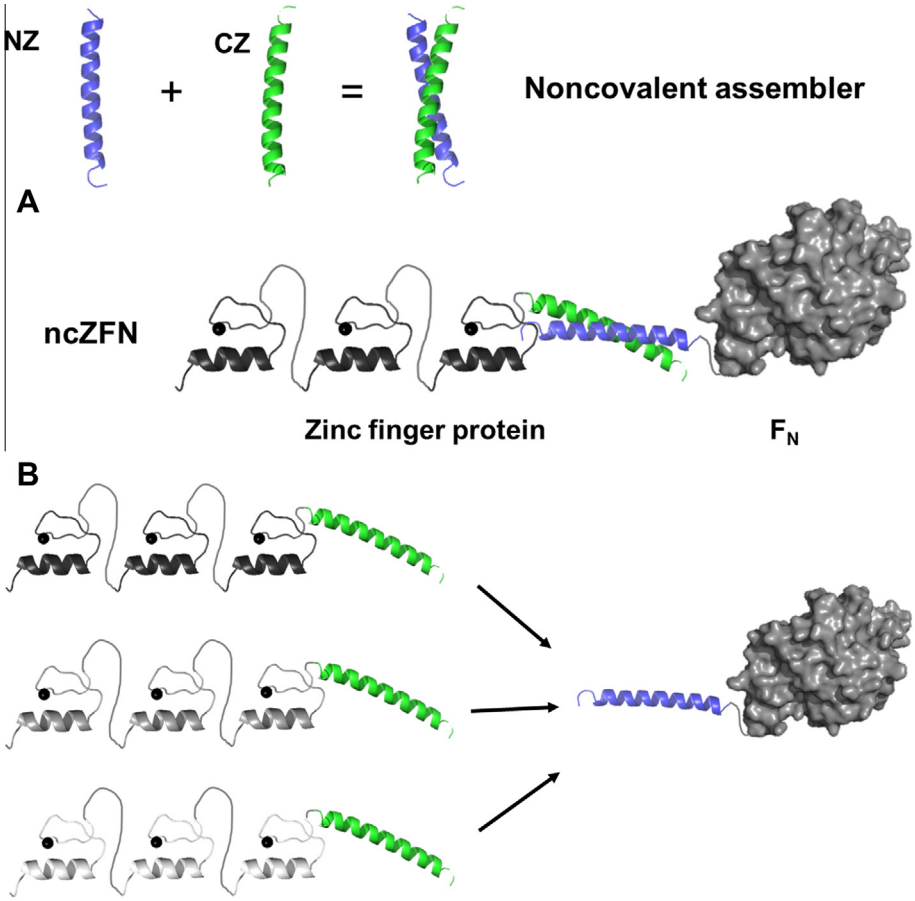

Fig. 1. Strategy for noncovalently assembled zinc finger nuclease. (A) *Fok*I nuclease domain (F_N) and zinc finger protein are fused to heterodimeric antiparallel leucine zipper pair (NZ and CZ), respectively. The noncovalently assembled ZFN (ncZFN) can be formed through the leucine zipper interaction. (B) Schematic illustration of mix-and-assemble approach for ncZFN formation using the noncovalent assembler. Diverse zinc finger proteins fused to the CZ were used to form ncZFNs simply by mixing one with the NZ-F_N.