Correlation between functional and binding activities of designer zinc-finger proteins

Jong Seok KANG¹

Department of Cell and Tissue Biology, University of California San Francisco, San Francisco, CA 94143, U.S.A.

Rapid progress in the ability to develop and utilize zinc-finger proteins with customized sequence specificity have led to their increasing use as tools for modulation of target gene transcription in the post-genomic era. In the present paper, a series of *in vitro* binding assays and *in vivo* reporter analyses were used to demonstrate that a zinc-finger protein can effectively specify a base at each position of the target site *in vivo* and that functional activity of the zinc-finger protein as either a transcriptional repressor or activator is positively correlated with its binding affinity. In addition, this

correlation can be extended to artificial engineered zinc-finger proteins. These data suggest that the binding affinity of designer zinc-finger proteins with novel specificity might be a determinant for their ability to regulate transcription of a gene of interest.

Key words: designer transcription factor, repression, transcriptional regulation, zinc-finger protein, zinc-finger protein 268 (Zif268).

INTRODUCTION

The Cys₂His₂-type zinc finger, a common DNA-binding motif in eukaryotes, has ~30 amino acid residues that fold into a compact module stabilized by a zinc ion. Three amino acid residues within the α -helix contact a 3-bp target DNA site [1]. This compact modular structure with a remarkably simple DNA-recognition mode has led to the use of this zinc-finger motif as a framework for developing designer transcription factor with customized DNA-binding specificity [2,3]. Several reports using phage display or one-hybrid selection systems have demonstrated that novel zinc fingers could be selected to bind to a given binding site [4–7], and that multi-finger proteins could be constructed to recognize a unique target site within a complex genome by combining two three-finger proteins with a linker [8–10]. In addition, it has been well documented that transcription of a gene of interest could be controlled by either a zinc-finger protein itself or a chimaeric protein in which a zinc-finger protein is fused to other functional domains, such as an activator or repressor domain [11,12]. It has been shown that designer zinc-finger proteins could access binding sites and regulate expression of target genes integrated into the genome [10,13].

Despite such progress in the capacity to select and utilize novel zinc-finger proteins, little is known about whether zinc-finger proteins have *in vivo* specificity comparable with *in vitro* specificity. Neither is it clear how the binding affinity of a zinc finger is related to its ability to modulate gene expression. The present study addresses these issues using a series of *in vitro* binding assays and *in vivo* reporter analyses with a prototype three-finger protein from Zif268 (zinc-finger protein 268), Zif268–GAL4 (galactosidase 4) chimaeric protein or various novel zinc-finger proteins selected using phage display.

EXPERIMENTAL

Plasmids

The Zif268 expression plasmid, pCS-Zif268, used for transfection studies in human cells, was described previously [10]. The ex-

pression plasmids for zinc-finger proteins selected by phage display were constructed by site-directed mutagenesis using pCS-Zif268 plasmid as a template. Variants of reporter plasmids containing various binding sites near the TATA box were made using site-directed mutagenesis using pGL3-TATA/Inr (initiator) as a template [11]. The expression vector pYTC-Zif268-GAL4, used in yeast transformation studies, was constructed by subcloning a DNA fragment encoding Zif268 fused to the activation domain of GAL4 into pYTC that was generated by removing the 2- μ origin and inserting the CEN (centromere)/ARS (autonomously replicating sequence) origin into pYESTrp2. The reporter plasmid, pLZ3, was made by inserting the 2- μ origin of pYESTrp2 into pLacZi, and subcloning double-strand oligonucleotides containing various binding sites upstream of the CYC1 (iso-1-cytochrome) minimal promoter.

Transient transfection

The HEK-293 (human embryonic kidney) cells were transiently transfected with either an empty expression vector or an expression vector encoding zinc-finger protein, a reporter plasmid encoding firefly luciferase, an activator plasmid [GAL4–VP16 (viral protein 16)] and an internal control plasmid encoding *Renilla* luciferase. The luciferase activities were measured as described previously [10].

β -Galactosidase assay

β -Galactosidase activities were measured in protein extracts and expressed as units per mg of protein as described previously [14].

Protein production and purification

The zinc-finger proteins were expressed in *Escherichia coli* as fusions with GST (glutathione S-transferase) and were purified by using affinity chromatography according to the manufacturer's protocol. GST was subsequently removed by digestion with thrombin protease. Protein concentrations were estimated by using SDS/PAGE with RNase A as a standard.

Abbreviations used: CYC1, iso-1-cytochrome; GAL4, galactosidase 4; GST, glutathione S-transferase; HEK-293, human embryonic kidney; VP16, viral protein 16; Zif268, zinc-finger protein 268.

¹ To whom correspondence should be addressed (email jong.kang@ucsf.edu).

Gel mobility-shift assay

To determine dissociation constants (K_d values), 2-fold serial dilutions of the zinc-finger proteins were incubated with a labelled probe (0.1–8 pM) at room temperature (25 °C) for 1 h in a binding solution [8], and then the reaction mixture was subjected to gel electrophoresis. The radioactive signals were quantified using a phosphorimager. Apparent K_d values were determined as described previously [8].

RESULTS AND DISCUSSION

In vivo binding specificity of zinc-finger protein

As the first step towards evaluating the DNA-binding specificity of zinc-finger protein *in vivo*, transient transfection experiments were performed with the three-finger protein from Zif268, which is structurally the most well characterized three-finger protein and recognizes the site 5'-GCGTGGGCGG-3' (Figure 1A) [1]. Since Zif268 efficiently represses transcription of a reporter gene when it binds to its binding site near the TATA box [11], this transcriptional repression assay was employed to test *in vivo* specificity of Zif268. To rigorously examine DNA-binding specificity at the level of base-by-base, various single-base substitutions were introduced within a 4-bp subsite that is recognized by finger 1 of Zif268 (Figure 1A). Then each of the various forms of binding site was incorporated near the TATA box of reporter plasmid, which contained five GAL4-binding sites upstream of the promoter of the reporter gene, firefly luciferase (Figure 1B). Each of the reporter plasmids was co-transfected with a plasmid encoding Zif268 and a plasmid expressing GAL4–VP16 to activate transcription.

When transfection was performed with a reporter plasmid having a promoter with the wild-type binding site GCGG (5'-GCGTGGGCGG-3') near the TATA box, Zif268 repressed VP16-activated transcription 19-fold (Figure 1C), consistent with previous transfection experiments [10]. On a promoter with the mutated binding site ACGG (5'-GCGTGGACGG-3') and CCGG (5'-GCGTGGCCGG-3'), in which a G base at position 7 of the binding site was replaced by an A or C base (underlined) respectively, the ability of Zif268 to inhibit VP16-activated transcription was greatly reduced (to 1.2 and 1.1-fold respectively). This result indicates that the Zif268 protein has a strong preference for G over A or C at position 7 by a factor of at least 15.8 *in vivo*. Similarly, the ability of Zif268 to inhibit transcription was greatly reduced (to 2-fold) on a promoter containing the mutated GGGG site (5'-GCGTGGGGGG-3'), in which a C base was replaced by a G base. However, reduction of repressor activity was only slight (to 16-fold) on a promoter containing the mutated GTGG site (5'-GCGTGGGTGG-3'), in which a C base at position 8 was mutated to a T base. This indicates that Zif268 does not efficiently discriminate between C and T bases at position 8, but it can strongly distinguish G from a C base at this position, despite the lack of physical contact between the C base at position 8 and the amino acid residues of finger 1 in the structure of the Zif268–DNA complex (Figure 1A) [1]. In the case of a promoter containing the mutated site GCAG (5'-GCGTGGGCAG-3') and GCCG (5'-GCGTGGGCCG-3'), in which a G base at position 9 was replaced by an A or C base respectively, fold repression was reduced from 19 to 8.1 and 5.6 respectively. This indicates that Zif268 has to some extent preference for G over A and C at position 9. Mutation of the promoter-binding site to GCGA (5'-GCGTGGGCGA-3') and GCGC (5'-GCGTGGGCGC-3'), in which a G base at position 10 was replaced by an A or C base respectively, also resulted in reduced ability of Zif268 protein to repress transcription (11.5- and 7.6-fold respectively). Thus it appears that Zif268 has

modest preference for G at position 10 *in vivo*. Taken together, these results demonstrate that a three-finger protein Zif268 can efficiently discriminate among the very closely related sequences and can effectively specify bases at each position of its binding site *in vivo*. Interestingly, one group has reported *in vitro* specificity of Zif268 using an *in vitro* selection method [15]. The data presented here are consistent with the reported *in vitro* selection data with respect to base-preference at each position by Zif268, implying that this zinc-finger protein have the ability to discriminate against the closely related target sites *in vivo* as well as *in vitro*, although it would face much more steric hindrance *in vivo* rather than *in vitro*.

Correlation between transcriptional repressor activity and binding affinity of zinc-finger protein

To examine whether the *in vivo* binding specificity of Zif268 observed in the experiments described above could be explained by its difference of affinity toward the closely related sites, the Zif268 protein was expressed and purified from *E. coli*, and gel mobility-shift assays were performed to determine the apparent K_d of Zif268 for each of the various binding sites.

As shown in Table 1, Zif268 protein bound to wild-type binding site GCGG with an apparent K_d of 18.8 pM, consistent with the previous report from Kim and Pabo [8], although the previous reports of K_d values for Zif268 have varied from 0.01 to 10 nM, depending on the measurement conditions. When DNA-binding affinity was determined with the mutated CCGG site, Zif268 bound 212-fold less tightly to the mutated site than to the wild-type binding site. Thus this protein bound to the mutated site with an apparent K_d of 4.0 nM. Similarly, Zif268 bound 106-fold less tightly to the mutated CCCG site than to the wild-type binding site (that is, a K_d of 2.1 nM). These results were consistent with the observation that Zif268 gave significantly less repression from a promoter containing these mutated binding sites than that from a promoter containing the wild-type site (Figure 1C). In contrast, Zif268 bound to the mutated GTGG and GCGA slightly less tightly (1.2- and 1.7-fold respectively) than to the wild-type GCGG site: Zif268 bound to the mutated GTGG and GCGA site with an apparent K_d of 23.8 pM and 32.1 pM respectively. These results were also consistent with the observation that mutation of the promoter to GTGG and GCGA resulted in slightly reduced repression by Zif268 (Figure 1C). When the apparent K_d of Zif268 for other binding sites was determined, it also showed a similar relationship between *in vitro* DNA-binding affinity and *in vivo* DNA specificity. Taken together, these results clearly imply that the differences in binding affinity can account for the *in vivo* specificity of Zif268 observed from transcriptional repression assays, and thus the ability of Zif268 to discriminate among the closely related sites appears to be derived from the relative affinity of this protein for the different binding sites. Furthermore, there was excellent correlation between the degree of transcriptional repression by Zif268 in human cells and the K_d values measured *in vitro* when a quantitative comparison was performed (Figure 1D). These results indicate that the repression of target gene expression is highly dependent on the DNA-binding affinity of zinc-finger proteins, and suggests conversely that the *in vitro* binding affinity of the zinc-finger protein for the target site may be indicative of its efficacy as a transcriptional repressor on a given target gene.

Correlation between transcriptional activator activity and binding affinity of zinc-finger protein

Since zinc-finger proteins are able to function as transcriptional activators when fused to an activation domain, there is a question

Figure 1 *In vivo* binding specificity and correlation between transcriptional repressor activity and binding affinity of zinc-finger protein

(A) Schematic representation of the base contacts in the Zif268–DNA complex. (B) Promoters of luciferase reporter genes. The nucleotide sequences of the promoters of the luciferase reporter gene are aligned to show the location of zinc-finger binding sites; the positions are numbered with respect to the transcription start point (+1). Identical nucleotides are shown as dots, and mutated nucleotides are underlined. (C) Transcriptional repression of various promoters with a series of altered binding sites by Zif268. HEK-293 cells were transiently transfected with the indicated reporter plasmid, an activator plasmid encoding GAL4–VP16 and an effector plasmid encoding Zif268. (D) Correlation between transcriptional repressor activities of Zif268 and its target site-binding affinities. The plotted data were derived from (C) and Table 1.

Table 1 Apparent dissociation constants of Zif268, its mutant, or zinc-finger proteins selected via phage display at the various binding sitesResults are means $\pm$ S.D. ND, not determined.

Zinc-finger protein	Binding site	Apparent K_d (pM)	K_d/K_d (wild-type)
Zif268	GCGTGGGCGG	18.8 ± 6.9	1
	GCGTGGACGG	ND	
	GCGTGGCCGG	4043 ± 1569	212.7
	GCGTGGGTGG	23.8 ± 6.4	1.2
	GCGTGGGGGG	ND	
	GCGTGGGCAG	ND	
	GCGTGGGCCG	80.4 ± 31.4	4.2
	GCGTGGGCGA	32.1 ± 9.4	1.7
	GCGTGGGCGC	40.7 ± 5.1	2.1
	GCGTGGGCAC	79.8 ± 11.0	4.2
	GCGTGGCCCG	2130 ± 337	106.3
	GCGTGGGACC	445.8 ± 19.2	23.7
	GCGTGGGCGG	18.8 ± 6.9	1
	Zif268(RDEA)	1760 ± 220	93.6
	Zif268(ADER)	371.9 ± 38.4	19.7
Zif268(RAER)	GCGTGGGCGG	42.65 ± 0.45	2.2
DSNR	GCGTGGGACC	2.5 ± 0.5	1
	GCGTGGGCAC	34.4 ± 5.6	13.7
	GCGTGGGCGC	36.0 ± 6.9	14.4
RADR	GCGTGGGCAC	4.4 ± 0.1	1
	GCGTGGGCGC	3.1 ± 0.07	0.7
	GCGTGGGACC	57.9 ± 5.8	13.1
QGSR	GCGTGGGCAC	1.6 ± 0.07	1
	GCGTGGGCGC	14.9 ± 0.1	9.3
	GCGTGGGACC	38.3 ± 3.1	23.9

of whether transcription activation by zinc-finger protein is likewise related to its binding affinity. To test this, transcription apparatus in yeast was used for transcriptional activation analyses. An expression plasmid encoding a chimaeric protein Zif268–GAL4, in which the three-finger protein from Zif268 was fused to the GAL4 activation domain under the control of a galactose-inducible GAL1 promoter, was transformed into yeast along with various reporter plasmids, in which wild-type or mutant binding sites for Zif268 protein were placed upstream of the yeast minimal promoter CYC1 of the reporter gene, β -galactosidase (Figure 2A). These transformed cells were then plated on X-Gal (5-bromo-4-chloroindol-3-yl β -D-galactopyranoside) plates containing galactose to induce expression of Zif268–GAL4, and the activity of the reporter gene in cell extracts was measured.

As shown in Figure 2(B), the degree of transcriptional activation by Zif268–GAL4 correlated positively with the binding affinity of Zif268. Taken together with the data derived from the transcriptional repression studies, these results clearly suggest that the function of a zinc-finger protein as either a transcriptional repressor or activator strongly depends on its binding affinity for target site. Interestingly, binding affinity positively correlates with function in two mechanistically different systems: in transcriptional repression, in which the zinc finger is able to directly inhibit transcription pre-initiation complex formation by binding to a nearby TATA box; and in transcriptional activation, in which the zinc finger can function indirectly by associating with an activator domain, which in turn functions through interaction with transcriptional machinery.

Contribution of DNA-contacting residues to functional activity and binding affinity of zinc-finger protein

From structural studies of the Zif268–DNA complex, it is known that four residues at positions –1, 2, 3 and 6 of the recognition helix of zinc finger are primarily involved in specific base-contacts with an overlapping 4-bp subsite (Figure 1A) [1]. To examine how

Figure 2 Correlation between transcriptional activator activity and binding affinity of zinc-finger protein

(A) Schematic representation of the chimaeric protein Zif268–GAL4, in which Zif268 is fused with a yeast GAL4 activation domain and reporter plasmids, which contains one of various forms of a binding site for Zif268 protein upstream of yeast minimal promoter CYC1 of the β -galactosidase gene. (B) Correlation of the transcriptional activation by the zinc-finger protein with its DNA-binding affinity. Each of the reporter plasmids containing binding site variants for Zif268 was transformed into yeast along with a plasmid encoding the Zif268–GAL4 under the control of the GAL1 minimal promoter. These transformed cells were cultured in medium containing galactose. The activities of β -galactosidase were measured in cell extracts and plotted as a function of the K_d value of Zif268 for its binding site variants.

Table 2 Requirement of DNA-contacting residues for transcriptional repressor activity of Zif268

HEK-293 cells were transiently transfected with a reporter plasmid containing promoter with wild-type binding site GCGG, an activator plasmid encoding GAL4–VP16 and the effector plasmid encoding either wild-type Zif268 or the indicated mutant Zif268 in which each of three base-contacting residues (arginine, aspartate, and arginine at positions –1, 2 and 6 respectively) of the recognition helix of finger 1 was replaced by alanine. Results are means $\pm$ S.D.

Reporter	Zinc-finger protein	Fold repression
GCGG	Zif268	18.3 ± 3.3
	Zif268(RDEA)	1.1 ± 0.1
	Zif268(RAER)	4.1 ± 0.6
	Zif268(ADER)	2.8 ± 0.4

each of the base-contacting residues contributes to the efficient repression of transcription of the target gene by a zinc-finger protein, a series of mutants of Zif268, in which each of the three base-contacting residues (arginine, aspartate and arginine at positions –1, 2 and 6 respectively) of finger 1 was replaced by alanine, was constructed, and then their functional activities were tested using the reporter with the wild-type binding site.

As shown in Table 2, mutant protein Zif268(RDEA), in which the arginine residue at position 6 was replaced by alanine, gave remarkably less (1.1-fold) repression than wild-type Zif268 on a promoter with the wild-type binding site. Other mutant proteins Zif268(ADER) and Zif268(RAER), in which arginine and aspartate at positions –1 and 2 respectively were mutated to alanine, showed much weaker, 2.8-fold and 4.1-fold, repression of VP16-activated transcription respectively. These data suggest that each of three base-contacting residues may play an important role in the efficient repression of transcription by Zif268.

To evaluate whether each of these base-contacting residues makes a contribution to the overall binding affinity of Zif268, the apparent K_d value of mutants for a wild-type binding site was determined (Table 1). Results obtained by comparing the apparent

K_d value of wild-type Zif268 with that of its mutants indicate that each of three base-contacting amino acid residues of finger 1 appears to make considerable contribution to overall binding affinity for Zif268 protein. The relative contribution of each residue follows the order arginine 6 > arginine -1 > aspartate 2 of finger 1 (Table 1). Thus, when arginine at position 6 was replaced by alanine, mutant protein Zif268(RDEA) bound with an apparent K_d of 1.7 nM, and thus it bound 93.6-fold less tightly than wild-type Zif268. The mutant protein Zif268(ADER), in which arginine at position -1 was mutated to alanine, bound 19.7-fold less tightly than wild-type protein. In contrast, substitution of alanine for aspartate at position 2 resulted in only slight (2.2-fold) loss of binding affinity. Taken together, these results imply that each base-contacting residue in the recognition helix of zinc-finger proteins makes a significant contribution to both overall binding affinity and functional activity of these proteins to repress transcription of target gene *in vivo*.

In vivo and *in vitro* activities of novel zinc-finger proteins selected via phage display

To date, many research groups have developed novel zinc-finger proteins for a given predetermined binding site using various design and/or selection methods [4–7]. However, there has been little information reported on the relationship between *in vivo* and *in vitro* activities of such novel zinc-finger proteins. Since the data presented above demonstrated a good correlation between *in vivo* and *in vitro* activities of Zif268, it therefore was important to ask whether this relationship might also be applied to the engineered zinc-finger proteins. To address this, the novel zinc-finger proteins DSNR, RADR and QGSR were employed, which were selected for the Zif268-binding site variants GACC (5'-GCGTGGGACC-3'), GCAC (5'-GCGTGGGCAC-3') and GCAC (5'-GCGTGGGCAC-3') respectively using phage display with a library with the randomized positions (-1, 2, 3 and 6) of finger 1 of Zif268 [5].

When the transcriptional repression assay described above was used to examine *in vivo* activities of these zinc-finger proteins in human cells, DSNR and RADR proteins gave significant fold repression (52- and 27-fold respectively) of activated transcription from a promoter containing their respective binding sites, GACC and GCAC, near the TATA box (Figure 3A). In contrast, the QGSR protein gave slightly weaker 8.4-fold repression of a promoter containing its binding site GCAC. By examining *in vivo* specificity of these proteins using a promoter with each of three binding sites (GACC, GCAC and GCGC), it showed that these proteins could efficiently discriminate against the closely related sequences (although RADR protein could not efficiently discriminate between an A and a G residue at position 9). Next, when an apparent K_d value for each of three binding sites was determined (Table 1) and the relationship between *in vivo* activity and *in vitro* binding affinity in these proteins was evaluated (Figure 3B), there appeared to be a good correlation between *in vivo* activity and *in vitro* DNA-binding affinity for each of the engineered zinc-finger proteins, although there were differences among zinc-finger proteins in terms of the strength of this relationship. This difference did not appear to result from difference in the amount of protein expression in human cells since these proteins were expressed at comparable levels (results not shown). Overall, however, the data suggest that the relationship between functional activity and binding affinity of zinc-finger proteins may be generally applied to an engineered zinc finger with novel specificity.

In conclusion, the present study demonstrates that: (i) zinc-finger proteins have an ability to discriminate against closely related sequences and thus efficiently specify each base of their tar-

Figure 3 Correlation between functional and binding activities of novel zinc-finger proteins

(A) Transcriptional repression by novel zinc-finger proteins selected using phage display. HEK-293 cells were transiently transfected with a reporter plasmid containing the indicated binding site, an activator plasmid encoding GAL4-VP16 and the indicated effector plasmid encoding a novel zinc-finger protein that was selected by phage display. (B) Correlation of transcriptional repressor activities with the DNA-binding affinities of novel zinc-finger proteins.

get sites *in vivo*; (ii) this ability originates mainly from the relative affinity of zinc-finger proteins towards their closely related target sites; (iii) each of the base-contacting residues of a zinc-finger protein is significant for providing the overall energy to DNA binding and remarkably contributes to its ability to regulate transcription of target gene *in vivo*; and (iv) the *in vivo* functional activity of a zinc-finger protein as either a transcriptional repressor or activator is positively correlated with its DNA-binding affinity, a finding which can be extended to engineered zinc-finger proteins selected by phage display. These studies clarify several important questions regarding the *in vivo* and *in vitro* activities of zinc-finger proteins, and will contribute to future development of these transcription factors as potent tools for controlling specific target gene expression in the post-genomic era.

I thank Chongsuk Ryou (University of Kentucky, Lexington, KY, U.S.A.) and Lisa Choy (University of California San Francisco, San Francisco, CA, U.S.A.) for critical reading of the manuscript. I also thank Jin Soo Kim (Seoul National University, Seoul, South Korea) and Rik Derynck (University of California San Francisco) for support and encouragement. I am a recipient of a Scientist Development Award from the National American Heart Association.

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Received 2 November 2006/27 November 2006; accepted 18 December 2006

Published as BJ Immediate Publication 18 December 2006, doi:10.1042/BJ20061644