Alteration of zif268 zinc-finger motifs gives rise to non-native zinc-co-ordination sites but preserves wild-type DNA recognition

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Zinc fingers are among the major structural motifs found in proteins that are involved in eukaryotic gene regulation. Many of these zinc-finger domains are involved in DNA binding. This study investigated whether the zinc-co-ordinating $(Cys)_2(His)_2$ motif found in the three zinc fingers of zif268 could be replaced by a $(Cys)_4$ motif while still preserving DNA recognition. $(Cys)_2(His)_2$-to-$(Cys)_4$ mutations were generated in each of the three zinc fingers of zif268 individually, as well as in fingers 1 and 3, and fingers 2 and 3 together. Whereas finger 1 and finger 3 tolerate the switch, such an alteration in finger 2 renders the polypeptide incapable of DNA recognition. The protein–DNA interaction was examined in greater detail by using a methylation-interference assay. The mutant polypeptides containing the $(Cys)_4$ motif in fingers 1 or 3 recognize DNA in a manner identical to the wild-type protein, suggesting that the $(Cys)_4$ motif appears to give rise to a properly folded finger. Additional results indicate that a zif268 variant containing a $(Cys)_2(His)(Ala)$ arrangement in finger 1 is also capable of DNA recognition in a manner identical to the wild-type polypeptide. This appears to be the first time that such alterations, in the context of an intact DNA-binding domain, have still allowed for specific DNA recognition. Taken together, the work presented here enhances our understanding of the relationship between metal ligation and DNA-binding by zinc fingers.

INTRODUCTION

The zinc finger is among the major structural motifs involved in eukaryotic protein–nucleic acid interactions [1]. Proteins with zinc-finger domains are involved in many aspects of eukaryotic gene regulation. To date, an ever-growing portion of the human genome has been found to encode zinc-finger-containing proteins, including proteins having the 'classical' zinc finger, such as that in *Xenopus laevis* TFIIIA, the steroid hormone receptors, the more-recently discovered GAL4 family of proteins, LIM-domain proteins and RING-finger proteins. The number of transcription factors reported to contain a zinc-finger motif has increased rapidly over the last decade. It has been estimated that those proteins that make up the classical zinc-finger family alone may constitute up to 1 % of all human gene products [2].

As our knowledge of zinc fingers increases, many interesting aspects of zinc-finger biochemistry are beginning to emerge. First, it is now becoming clear that certain transition metals, some of which are known to be toxic, can replace zinc and can also function as structural centres in certain zinc fingers [3–5]. This could lead to aberrant DNA binding by these zinc-finger proteins and could potentially have profound effects on gene regulation. Secondly, it is now clear that zinc finger–DNA interactions are extremely specific and can even involve co-operative protein interactions. This is especially true in the case of the oestrogen receptor [6]. Thirdly, an interesting question arises when one considers why such a variety of Zn-ligating motifs (various arrangements of four amino acids that are responsible for tetrahedral zinc ligation in zinc fingers) have evolved as structural centres in zinc-finger proteins.

The TFIIIA-like group of zinc-finger proteins includes a subset that binds to relatively guanine-rich binding sites. This subset includes TFIIIA, Sp1, the zif268/NGF-IA/Krox-20,24/Egr-1,2/Wilm tumour family and yeast ADR1, all of which utilize the $(Cys)_2(His)_2$ metal-binding motif. The crystallographic studies of a polypeptide containing the three zinc-finger DNA-binding domain (DBD) of zif268, a murine DNA-binding protein, bound to its consensus DNA site have provided the most detailed structural information to date concerning 'classical' zinc-finger protein–DNA interactions [7]. Zif268 is a 533 amino acid protein that contains three tandem $(Cys)_2(His)_2$ zinc fingers [8]. The cDNA for zif268 was cloned from mouse 3T3 fibroblast cells, and is also known as Egr-1, Krox-24 and NGF-IA. The functional aspects of zif268 have been studied little thus far, although it has been shown that this protein is capable of activating an Epstein–Barr virus immediate-early promoter [9]. Furthermore, zif268 has been demonstrated to have a possible role in regulating synapsin II gene expression [10].

It is now apparent that there is a degree of flexibility in terms of the transition metals that can be ligated by zinc-finger motifs [3–5]. But why do different zinc-finger motifs use differing combinations of Cys and His residues to co-ordinate zinc in these proteins? At first glance, all of the motifs, whether $(Cys)_2(His)_2$, $(Cys)_4$, $(Cys)_6(Zn)_2$ or $(Cys)_3(His)$, seem to serve the identical function of tetrahedral co-ordination of zinc and subsequent maintenance of a global tertiary structure. Therefore, it is interesting to ask why distinct zinc-co-ordinating motifs have evolved in zinc-finger proteins. Perhaps subtle differences in secondary structure in the area immediately adjacent to zinc ligation are possible only through the use of different combinations of Cys and His residues. Conversely, it may be that required electrostatic interactions between zinc-ligating residues and other regions of the protein or the DNA itself, for a given zinc-finger protein, are only achievable through the use of various zinc-ligation arrangements. Research addressing this question, specifically in the case of the well-defined zinc-finger proteins, is

Abbreviations used: GST, glutathione S-transferase; MSA, mobility-shift assay; Zn-ligating motif, various arrangements of four amino acids that are responsible for tetrahedral zinc ligation in zinc fingers; DBD, DNA-binding domain.

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lacking. The observation that different Zn-ligating motifs all coordinate this metal ion with tetrahedral geometry suggests that Cys and His residues might be functionally interchangeable in proteins of different zinc-finger classes. Alternatively, it may be that the diversity of Zn-ligating motifs exists to provide a corresponding diversity of protein conformations in and around the site of metal ligation; this in turn would allow for specific protein–protein or protein–DNA interactions.

In the present study we have demonstrated that these motifs were interchangeable between at least two zinc-finger proteins that utilized different arrangements of Zn-ligating Cys and His residues. An additional mutation, in which finger 1 was mutated to a $(Cys)_2(His)(Ala)$ arrangement, gave rise to an altered finger that also recognizes its DNA site in a manner identical to the native finger. This appears to be the first time that such alterations have been made to a zinc-finger DBD such that a wild-type interaction with DNA is maintained.

EXPERIMENTAL

Media, enzymes and reagents

Bacterial cultures were grown in Luria broth containing ampicillin at a concentration of 100 μg/ml. Techniques involving DNA manipulation have been described [11]. Restriction endonucleases were supplied by Pharmacia, New England Biolabs, Bethesda Research Laboratories and Boehringer-Mannheim. Radiolabelled nucleotides were supplied by Amersham and Dupont-New England Nuclear; unlabelled nucleotides were supplied by Boehringer-Mannheim. The plasmid containing the zif268 cDNA was obtained from the American Type Culture Collection. The glutathione S-transferase (GST) Protein Expression System from Pharmacia was used to produce all native and mutant polypeptides. The U.S.E. Mutagenesis kit from Pharmacia was used for the generation of all mutant zif268 DNA constructs. Metal salts were supplied by Fisher Scientific Company.

Subcloning of the zif268 DBD

The portion of zif268 cDNA (contained in the vector pBSZif) encoding the three zinc-finger DBD (nucleotides 1287–1558, amino acids 331–422) was amplified by PCR and subcloned into the pCR II plasmid, taking advantage of the adenosine overhangs, which are incorporated into the PCR product by *Taq* polymerase, and the thymidine overhangs present in the pCR II plasmid. The PCR primers used for amplification of this portion of zif268 had the following sequence: (upstream primer) 5′-CCCATGAACGCCCATATGCTTGCC-3′; (downstream

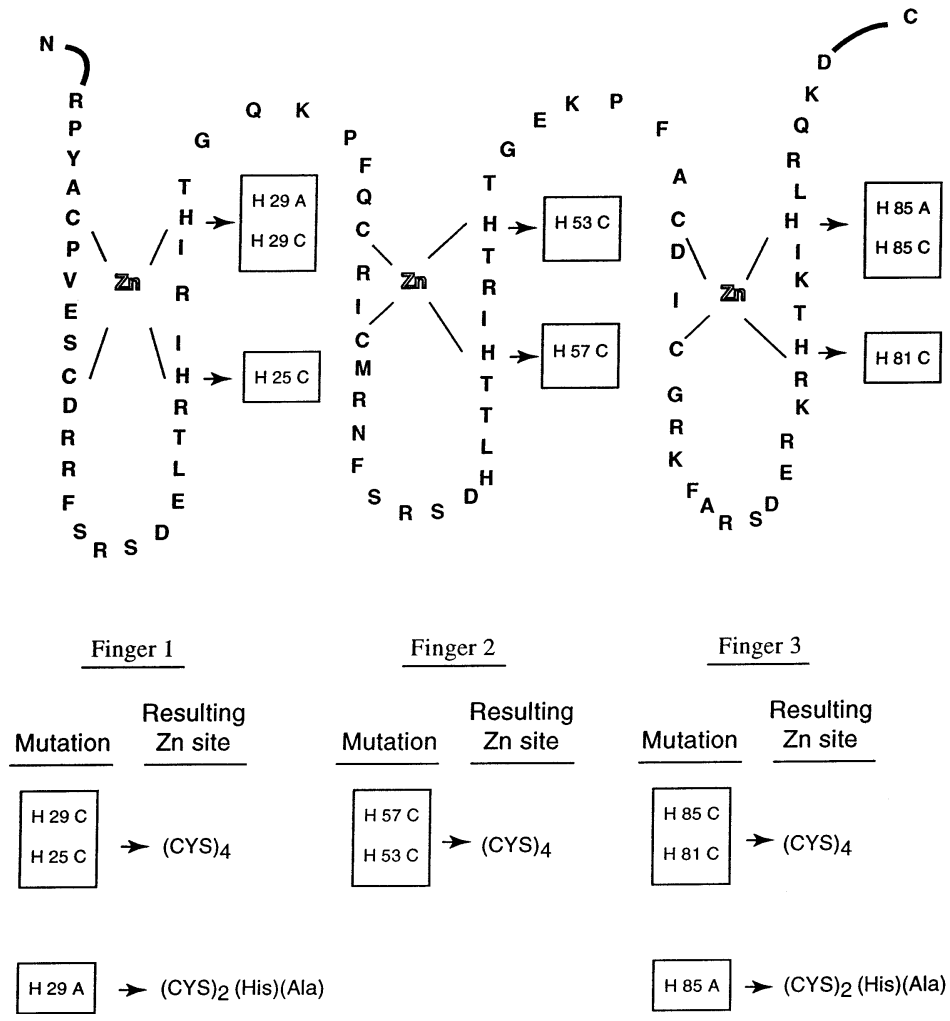

Figure 1 A schematic outline depicting the mutations made in the zif268 DBD to generate the $(Cys)_2(His)_2$-to-$(Cys)_4$ and -$(Cys)_2(His)(Ala)$ transitions

primer) 5′-GTCCTTCTGTCTTAAATGGATTTT-3′. The PCR product was then excised from the pCR II vector by *Eco*RI digestion and subcloned into the *Eco*RI site of the Pharmacia GST fusion-protein expression vector pGEX-3X. The resultant construct, denoted pGEX-zifDBD, was verified by di-deoxy sequencing.

Expression and purification of the GST-zif268 DBD fusion protein

The *Escherichia coli* bacterial strain BL21(DE3) was transformed with the plasmid pGEX-zifDBD. Cultures were grown at 37 °C to logarithmic phase ($A_{600} = 0.7-0.9$) and induced with 0.1 mM isopropyl β-D-thiogalactopyranoside for 4 h. The cells were then collected, resuspended in 1/20 of the volume of the original cell culture with PBS [140 mM NaCl/2.7 mM KCl/10 mM Na_2HPO_4/1.8 mM KH_2PO_4 (pH 7.3)], containing 1 mM PMSF and 1 % Triton X-100, and followed with cell lysis by sonication. The suspension was centrifuged (60000 ***g***, 45 min, 4 °C) and the supernatant was passed over a glutathione-Sepharose affinity column (equilibrated with PBS buffer), which accompanied the GST Expression System. The column was washed thoroughly with PBS and then re-equilibrated with Factor Xa cleavage buffer [50 mM Tris/HCl (pH 7.5)/150 mM NaCl/1 mM $CaCl_2$], followed by cleavage of the zif268 DBD from the GST moiety directly on the column with Factor Xa (50 mg/ml glutathione-Sepharose bed volume, 16 h, room temperature). The concentration of protein was determined by using the BioRad Bradford protein assay, and the purity of the cleaved zif268 DBD was assessed by SDS/PAGE.

Mobility-shift assays (MSAs)

Specific binding of the zif268 DBD to DNA was measured using a gel MSA. A ^{32}P-labelled double-stranded oligonucleotide that contained the zif268 DNA-binding site was obtained by annealing the complementary oligonucleotides after their 5′ ends had been phosphorylated in the presence of [γ^{32}P]ATP and T4 polynucleotide kinase. The oligonucleotide used was 5′-AGCTTT-GTGATCA**GCGTGGGCG**TAACTGACC-3′ (shown with consensus binding site in bold) and it was in duplex form. Approximately 250 ng of DBD was preincubated with the labelled oligonucleotide for 30 min at room temperature in 30 μl of sample running buffer [20 mM Hepes (pH 7.9)/100 mM NaCl/4 % (v/v) glycerol/1 mM dithiothreitol] containing 1.0 μg of non-specific DNA [poly(dI.dC).poly(dI.dC)]. This mixture (15 μl) was then loaded on a 5.4 % gel that had been prerun for 1.5 h at 100 V. The gel and running buffer was 70 mM Tris/HCl (pH 7.9), 30 mM NaOAc, 10 mM EDTA and 2.5 % (v/v) glycerol. The running buffer was circulated between chambers. The dried gel was exposed to X-ray film (16 h, −70 °C with intensifying screen). For gel-MSA involving the various zif268 polypeptides containing mutated zinc-binding sites, equivalent amounts of wild-type and mutated polypeptides (as determined by the BioRad Bradford protein assay) were used in the binding reactions prior to the gel-MSA.

Conversion of the $(Cys)_2(His)_2$ Zn-ligating motifs of zif268 into $(Cys)_4$ and $(Cys)_2(His)(Ala)$

Mutagenesis of the $(Cys)_2(His)_2$ motif of the individual zinc fingers of the zif268 DBD zinc fingers to a $(Cys)_4$ motif was performed using the Pharmacia U.S.E. Mutagenesis kit. Briefly, the pGEX-zifDBD construct described above was used as a template from which multiple rounds of mutagenesis could be performed in order to make the desired mutant polypeptides. Each of the three fingers was mutated individually, as were certain double mutants in which both fingers 1 and 3, or fingers 2 and 3, were altered. In addition, Ala mutants of fingers 1 and 3 of the zif268 DBD were constructed using this method, giving rise to $(Cys)_2(His)(Ala)$ motifs. Mutation-positive clones were verified by di-deoxy DNA sequencing. The positioning of the mutations made to the zif268 DBD is summarized in Figure 1. The mutant zif268 DBD polypeptides were purified in the same manner as the wild-type zif268 DBD.

Methylation-interference assays

Methylation-interference assays were performed essentially as described in [12], using the same ^{32}P-labelled double-stranded oligonucleotide, which contained the zif268 DNA-binding site (described above). The DNA (the 5′ end ^{32}P-labelled on the strand containing the consensus 5′-GCGTGGGCG-3′ binding site, 1×10^6 cpm) was G-methylated by exposure to 0.5 % (v/v) dimethyl sulphate for 10 min at room temperature. The reaction was stopped by the addition of 1/10 volume of stop buffer [10 mM Tris/HCl/1 mM EDTA/5 mM β-mercaptoethanol (pH 8.0)]. Wild-type zif268 DBD and the mutated derivatives (500 ng) were preincubated with the methylated DNA, and bound and free DNA was separated by gel-MSA. The wet gel was exposed to X-ray film to identify the bands. The bound and free DNA for each polypeptide was eluted from the gel by band excision, followed by utilization of the 'crush and soak' method, in which the gel pieces were incubated in 400 ml of elution buffer [0.5 M NH_4OAc/10 mM MgOAc/0.1 % (w/v) SDS] for 12 h at 37 °C. Two phenol/chloroform extractions were performed, and the DNA was precipitated with ethanol. This DNA was then treated with 1 M piperidine for 30 min at 90 °C and subsequently lyophilized. The lyophilization procedure was repeated three more times. Equivalent cpm of free and bound DNA were electrophoresed on a 20 % acrylamide denaturing gel at 2400 V for 2.5 h. The gel was dried and exposed to X-ray film overnight at −70 °C with an intensifying screen.

RESULTS

Construction, expression and purification of $(Cys)_2(His)_2$-to-$(Cys)_4$ and $(Cys)_2(His)_2$-to-$(Cys)_2(His)(Ala)$ mutated zif268 DBD zinc-finger polypeptides

Several mutated forms of the zif268 DBD polypeptide were generated in order to test the ability of a $(Cys)_4$ Zn-ligating motif to substitute for the native $(Cys)_2(His)_2$ motif. We used site-directed mutagenesis of the pGEX-zif DBD vector to obtain forms of the zif268 DBD with a $(Cys)_4$ motif in the first, second and third fingers individually, and in the first and third, and second and third fingers together. We also constructed mutants in which finger 1 or finger 3 was disrupted by the incorporation of an Ala residue in place of the second zinc-ligating His residue. Expression and purification of these mutant polypeptides were carried out in a manner identical to that of the wild-type zif268 DBD.

Assessment of the ability of $(Cys)_2(His)_2$-to-$(Cys)_4$ mutant zif268 DBD polypeptides to bind to the zif268 consensus DNA site

In order to test the ability of the mutated zif268 polypeptides to bind DNA, gel-MSA was employed. This analysis demonstrated that the mutated zif268 DBD polypeptides, in which the first (most N-terminal) and third (most C-terminal) fingers were mutated separately from the $(Cys)_2(His)_2$ motif into the $(Cys)_4$ motif, were capable of binding to the zif268 consensus DNA site

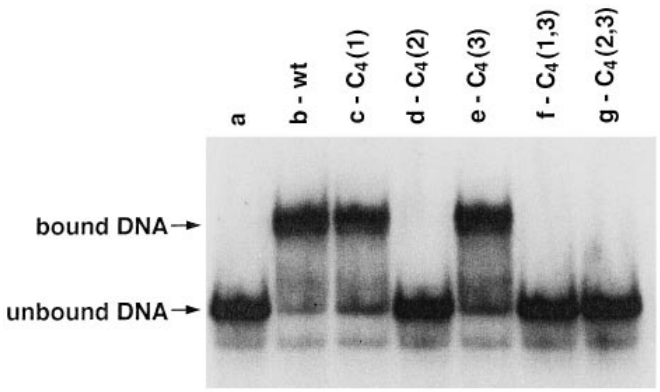

Figure 2 DNA-binding analysis of mutant zif268 polypeptides containing $(Cys)_2(His)_2$-to-$(Cys)_4$ mutations: individual fingers and combinations of fingers

Each zinc finger of zif268 was subjected to mutagenesis such that the Zn-ligating motif was altered from $(Cys)_2(His)_2$ to $(Cys)_4$. Additionally, polypeptides with $(Cys)_4$ motifs in both fingers 1 and 3, and both fingers 2 and 3, were studied, as described in the Experimental section. Gel-MSA analysis was used to test the ability of these mutant forms of the DBD of zif268 to bind to a ^{32}P-labelled oligonucleotide containing the zif268 consensus site. (a) No protein, (b) wild-type zif268 DBD, (c) polypeptides with a $(Cys)_4$ motif in finger 1, (d) finger 2, (e) finger 3 ,(f) both fingers 1 and 3, and (g) both fingers 2 and 3. Approx. 1 μg of each polypeptide was added to the binding reactions that preceded the gel-MSA.

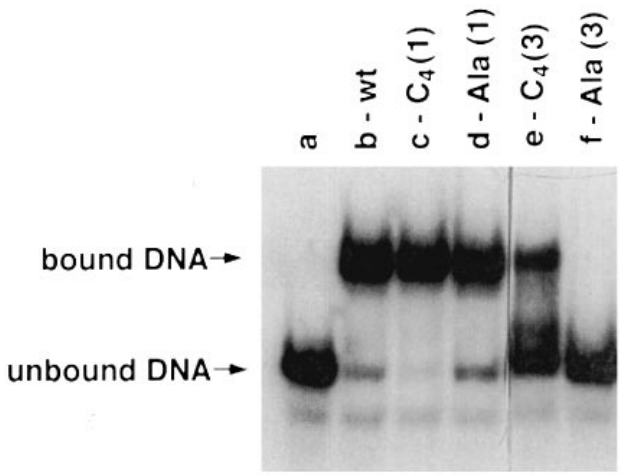

Figure 3 DNA-binding analysis of mutant zif268 polypeptides containing $(Cys)_2(His)_2$-to-$(Cys)_2(His)(Ala)$ mutations

Fingers 1 and 3 of zif268 were subjected to mutagenesis such that the Zn-ligating motif was altered from $(Cys)_2(His)_2$ to $(Cys)_2(His)(Ala)$, as described in the Experimental section. Gel-MSA analysis was then employed to determine the effect such alterations have on the DNA-binding activity of these mutant polypeptides. (a) No protein, (b) wild-type zif268 DBD, (c) finger 1 $(Cys)_4$ polypeptide, (d) finger 1 $(Cys)_2(His)(Ala)$ polypeptide, (e) finger 3 $(Cys)_4$ polypeptide, and (f) finger 3 $(Cys)_2(His)(Ala)$ polypeptide. Approx. 1 μg of each polypeptide was added to the binding reactions that preceded the gel-MSA.

(Figure 2, lanes c and e). Polypeptides with the same mutation in the second finger, or in both fingers 1 and 3, or fingers 2 and 3, were incapable of binding to the zif268 consensus DNA site (Figure 2, lanes d, f, and g). This suggested that co-ordination of zinc by $(Cys)_4$ in finger 1 or finger 3, but not in finger 2 or in combinations of fingers 1 and 3 or fingers 2 and 3, led to a properly folded polypeptide that could interact specifically with DNA. The possibility that finger 1 or finger 3 is not required for

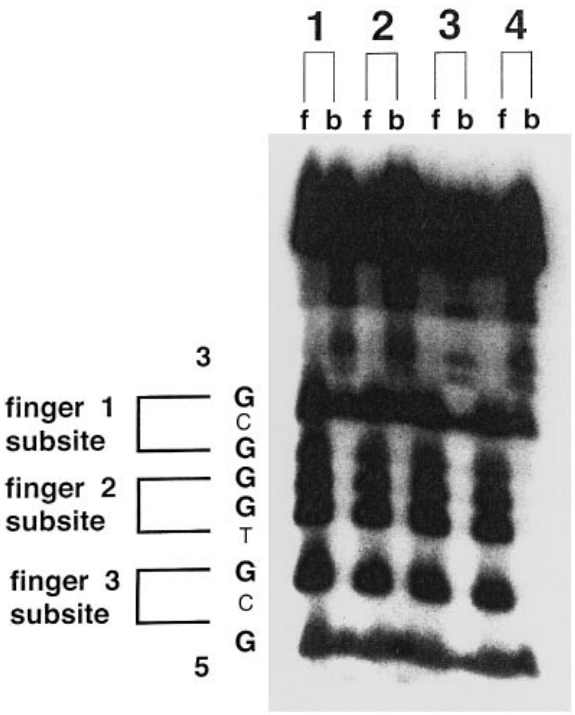

Figure 4 Methylation-interference assay of zif268 Zn-ligating-motif mutants

Zif268 DBD variants which retained DNA-binding activity were assayed to characterize any differences in specific protein–DNA base interactions that arose due to alteration of the Zn-ligating motif, as described in the Experimental section. Abbreviation used: f, DNA that was not selected by zif268 DBD variants for binding; b, DNA that was selected by zif268 DBD variants for binding. (1) Wild-type zif268 DBD, (2) finger 1 $(Cys)_4$ polypeptide, (3) finger 1 $(Cys)_2(His)(Ala)$ polypeptide, and (4) finger 3 $(Cys)_4$ polypeptide. The bases that constitute the zif268 consensus site are highlighted to the left of the figure, as is the orientation of this site. Also shown are the subsites with which each finger of zif268 specifically interacts [7].

binding to DNA, in which case the presence of the $(Cys)_4$ motif in either one of these fingers would be inconsequential, was next examined by the generation of disruptive Ala mutations in finger 1 and finger 3.

Assessment of the ability of $(Cys)_2(His)(Ala)$ zif268 DBD polypeptides to bind to the zif268 consensus DNA site

In order to determine the effect that disruption of zinc ligation in finger 1 and finger 3 of the zif268 DBD would have on the ability of these mutant polypeptides to bind to the zif268 consensus DNA site, the second zinc-ligating His residues of fingers 1 and 3 were separately mutated to Ala. The resultant $(Cys)_2(His)(Ala)$ motif was assumed to not provide the correct geometry for a wild-type zinc finger [12]. The $(Cys)_2(His)(Ala)$ motif allowed for DNA binding by the mutated polypeptide when incorporated into finger 1 (Figure 3, lane d), but not when incorporated into finger 3 (Figure 3, lane f). These results suggest that the $(Cys)_4$ motif was functional in finger 3, since disruption of this finger by incorporation of the Ala residue abolishes DNA binding.

Methylation-interference assay of mutant zif268 DBD polypeptides that are capable of binding to DNA

In order to probe in more detail the ability of the finger 1 $(Cys)_4$ polypeptide, the finger 3 $(Cys)_4$ polypeptide and the finger 1

$(Cys)_2(His)(Ala)$ polypeptide to interact with DNA, we employed a methylation-interference assay. This assay is capable of pinpointing specific bases in a DNA site that are necessary for protein–DNA interactions. Thus it was possible to determine if the $(Cys)_4$ motif (in finger 1 and finger 3) or the $(Cys)_2(His)(Ala)$ motif in finger 1 still allowed for finger interactions with the guanosine bases of the DNA site, which are known to be necessary for zif268 binding to DNA [6]. As such interactions with DNA bases require a specific tertiary structure of the finger that is stabilized by the ligation of zinc, this assay would indirectly determine if the mutated fingers retained the ability to fold properly and subsequently recognize DNA. Guanosine residues which hydrogen-bond with the polypeptide, and whose methylation interferes with this interaction, are detected by differences between 'free' and 'bound' lanes for each polypeptide; a reduced band signal appearing in the 'bound' lane as compared with the corresponding band in the 'free' lane indicates a guanosine residue involved in interactions with the protein. As shown in Figure 4, there appears to be no significant difference between the various mutant polypeptides in terms of which guanosine residues are taking part in polypeptide binding. The mutant $(Cys)_4$ finger 1 polypeptide, $(Cys)_2(His)(Ala)$ finger 1 polypeptide and the $(Cys)_4$ finger 3 polypeptide appear to interact with specific guanosine residues in the DNA site in a manner that is identical with that of the wild-type polypeptide. This indicates indirectly that these mutations still allowed for the proper folding of their corresponding fingers, such that interactions with DNA were maintained, and indicates subsequently that this correct folding was most likely made possible through zinc ligation.

DISCUSSION

To address the question of interchangeability between various zinc-finger metal-ligating motifs, we looked at the consequences on DNA binding of sequentially replacing each of the $(Cys)_2(His)_2$ motifs found in zif268 with the $(Cys)_4$ motif found in the zinc fingers of the steroid hormone receptors (as well as GATA-1 and related proteins). The conversion of all three $(Cys)_2(His)_2$ Zn-ligating motifs of zif268 into the $(Cys)_4$ motif indicates that such a conversion is not possible across all three zinc fingers while still maintaining the ability of the polypeptide to bind DNA. Whereas fingers 1 and 3 of zif268 could undergo this conversion and still allow for the binding of the polypeptide to DNA, DNA-binding was abolished when finger 2 underwent this change of motif. Furthermore, attempts to convert more than one finger at a time (i.e. fingers 1 and 3 or fingers 2 and 3 simultaneously) resulted in a polypeptide that was incapable of recognizing the consensus zif268 DNA binding site. Such a conversion of the zinc-binding motif of finger 2 most probably results in an intolerable disruption of the secondary and tertiary protein structure in this region of the zif268 DBD, which is crucial for DNA recognition. There are two possible outcomes that could arise, in terms of zinc ligation, with the introduction of the new $(Cys)_4$ motif in the zif268 zinc fingers. First, the new site might be incapable of ligating zinc simply due to the unfavourable geometric constraints imposed on the new motif in the context of the existing primary structure of the zif268 zinc fingers. Indeed, those zinc fingers that normally utilize the $(Cys)_4$ motif generally have fewer intervening residues between the N-terminal pair of zinc-ligating Cys residues than those zinc fingers that utilize His residues in this portion of a zinc-finger motif. Thus it is possible that the smaller Cys residues are not capable of overcoming the larger size of the zinc-co-ordination sphere encountered in the zif268 zinc fingers. This would presumably result in a site with little ability to co-ordinate zinc. Alternatively, it is possible that the new site might still be capable of zinc ligation, but such ligation is only possible through alterations of the immediate secondary and tertiary structure in the area surrounding metal ligation. This was the case in experiments where His-to-Cys mutations in a consensus zinc-finger peptide still allowed for metal ligation but caused structural distortions around the metal centre [13,14]. Such modifications could be detrimental to the specific finger structure necessary for DNA recognition. It is possible that similar structural distortions are generated by the $(Cys)_4$ modification in finger 2 of zif268, giving rise to altered side-chain spatial orientations and perhaps more importantly to changes in the relative positioning of the neighbouring fingers, which would preclude DNA-binding.

The methylation-interference assay indicated that both the finger 1 $(Cys)_4$ polypeptide and the finger 3 $(Cys)_4$ polypeptide were interacting with their DNA subsites in a manner essentially identical to the wild-type polypeptide. This most probably rules out the possibility that the $(Cys)_4$ finger 1 and finger 3 mutant polypeptides were binding DNA due to the remaining two wild-type fingers compensating for the mutations by providing essentially all of the DNA-binding affinity. Taken together, these results indicate that the mutant $(Cys)_4$ finger 1 and finger 3 polypeptides are indeed folding properly and allowing for DNA recognition. If the $(Cys)_4$ mutations in finger 1 and finger 3 are resulting in proper folding and allowing for DNA recognition, then this appears to be the first instance where such an alteration of Zn-ligating motifs (in the context of an intact zinc-finger DBD) has retained specific DNA recognition.

In parallel with the methylation-interference assays, further experiments were conducted in an effort to determine if the mutant $(Cys)_4$ finger 1 and finger 3 polypeptides were binding DNA due to the two wild-type fingers compensating for these mutations by providing the necessary DNA-binding affinity. Two additional mutant polypeptides were generated in which the second zinc-ligating His residue of fingers 1 and 3 were switched separately to Ala. The results of such Ala substitutions in the zif268 DBD fingers 1 and 3 were interesting, in that these fingers responded differently to this mutation in terms of polypeptide binding to DNA. This mutation in finger 3, which tolerated the $(Cys)_4$ motif, abolished DNA binding. For finger 3, these results provide further evidence that the $(Cys)_4$ motif was functioning in terms of providing for the proper folding of finger 3 and allowing for DNA-binding by the polypeptide. In addition, it appears that a properly folded, zinc-ligating finger 3 is necessary for overall DNA recognition by the polypeptide. The results of the switch to the $(Cys)_2(His)(Ala)$ motif in finger 1 were even more interesting. Not only did the presence of the $(Cys)_4$ motif not preclude DNA-binding, but neither did the Ala mutation in the second zinc-ligating His residue of this finger. A methylation-interference assay of the $(Cys)_2(His)(Ala)$ finger 1 mutant polypeptide indicated that it was interacting with its DNA subsite in a manner essentially identical to that of the wild-type polypeptide as well as the mutant $(Cys)_4$ finger 1 polypeptide. This suggests that it almost certainly has the correct folding arrangement for such an interaction. The most likely explanation for the ligation of zinc in this case is that tetrahedral co-ordination of zinc is occurring through the use of the remaining Cys and His residues, with the fourth ligand being supplied exogenously, perhaps by H_2O. Studies by Cook et al. [15] on mutations in the zinc-finger region of yeast ADR1 resulted in the isolation of a mutant *ADR1* allele that resulted in the second zinc-ligating His residue of the C-terminal zinc finger being replaced by Tyr. The authors postulated that in the case of this particular mutant ADR1, three zinc chelators are minimally sufficient to bind zinc and maintain the finger in an active form. It is possible that the Ala mutation incorporated into finger 1 of zif268 is also minimally sufficient to

allow for the chelation of zinc by the remaining two Cys residues and the His residue.

The results obtained here regarding the mutagenesis of the individual zinc fingers of zif268 seem to indicate that all three fingers behave differently in terms of tolerating both the new $(Cys)_4$ motif and the Ala mutation. This is consistent with a number of studies, which demonstrate that identical mutations in sequential zinc fingers of a given zinc-finger protein produce different effects in subsequent assays. For example, Thukral et al. [16] used Ala-scanning mutagenesis to identify important amino acids in the two $(Cys)_2(His)_2$ zinc fingers of transcription factor ADR1. Most notably, these authors observed that the severity of the effect of Ala substitution on DNA binding varied greatly at equivalent positions in the fingers. Similarly, studies on TFIIIA have shown that the three most N-terminal zinc fingers of this protein do not contribute equally to the overall DNA-binding energy [17].

We believe the work presented here will contribute to our knowledge of the intricate relationship between the amino acids and metals that constitute zinc-finger motifs and their interaction with specific DNA bases. This should ultimately lead to a better understanding of how these important motifs regulate the transcription of gene sequences.

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REFERENCES

1 Klug, A. and Schwabe, J. W. R. (1995) FASEB J. **9**, 597–604
2 Hoovers, J. M., Mannens, M., John, R., Bliek, J., van Heyningen, V., Porteous, D. J., Leschot, N. J., Westerveld, A. and Little, P. F. (1992) Genomics **12**, 254–263
3 Predki, P. F. and Sarkar, B. (1992) J. Biol. Chem. **267**, 5842–5846
4 Conte, D., Narindrasorasak, S. and Sarkar, B. (1996) J. Biol. Chem. **271**, 5125–5130
5 Omichinski, J. G., Trainor, C., Evans, T., Gronenborn, A. M., Clore, G. M. and Felsenfeld, G. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 1676–1680
6 Predki, P. F. and Sarkar, B. (1995) Biochem. J. **305**, 805–810
7 Pavletich, N. P. and Pabo, C. (1991) Science **252**, 809–816
8 Christy, B. A., Lau, L. F. and Nathans, D. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 7857–7861
9 Zalani, S., Holley-Guthrie, E. and Kenny, S. (1995) J. Virol. **69**, 3816–3823
10 Petersohn, D., Schoch, S., Brinkman, D. R. and Thiel, G. (1995) J. Biol. Chem. **270**, 24361–24369
11 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning, A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
12 Baldwin, A. S. (1989) in Current Protocols in Molecular Biology (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K., eds.), pp. 12.3.1–12.3.6, John Wiley, New York
13 Krizek, B. A., Amann, B. T., Kilfoil, V. J., Merkle, D. L. and Berg, J. M. (1991) J. Am. Chem. Soc. **113**, 4518–4523
14 Krizek, B. A. and Berg, J. M. (1992) Inorg. Chem. **32**, 937–940
15 Cook, W. J., Mosley, S. P., Audino, D. C., Mullaney, D. L., Rovelli, A., Stewart, G. and Denis, C. L. (1994) J. Biol. Chem. **269**, 9374–9379
16 Thukral, S. K., Morrison, M. L. and Young, E. T. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 9188–9192
17 Del Rio, S., Menezes, S. R. and Setzer, D. R. (1993) J. Mol. Biol. **233**, 567–579