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Zinc Finger Transcription Factor Zn_3-Sp1 Reactions with Cd^{2+}

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Introduction

Cadmium is recognized as a major pollutant and threat to health in industrialized countries (1,2). In humans, chronic Cd^{2+} exposure causes toxicity that targets kidney and bone (1-3). Suggestive evidence implicates other sites such as lung (4). Experimental toxicological studies have largely focused on the role of metallothionein (MT) in the protection of tissues against Cd^{2+}-induced injury (5).[1] Using a variety of approaches including investigations with mice that bear the MT null genotype or contain extra, transgenic MT, it is clear that metallothionein induction by Cd^{2+} protects against acute exposure to Cd^{2+} and probably chronic exposure as well (5,6).

Compared with the sustained interest in metallothionein as a site of reaction of Cd^{2+}, little progress has been made until recently in identifying and characterizing intracellular sites that react with Cd^{2+} and produce cell injury related to human toxicity. Moreover, few reports have attempted to make the link between functional phenotypic responses induced by Cd^{2+} and the chemical sites where it binds and initiates the biochemistry that causes these effects, admittedly a difficult challenge.

[1]Abbreviations

ChIP	chromosomal immunoprecipitation
DIG	digoxigenin
EMSA	electrophoretic mobility shift assay
GC, GC1	*SGLT1* promoter binding sequence for Zn_3-Sp1 (Figure 4)
$GC_{CONSENSUS}$	DNA binding sequence for Zn_3-Sp1 (Figure 4)
DTT	dithiothreitol
IR	proprietary infrared label for DNA (LiCor)
mF3	modified finger 3 of transcription factor IIIA
MT	metallothionein
SGLT1 and 2	sodium-glucose transporter proteins
SGLT1* or *2	sodium-glucose transporter gene for isoform 1 or 2
SGLT2-GC	Zn^3-Sp1 binding site in *SGLT2* promoter
Sp1	transcription factor name

In this context, a series of papers on the reaction of Cd^{2+} with mouse kidney cortical cells that have the properties of proximal tubule cells show that Cd^{2+} down-regulates Na^{+}-glucose cotransporter (SGLT) activity as a result of inhibition of *SGLT1* and *2* mRNA synthesis (7-9). The *SGLT 1* and *2* gene promoters contain transcription factor binding elements for HNF-1 and Sp1 that have been shown to mediate gene expression in other systems (10). Sp1 binding to such elements declines in cells exposed to Cd^{2+} and human recombinant Sp1 exposed to Cd^{2+} also loses its affinity for these cognate DNA sequences (7).

Sp1 is a Zn-finger transcription factor, that associates with DNA through three tandem Zn-fingers (11). Each finger exists as in a ββα peptide conformation that is stabilized by Zn^{2+} complexed to 2 sulfhydryl groups and 2 imidazole nitrogens (C_2H_2 coordination) (12). Studies with other Zn-finger proteins demonstrate that Cd^{2+} exposure can diminish DNA selective binding as measured by the electrophoretic mobility shift assay (EMSA) (13,14). Titration of Zn_3-Sp1 with Cd^{2+} also depresses its DNA binding capacity in a Cd^{2+} concentration dependent manner (15). These results are consistent with the operation of a reversible metal ion exchange reaction

$$3\,Cd^{2+} + Zn_3\text{–}Sp1 \rightleftharpoons Cd_3\text{–}Sp1 + 3\,Zn^{2+} \quad (1)$$

In turn, they support the hypothesis that Cd^{2+} down-regulates *SGLT1* and *2* mRNA synthesis by converting Zn_3-Sp1 to Cd_3-Sp1. The present report advances the study of reaction 1 *in vitro*.

Materials and Experimental Methods

Reagents

Unlabeled oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA), and labeled with DIG using the DIG Gel Shift Kit, 2nd Generation from Roche (Indianapolis, IN). Infrared (IR) dye labeled oligonucleotides labeled dye were synthesized by LiCor (Lincoln, Nebraska). Oct2A protein and its DNA binding oligonucleotide were provided in the DIG labeling kit. Promega (Madison, WI) was the source of recombinant human Sp1. Rabbit polyclonal antibody against Sp1 (sc-59×, 2 mg/ml) came from Santa Cruz Biotechnology (Santa Cruz, CA). Protein concentration was quantified with the DC Protein Assay (Bio-Rad Laboratories; Hercules, CA). All other reagents or chemicals were molecular biology grade and were purchased from either Thermo Fisher Scientific (Waltham, MA) or Sigma (St. Louis, MO).

Electrophoretic mobility shift assay

In the electrophoretic mobility shift assay, the DNA binding capacity of designated proteins is measured. The reaction was monitored with DNA labeled with DIG or, later, with an improved infrared (IR) dye molecule (9,15). Reactant protein (45 nM) and DNA (3.6 nM) were electrophoresed through a polyacrylamide gel with 6% cross-linking in a reaction buffer that contains 85 μM base pairs of DNA (polydI-dC) and 400 μM peptide (poly-lysine) that serve as nonspecific competitors to enhance the selectivity of the observed reaction. In the reported experiments, the reaction of recombinant human Sp1 (Zn_3-Sp1) and DIG- or IR-labeled DNA oligomer sequence from the promoter of mouse *SGLT1* (GC1) and specific for Zn_3-Sp1was assayed under different conditions. DNA and protein·DNA migration were visualized by chemiluminescence (alkaline phosphatase) or infrared (IR) detection.

The binding reaction was conducted in a standard reaction buffer that included 20 mM Hepes, pH 7.6, 10 mM $(NH_4)_2$ $S0_4$, Tween 20, 0.2% (w/v), 30 mM KCl and minor contributions from

the Zn_3-Sp1 stock solution and the reaction mixture used to synthesize DIG or IR labeled DNA (9,15). EDTA, commonly used in the reaction buffer (e.g. Promega Inc.), was deliberately omitted in order that Zn_3-Sp1 not be confronted with a powerful metal ion chelating agent during the EMSA (16). DTT was also removed from the reaction buffer, because as a vicinal dithiol, it, too, has some affinity for Cd^{2+}. All results reported here were averages ± standard error for 3 or more runs.

Commercially available EMSA kits employ EDTA as the quenching agent in the DNA labeling reaction and as a major component of the reaction mixture (0.2 mM) (16). It is also part of the electrophoresis running buffer.

Equilibrium constant for the reaction of Cd^{2+} with Zn_3-Sp1

EMSA data were used to calculate the equilibrium constant for the reaction,

$$Cd^{2+} + Zn\text{-}Sp1 \rightleftharpoons Cd\text{-}Sp1 + Zn^{2+} \quad (2)$$

This is a simplification of the full reaction 1 above based on the assumption that each Zn-finger is identical and reacts independently with Cd^{2+}. The equilibrium constant (K) is written as

$$K = [Cd\text{-}Sp1]\left[Zn^{2+}\right] / [Zn\text{-}Sp1]\left[Cd^{2+}\right] \quad (3)$$

in which $[Zn^{2+}]$ and $[Cd^{2+}]$ are the initial concentrations added into the reaction mixture, [Zn-Sp1] represents the percent of untreated control Zn-Sp1 that is quantified in the EMSA as Zn-Sp1•GC1, and [Cd-Sp1] is 100 minus this percentage.

Molecular visualization

The average NMR structures in Protein Data Bank file format of Zn mf3 and Cd mf3 were aligned using the structural alignment tool in Swiss PDB Viewer and in PyMol, two molecular visualization software suites. Comparison of the back bone and side chain alignments of the two structures was done by each software package, using root mean square deviation of backbone atoms as the criterion for best alignment. The secondary structure distribution of alpha helix and beta sheet was visualized by cartoon representation with the PyMol program.

Data analysis

Results are shown as averages ∀ standard error for 3 independent replicates of experiments.

Results

Reaction of Cd-EDTA with Zn_3-Sp1

Initially, the EMSA reactions were conducted with a standard commercial kit that contained EDTA. Cd^{2+} and Zn_3-Sp1 were incubated together, then DNA was added, and the EMSA conducted, showing a Cd^{2+} concentration dependent loss of DNA binding activity by the transcription factor in comparison with control Zn_3-Sp1 (Figure 1). The reaction of Cd^{2+} with preformed Zn_3-Sp1 DNA is discussed below.

It was subsequently recognized that the reaction and electrophoresis running buffers contain large concentrations of 200 μM EDTA (16). Under the conditions of the standard EMSA experiment in the presence of EDTA, about 50% of native Zn_3-Sp1 reacted with EDTA (Figure 1, 0 μM Cd^{2+}). As a control for the possibility of non-specific Cd^{2+}-protein reaction in this buffer, the effect of Cd^{2+} on the binding of OCT2A, a non- zinc finger transcription factor,

with its cognate DNA, OT1, was determined under the same conditions for protein and DNA. In data not shown, there was no observable change in OCT2A·OT1 formation in the presence of 0-15 μM Cd^{2+}.

Since EDTA readily competes with Sp1 for Zn^{2+}, we previously explored how EMSA experiments managed to reveal the binding of Zn_3-Sp1 to cognate DNA, despite the reaction of EDTA with Zn_3-Sp1 (16). The conclusion was that, fortuitously, the simultaneous competitive reaction of Zn_3-Sp1 with cognate DNA protects against the formation of Zn-EDTA (reactions 4 and 5) (16).

$$3\,\mathrm{EDTA}+\mathrm{Zn_3}-\mathrm{Sp1} \rightleftharpoons 3\,\mathrm{Zn}-\mathrm{EDTA}+\mathrm{Sp1} \tag{4}$$

$$\mathrm{Zn_3}-\mathrm{Sp1}+\mathrm{DNA} \rightleftharpoons \mathrm{Zn}-\mathrm{Sp1}\cdot\mathrm{DNA} \tag{5}$$

The addition of Cd^{2+} to the mixture raised the extent of protein inactivation to nearly 100% at 10 μM Cd^{2+} and appeared at least as effective in the presence of EDTA as in its absence. This result indicated that even in the presence of EDTA, Cd^{2+} exerted a significant inhibitory effect on the reaction of the protein with DNA. In another experiment, preformed Zn_3-Sp1·GC1 was reacted with Cd^{2+}. According to Figure 1, no reaction occurred, showing that the adduct was unreactive with both EDTA and Cd^{2+}.

Considering the concentrations of Cd^{2+} employed in previous experiments in comparison with 200 μM EDTA, it was evident that Cd-EDTA must have been the actual species presented to Zn_3-Sp1 in reaction 1. That Cd-EDTA, a high affinity complex, was actually reactive with Zn_3-Sp1 in a background of direct reaction of EDTA with Zn_3-Sp1 was not anticipated (reaction 6).

$$3\,\mathrm{Cd}-\mathrm{EDTA}+\mathrm{Zn_3}-\mathrm{Sp1} \rightleftharpoons \mathrm{Cd_3}-\mathrm{Sp1}+3\,\mathrm{Zn}-\mathrm{EDTA} \tag{6}$$

In order to establish the reactivity of Cd-EDTA through direct experiment, the extent of inhibition of reactions 1 and 6 by Cd^{2+} and Cd-EDTA, respectively, was compared, using standard buffer minus EDTA. According to Figure 2, Cd-EDTA caused a loss in Zn_3-Sp1 binding to GC1, with 60% reduction occurring at 0.5 μM Cd-EDTA, comparable to the concentration required with Cd^{2+} alone. That down-regulation of Zn_3-Sp1 occurred rapidly upon incubation with Cd-EDTA as well as Cd^{2+} is consistent with Cd^{2+}-Zn^{2+} exchange in each reaction (reactions 1 and 6). This result implies both that a favorable equilibrium state existed under the conditions of the reaction and that fully chelated Cd-EDTA and Zn_3-Sp1 found a facile kinetic path of reaction.

Participation of Zn^{2+} in reaction 1

A previous study demonstrated that the simultaneous exposure of Zn_3-Sp1 to Cd^{2+} and Zn^{2+} resulted in a Zn^{2+} concentration dependent increase in Zn_3-Sp1 association with DNA in comparison with the reaction in which no Zn^{2+} was added (15). The behavior was consistent with a mass action effect in reaction 1 that favored the retention of Zn_3-Sp1 at higher Zn^{2+} concentrations (Table 1). Assuming each Zn-finger was identical and reacted independently with Cd^{2+}, the equilibrium constant (K_1) for each reaction was calculated. The average ± standard error was 14 ± 3. In another experiment, in which *SGLT2-GC*, a Zn_3-Sp1 binding sequence in the *SGLT2* promoter was substituted for GC1, a similar value of 7 ± 4 was obtained.

The metal ion competition experiment was redone using sequential addition of Cd^{2+} and Zn^{2+} with an interval of 15 min between additions. According to Figure 3, no concentration of Zn^{2+} was able to reverse the negative impact of Cd^{2+}. This result suggested that in the absence of excess Zn^{2+}, reaction 1 proceeded further to form another species:

$$Cd_3-Sp1 \rightarrow Cd_{3-n}-Sp1'+nCd^{2+} \quad (7)$$

The reaction was repeated with decreasing periods between addition of Cd^{2+} and Zn^{2+}. Even 1 min reaction of Cd^{2+} with Zn_3-Sp1 was sufficient to ablate the protective effect of Zn^{2+} seen in the simultaneous reaction of Zn_3-Sp1with Cd^{2+} and Zn^{2+}. This experiment clearly demonstrated that reaction 3 was followed by a second process that rendered reaction 7 irreversible in the presence of added Zn^{2+}. The short time frame that prevented the antagonistic effect of Zn^{2+} on the reaction of Cd^{2+} with Zn_3-Sp1 demonstrated that both steps 3 and 7 take place rapidly and that Zn_3-Sp1is kinetically reactive with Cd^{2+}.

Metallothionein is recognized as a central component of the cell's capacity to cope with Cd^{2+} (5,6). It has been hypothesized that Zn-MT restores native function to proteins that have undergone Cd^{2+}-Zn^{2+} exchange.

$$Zn-MT+Cd-Protein \rightleftharpoons Zn-Protein+Cd-MT \quad K_2 \quad (8)$$

Indeed, this reaction has been observed when the Cd-substituted protein was carbonic anhydrase or another zinc-finger transcription factor, tramtrack (13,17). Nevertheless, Zn_7-MT in ratios of 2.5-45 μM protein to 45 nM Zn_3-Sp1was not able to reverse Cd^{2+} dependent (5 μM) inhibition of Zn_3-Sp1binding to DNA (data not shown). Perhaps, this is because Cd_{3-n}-Sp1 generated in reaction 7 binds Cd^{2+} strongly enough to withstand the favorable Zn-MT to Cd-MT transition in reaction 8.

Reaction of Cd^{2+} with Zn_3-Sp1·DNA Adducts

It had previously been observed that Cd^{2+} did not depress the DNA binding activity of Zn_3-Sp1 already associated with GC1 (Figure 1) or $GC_{CONSENSUS}$ DNA probes (data not shown) over a 15 min incubation period (Figure 4). This led to the hypothesis that Zn_3-Sp1·DNA adducts were unreactive with Cd^{2+} and, thus, that any intracellular reaction of Cd^{2+} with Zn_3-Sp1 must involve a pool of the transcription factor that is not bound to DNA (15).

In order to examine the latter hypothesis further, Zn_3-Sp1 was allowed to bind to GC1 or $GC_{CONSENSUS}$ (**Figure 4a** and **b**) for 15 min; then the adducts were reacted for an additional 0-60 min with 5 μM Cd^{2+}. In tandem, the same concentration of Cd^{2+} was reacted with Zn_3-Sp1. With both probes, DNA-bound Zn_3-Sp1less reactivity than the free protein with Cd^{2+}.

A kinetic analysis of the reaction of Cd^{2+} with Zn_3-Sp1·GC1 showed that after an induction period including the 15 min time point, Cd^{2+} slowly reacts with GC1-bound Zn_3-Sp1 and dissociates it from the protein·DNA complex with 50% of the reaction complete at 60 min (Figure 4a). In contrast, free Zn_3-Sp1 was much more reactive with about 60% of its GC1 binding capacity abolished at the first time point (15 min). In the comparison study using the consensus DNA binding sequence for Zn_3-Sp1, that is embedded in the GC1 sequence, exerted no impact on the stability of that Zn_3-Sp1·DNA adduct over a 60 min incubation period (Figure 4b).

The possible importance of site specific DNA interactions in this reaction was shown by comparing the sequences of GC1 and $GC_{CONSENSUS}$ (Figure 5). The latter sequence is flanked

by non-specific DNA in GC1 that might enhance the rate of reaction of Zn_3-Sp1·GC1. We modeled the reactivity of this part of the GC1 sequence with poly-dI-dC. According to **Figures** 1 and 4, GC1 inhibited the Cd^{2+}-Zn^{2+} exchange reaction. In contrast, Zn_3-Sp1·(polydI-dC) was just as susceptible to reaction with 2.5 μM Cd^{2+} as free Zn_3-Sp1(Figure 6).

NMR structure comparion of Cd- and Zn-fingers

A previous NMR study reported on the 3-dimensional conformation of Zn-mF3, a modified finger 3 (mF3) of transcription factor IIIA, and on changes in backbone conformation upon Cd^{2+} substitution for Zn^{2+} (18). According to Figure 7, Sp1 fingers are analogous to mF3 in sequence position of the cysteinyl and histidinyl metal binding ligands, the length of the intervening sequence between the second cysteine and first histidine metal ion binding sites, and in the number of inter-histidine amino acid residues. Thus, the interaction of the histidine imidazole ligands with Zn^{2+} should be similar in the two structures. Considering that the histidine ligand-containing helix is primarily responsible for selective binding to the DNA major groove, we reexamined the Zn- and Cd-mF3 structures to provide insight into the effect of Cd^{2+} on Zn_3-Sp1 finger motifs as represented in reaction 3. As seen in Figure 8, exchange of Cd^{2+} for Zn^{2+} perturbed the orientation of the histidine imidazole ligands about the metal ion, such that the tetrahedral geometry of the coordinated ligands became distorted and the plane of each imidazole twisted about 90° (Table 2). This conformational shift requires reorientation of the adjoining methylene group and, secondarily, the peptide backbone. With the perturbation of the DNA-binding helix backbone into a distorted helix in Cd-mF3, side chain residues involved in the DNA binding helix also reorganized shifted (Figure 8). This alteration made stabilizing side chain-base pair interactions less favorable.

Discussion

Cadmium selectively causes kidney toxicity. It accumulates in the kidney cortex and compromises the function of the sodium dependent nutrient transporters in the proximal tubule, resulting, for example, in the development of glucosuria, defined as the inability to reabsorb glucose from the glomerular filtrate (19,20). Cadmium-dependent inhibition of glucose reabsorption in the kidney has been investigated in a mouse kidney cell culture system (21). Down regulation of sodium-dependent glucose uptake by Cd^{2+} is correlated with depression of the mRNA levels of glucose transporters SGLT1 and SGLT2 (7,22). Zn_3-Sp1 is a key transcription factor for the expression of SGLTs in human cells (10). Mouse and human *SGLT1* promoters contain homologous Zn_3-Sp1 binding sites; (human *SGLT2* promoter has GC site but its location is not conserved) (8,10). Thus, we hypothesized that Zn_3-Sp1 is the direct or indirect cellular target of Cd^{2+}. Supporting this idea was a report that Cd^{2+} down-regulated Sp1 in alveolar lung cells (23). That paper suggested an indirect effect of Cd^{2+} on Zn_3-Sp1 that resulted from the activation of protein kinase C, such as a phosphorylation event. Because Sp1 is a zinc-finger protein, we also entertained the alternative hypothesis that Zn_3-Sp1 is down-regulated by direct Cd^{2+}- Zn^{2+} exchange in the protein.

Sp1 is a Zn-finger transcription factor of the most common class that binds Zn^{2+} with a C_2H_2 set of side chain ligands (12,24-26). Recent ChIP assays probing the *in vivo* occupancy of the *SGLT1*-GC1 and *SGLT2*-GC promoter sites by Zn_3-Sp1 protein demonstrated that cell Cd^{2+} exposure inhibits the normal association of Sp1 with these sites (15). Parallel experiments showed that Cd^{2+} inhibits the *in vitro* DNA binding activity of Zn_3-Sp1 in a concentration dependent manner (15). The present results develop the underlying chemical understanding of this reaction.

This study follows a history of experiments demonstrating that the reaction of Cd^{2+} with other Zn-finger structures such as tramtrack, Zn-MTF-1 and Zn-TFIIIA causes loss of their selective DNA binding activity (13,14,18,27). Cd^{2+} and Zn^{2+} are chemically similar since Cd^{2+} lies

below Zn^{2+} in the same family of the periodic table and, thus, Cd^{2+} is commonly substituted for Zn^{2+} in metalloproteins as, for example, in metallothionein (5,28).

The reaction of Cd^{2+} with Zn_3-Sp1 was originally thought to involve a simple metal ion exchange mechanism as in reaction 1. According to this reaction, addition of Zn^{2+} to the reaction mixture should favor Zn_3-Sp1 relative to Cd_3-Sp1. Indeed, data from a series of these reaction mixtures containing increasing concentrations of Zn^{2+} were used to calculate the equilibrium constant (K_1) for reaction 1 (Table 1). Values of $K_1 = 14 \pm 3$ and $K_1 = 7 \pm 4$ from EMSA experiments utilizing GC1 and *SGLT2*-GC, respectively, demonstrate a modest preference of Sp1 for Cd^{2+} over Zn^{2+}. Comparable affinities of transcription factor IIIA fingers for Zn^{2+} and Cd^{2+} has been demonstrated previously (14,18). Nevertheless, these results do not rationalize how Cd^{2+} might target Zn_3-Sp1 in cells where there are multiple competing binding sites for Cd^{2+}.

Addition of Zn^{2+} to the mixture of Cd^{2+} and Zn_3-Sp1 after the reaction was underway failed to reverse the observed Cd^{2+} inhibition of Sp1 binding to *SGLT1* and *2* GC probes (Figure 3). Clearly, Zn^{2+} behaved differently in this experiment than in the previous one, even though the final concentration of all the reactants was the same in both versions of the Cd^{2+}-Zn^{2+} competition experiments. Only the order of addition of reactants changed. Thus, equilibrium reaction 3 is not sufficient to explain the interaction of Cd^{2+} with Zn_3-Sp1. Other reactions must come into play as well. These results parallel ones for the reaction of transcription factor IIIA with Cd^{2+} (14).

We propose that Cd_3-Sp1 in reaction 1 retains normal Cys_2His_2 metal ion coordination and that Cd^{2+} only perturbs the peptide's usual conformation. This is consistent with NMR structural studies on a Zn- and Cd-finger related to finger 3 of transcription factor IIIA summarized above (18). Refinement of the NMR structural comparison of the two structures showed that the plane of each imidazole group bound to Cd^{2+} was twisted about 90° in relation to its conformation in the coordination sphere of Zn-mF3 (Figure 8). As the finger helix, which contains the histidine imidazole ligands, is altered, other helix side chains become misoriented (Figure 8). Plausibly, this perturbation causes a reduction in stabilizing protein-DNA base interactions that decreases DNA binding activity as seen in the previous model study (18).

It is further proposed that in the presence of Cd^{2+}, which has a stronger preference for sulfhydryl group coordination than Zn^{2+}, the structure undergoes rearrangement to form Cd_{3-n}-Sp1' (reaction 7). In this conformation, Cd^{2+} is bound to 4 cysteinyl sulfhydryl groups from adjacent finger domains. On the basis of reduced stoichiometry of binding of Cd^{2+} to transcription factor IIIA in comparison with Zn^{2+}, we made a similar proposal (14).

In order to account for the ability of simultaneously added Zn^{2+} to inhibit reaction 1 or 2, we propose that the overall reaction from Zn_3-Sp1 to Cd_{3-n}-Sp1' can be thought of as follows, based on the reactions of individual fingers (Zn-F) in the tandem 3-finger structure of Zn_3-Sp1:

$$(\mathrm{Zn{-}F})_2 + \mathrm{Cd}^{+2} \rightleftharpoons (\mathrm{Cd{-}, Zn{-}F}) + \mathrm{Zn}^{2+} \quad K_1 \sim 10 \tag{9}$$

(Table 1)

$$(\mathrm{Cd{-}F, Zn{-}F}) \rightleftharpoons \mathrm{Cd{-}F_2} + \mathrm{Zn}^{2+} \qquad K_3 = K_b K_c \tag{10}$$

$$(\mathrm{Cd-F,Zn-F}) \rightleftharpoons (\mathrm{Cd-F,F+Zn^{2+}} \quad K_b \sim 10^{-9} \tag{11}$$

$$(\mathrm{Cd-F,F}) \rightleftharpoons \mathrm{Cd-F_2} \quad K_c \gg 1 \tag{12}$$

Rearranging the equilibrium expression for reaction 10, the ratio of [Cd-F_2]/[Cd-F,Zn-F] = $K_bK_c/[Zn^{2+}] = 10^{-9}K_c/[Zn^{2+}]$, where the approximate value for K_b is the dissociation constant determined for a single Zn-Sp1 finger (29). In the absence of added Zn^{2+}, free $[Zn^{2+}] \sim 10^{-7}$ M after this reaction reaches equilibrium because the initial concentration of Zn_3-Sp1 is 45 nM and Cd-F_2 is highly favored. Values of $K_c \geq 10^4$ would be consistent with this outcome ([Cd-F_2]/[Cd-F,Zn-F] $\geq 10^2$). In contrast, when $[Zn^{2+}] \sim 10^{-5}$ M, reaction 10 is evidently not particularly favorable, suggesting that $K_c \sim 10^4$. Once tandem zinc fingers are reconfigured as Cd-F_2, it might be kinetically difficult for Zn^{2+} to drive the reaction back toward (Cd-F,Zn-F) because of the need to refold the peptide into the ββα conformation. This would account for the inability of sequentially added Zn^{2+} to reverse the formation of Cd-F_2.

We addressed the impact of EDTA on Cd^{2+}-dependent inhibition of Zn-Sp1·GC1 binding that we observed with a commercial EMSA kit. This was important to resolve because it was recognized that some of the steps in commercial DNA binding assay used in our initial studies as well as those of others in the literature include large concentrations of EDTA. In another paper, we showed that the observation of Zn_3-Sp1 binding to cognate DNA sequences in the standard EMSA reaction is a fortuitous outcome that results from comparable rates of the reactions of EDTA and DNA with Zn_3-Sp1 (reactions 4 and 5) (16).

The question then arose: how does the presence of EDTA affect these reactions and reaction 1 above? Clearly, some but not all Zn_3-Sp1 reacted with EDTA during the typical 15 min reaction time. Furthermore, any Cd^{2+} added to Zn_3-Sp1 would be chelated immediately by EDTA as the log stability constant of Cd-EDTA at pH 7.4 and 25° C is 13.6 and the kinetics of formation are rapid (30). Nevertheless, the reaction went nearly to completion with 2.5 μM added Cd^{2+} (Figure 2). By comparison, reaction of 5-200 μM EDTA with Zn_3-Sp1 under the same conditions resulted in 40-60% reaction (16). Remarkably, fully EDTA-bound Cd^{2+} was a more powerful inhibitor of Zn_3-Sp1 than EDTA, itself.

At the outset, it seemed counterintuitive that Cd^{2+} bound to a high affinity ligand such as EDTA could still react with Zn_3-Sp1. Yet, experiments showed that it did so to a comparable extent as Cd^{2+} reacting with Zn_3-Sp1 in a specially prepared, EDTA free buffer (Figure 2). Perusing reaction 6, simplified by the assumption that each finger is chemically identical and independent,

$$\mathrm{Cd-EDTA+Zn-Sp1} \rightleftharpoons \mathrm{Cd-Sp1+Zn-EDTA} \quad K_4 \tag{13}$$

it was evident that one had to consider the formation of a highly stable product, Zn-EDTA in the analysis. The equilibrium constant for reaction 13 is

$$K_4 = K_{\mathrm{Cd-Sp1}} K_{\mathrm{Zn-EDTA}} / K_{\mathrm{Zn-Sp1}} K_{\mathrm{Cd-EDTA}} \tag{14}$$

in which each component equilibrium constant is the stability constant at pH 7.4 for the formation of the subscripted species. The numerical value of the $K_{Zn\text{-}EDTA}/K_{Cd\text{-}EDTA}$ ratio is close to 1 (30). That of the $K_{cd\text{-}Sp1}/K_{Zn\text{-}Sp1}$ ratio as calculated above is about 10, similar to the results of studies of transcription factor IIIA and one if its constituent fingers (14,18). Since the Cd-EDTA concentration is large in comparison with Zn_3-Sp1, the reaction will be driven toward products as is observed.

Although the above results and discussion are consistent with direct Cd^{2+}-Zn^{2+} interactions as a basis for down regulation of Zn_3-Sp1 binding to GC1, alternative hypotheses could be entertained, including that Cd^{2+} non-specifically binds to Zn_3-Sp1, altering its conformation or that the metal ion exerts a similar effect on the DNA probe. Both were explored in a previous paper, in which it was shown that the presence of Cd^{2+} had no impact on the binding of a non-Zn-finger transcription factor, Oct2A to its cognate DNA [15]. Moreover, the capacity of simultaneously added Zn^{2+} to reduce the effect of Cd^{2+} on Zn_3-Sp1 binding to DNA (Table 1) is not readily explained by the non-specific interaction of Cd^{2+} with the protein:

$$Cd^{2+} + Zn_3 - Sp1 \rightleftharpoons Zn_3 - Sp1 - Cd \tag{15}$$

Zn^{2+} like Cd^{2+} could bind adventiously to the protein, causing a similar negative conformational change. Thus, competition between the two metal ions would not in general be expected to restore the protein's ability to associate specifically with DNA. Similarly, if Cd-EDTA replaced Cd^{2+} in reaction 15,

$$Cd - EDTA + Zn_3 - Sp1 \rightleftharpoons Zn_3 - Sp1 - Cd + EDTA \tag{16}$$

there would be no driving force to dissociate Cd-EDTA and form Zn_3-Sp1-Cd as in reaction 13. Thus, direct competition between Cd^{2+} and Zn^{2+} for Sp1 is the well supported hypothesis based on this work.

The mechanistic pathway of reaction 13 deserves comment. With both Zn^{2+} and Cd^{2+} fully bound to multidentate ligands, the mechanism of reaction requires multiple steps in which at least one of the reactants undergoes a metal-ligand bond dissociation that frees a ligating group to attack the other metal center. That the Zn^{2+} center in Sp1 undergoes exchange with another fully bound metal ion reveals unusual kinetic reactivity of a canonical Cys_2His_2 zinc finger site. This property deserves further study because it suggests that this type of Zn-binding site is an attractive site for reaction with xenobiotic metal ions.

The reactivity of Zn_3-Sp1 with Cd^{2+} was blocked during 15 min incubations when the protein was previously bound with cognate DNA sequences. Over the same concentration range that completely abolished GC1 binding by Zn_3-Sp1, Cd^{2+} failed to react with Zn_3-Sp1·GC1 (**Figures** 1 and 4). Similar results were obtained with Zn_3-Sp1 bound to *SGLT2*-GC and a GC consensus sequence (Figure 4) (15). This finding raised the possibility that *in vivo* Cd^{2+} could only react with free not DNA-bound Zn_3-Sp1. However, further experimentation revealed that longer reaction of Zn-Sp1·GC1 with Cd^{2+} resulted in gradual dissociation of the adduct. Thus, Zn_3-Sp1 associated with *SGLT* promoter elements are reactive with Cd^{2+}. In comparison, Zn_3-Sp1·$GC_{CONSENSUS}$ remained intact under similar conditions of reaction with Cd^{2+}. During the investigation of this differential behavior, experiments revealed that a non-specific DNA sequence, dI-dC, as found in the wings of the GC1 sequence afforded no protection against Cd^{2+}-Zn^{2+} exchange (Figure 5).

The general reaction of a DNA binding protein with cognate DNA can be considered as a combination of non-specific electrostatic protein-nucleic acid interactions and site specific

hydrogen bonding and van der Waals contacts (31). The differential behavior of the specific Zn_3-Sp1·GC1 adduct and non-specific Zn_3-Sp1·(dI-dC) with Cd^{2+} observed in Figure 5 indicates that the specific binding component that sterically organizes and stabilizes Zn_3-Sp1·GC1 or Zn_3-Sp1·$GC_{CONSENSUS}$ prevents the comparatively rapid reaction of Cd^{2+} with Zn_3-Sp1 that is available when the protein is bound non-specifically to poly(dI-dC). Some mix of thermodynamic and steric/kinetic factors result in the relative lack of reactivity of Zn-Sp1·GC1. We hypothesize that the difference in behavior of GC1 and $GC_{CONSENSUS}$ resides in the fact that GC1 places $GC_{CONSENSUS}$ between non-specific sequences (Figure 6). The presence of contiguous wings of non-specific DNA affords a kinetic pathway by which Zn_3-Sp1 can slide between specific and non-specific sites, permitting Cd^{2+} to react with Zn_3-Sp1 associated with non-specific DNA analogous to poly(dI-dC) (Figure 5) (32).

Such DNA protection against reaction of reagents that can attack the Zn-center of Cys_2His_2 zinc finger proteins has some generality. We have also demonstrated the strong oligonucleotide protection of Zn^{2+} in Zn-Sp1·GC1 against reaction with EDTA, TPEN, and other chelators including apo-MT (16,33). Similar observations were obtained in experiments with Zn-TFIIIA (34).

Is direct reaction of Cd^{2+} with Zn_3-Sp1 as in reactions 8-12 a reasonable cellular hypothesis? The answer to that question is not known with certainty. The ratio of [Cd_3-Sp1]/[Zn_3-Sp1] in reaction (1) depends on the ratio of free metal ions, [Cd^{2+}]/[Zn^{2+}]. Although the total concentration of cellular Zn^{2+} is on the order of 100 μM, the free, unbound Zn^{2+} concentration is generally thought to be much smaller, in the picomolar to nanomolar range (35). Under this condition, even nM free Cd^{2+} would be sufficient to drive reaction 1 toward the formation of Cd_3-Sp1.

A second consideration is the extent of competition among cellular binding sites for Cd^{2+} that might dilute out the impact of Cd^{2+} on Zn_3-Sp1. Metallothionein is one obvious site but when its preinduction concentration is low, as in kidney cortical cells, other sites such as Zn_3-Sp1 are clearly targeted, directly or, perhaps, indirectly through the involvement of other Cd binding sites. The existence of a second, highly favorable reaction that converts Cd_3-Sp1 to Cd_{3-n}-Sp1τ in reaction 5, both strengthens the chemical plausibility of direct reaction of Cd^{2+} with Zn_3-Sp1 and its possible cellular selectivity.

The results of this study support the hypothesis that the down-regulation of renal mRNA expression of SGLT1 and SGLT2 in response to Cd^{2+} may result from decreases in the pool of Zn-containing Sp1 and parallel increases in Cd-bound Sp1. With the current *in vitro* experiments clarifying the effect of Cd-binding on the DNA binding properties of Sp1, cellular experiments are under way to test this hypothesis.

Acknowledgments

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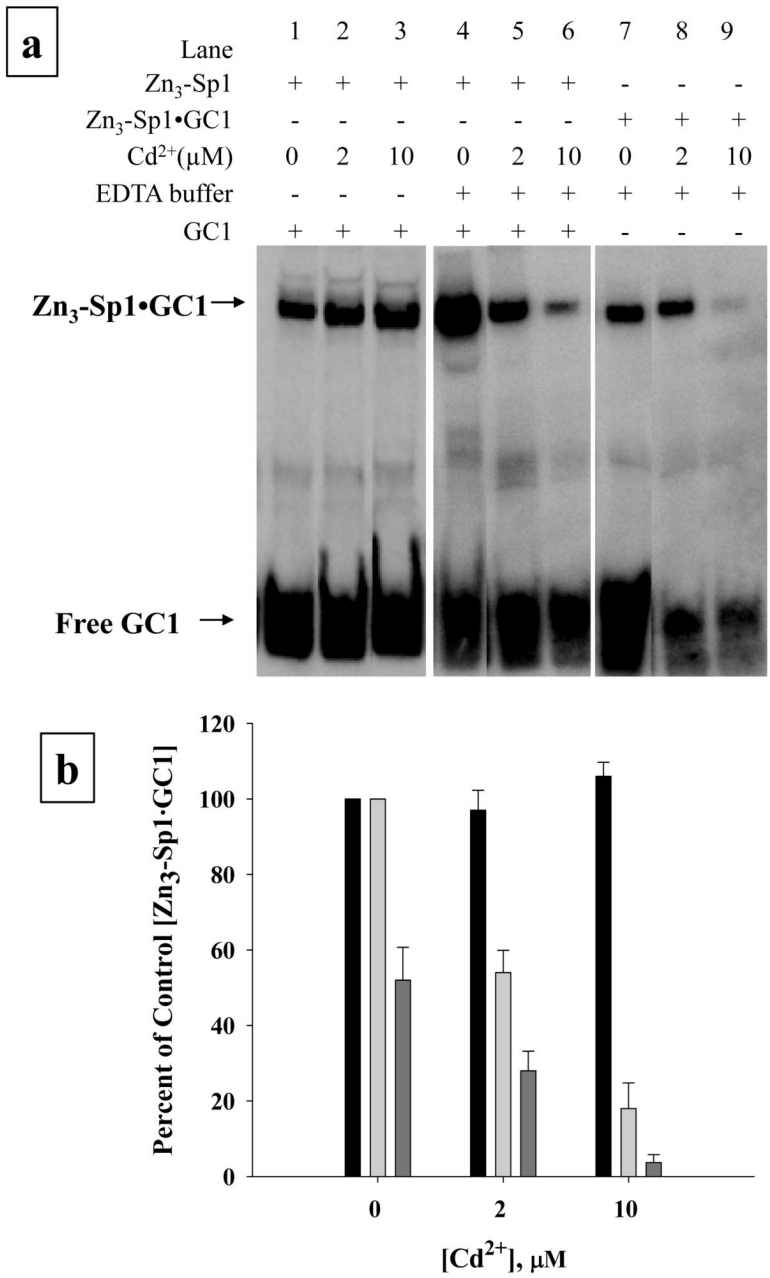

Figure 1.

EMSA analysis of reaction of Cd^{2+} with Zn_3-Sp1. a. Sample EMSA data. b. Data summary: Cd^{2+} + 45 nM Zn_3-Sp1 in absence of EDTA (light bar); Cd^{2+} + 45 nM Zn_3-Sp1 in presence of 200 :M EDTA (darker bar); Cd^{2+} + Zn_3-Sp1·GC1 (reaction product of 45 nM Zn_3-Sp1 and 3.6 nM GC1) in absence of EDTA (black bar). Results are averages ± standard error of three independent runs. Specific details of reaction are listed in the Methods.

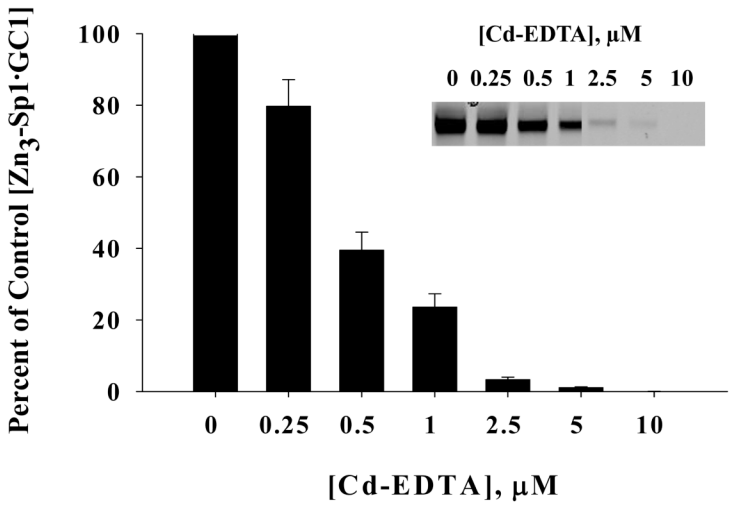

Figure 2.

Reaction of Cd-EDTA with Zn_3-Sp1 measured by the EMSA. Stock solution contains Cd^{2+}:EDTA of 1:1.1. Results are averages ± standard error of three independent runs.

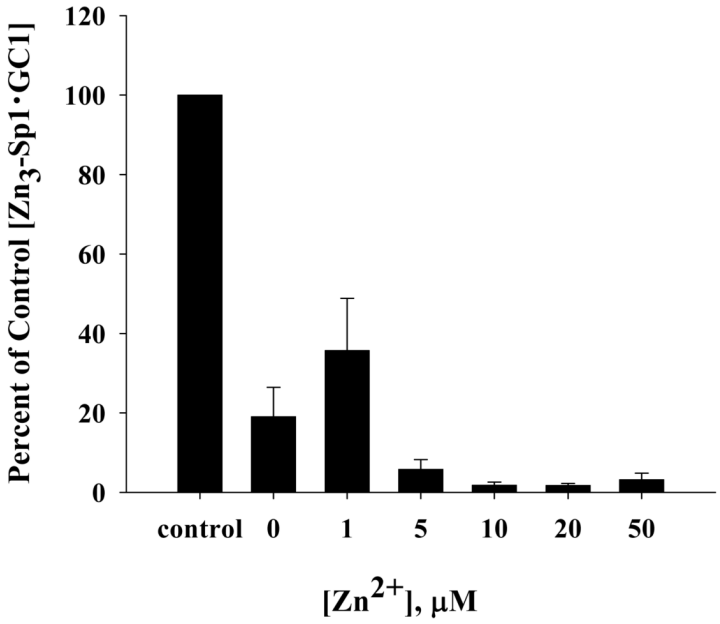

Figure 3.

Sequential reaction of Cd^{2+} and Zn^{2+} with Zn_3-Sp1. 5μM Cd^{2+} was reacted with 45 nM Zn_3-Sp1. After 15 min incubation at room temperature, Zn^{2+} was added and incubated with the reaction mixture for 15 min at room temperature. Then, the samples were electrophoresed using EMSA conditions. Results are averages ± standard error of three independent runs.

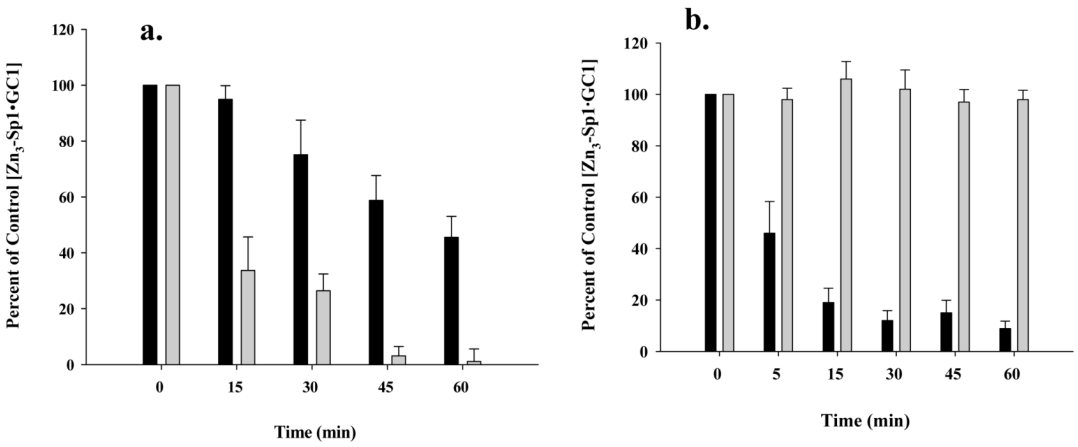

Figure 4.

Time dependent reaction of Cd^{2+} with Zn_3-Sp1, Zn_3-Sp1·GC1 and Zn_3-Sp1·$GC_{CONSENSUS}$. Reactions of 5 μM Cd^{2+} with 45 nM Zn_3-Sp1 (gray bar) (a, b), Zn_3-Sp1·GC1 (reaction product of 45 nM Zn_3-Sp1 and 3.6 nM GC1, black bar) (a) and Zn_3-Sp1·$GC_{CONSENSUS}$ (reaction product of 45 nM Zn_3-Sp1 and 85 :M $GC_{CONSENSUS}$, black bar) (b). Results are averages ± standard error of three independent runs.

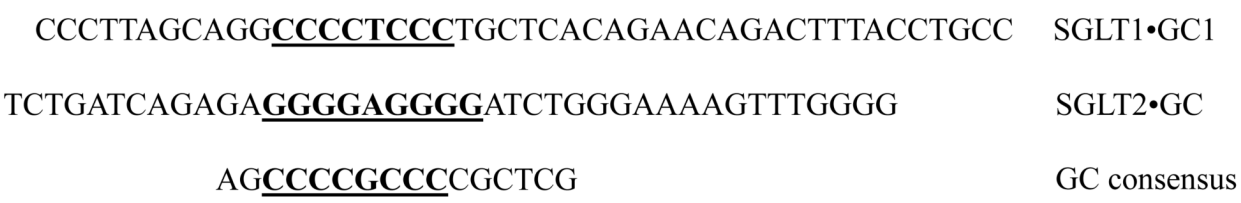

Figure 5.
DNA base sequences of GC1(*SGLT1*), GC (*SGLT2*) and $GC_{CONSENSUS}$.

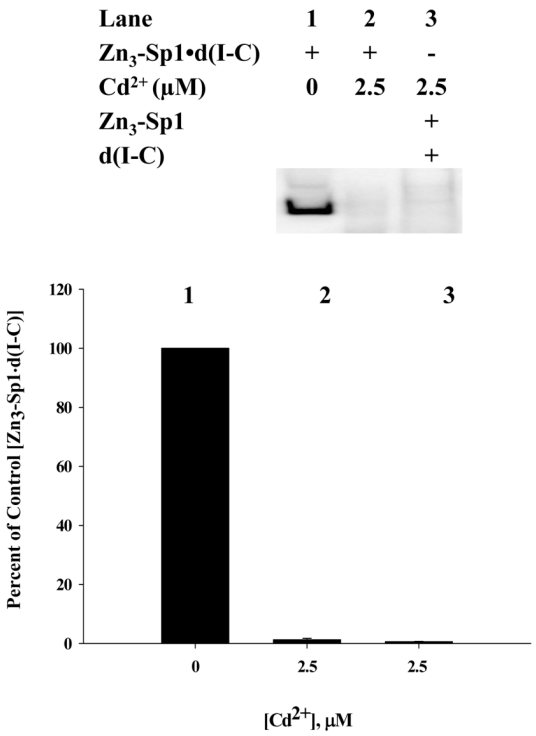

Figure 6.
EMSA analysis of reaction of Cd^{2+} with Zn_3-Sp1·(polydI-dC) (reaction product of 45 nM Zn_3-Sp1 and 3.6 nM poly dI-dC). a. Sample EMSA data. b. Data summary of three experiments. .

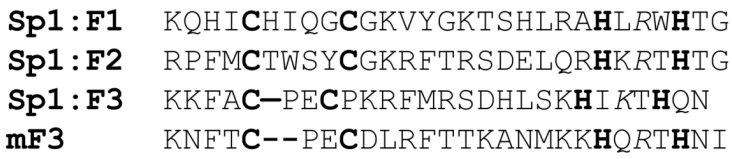

Figure 7.
Amino acid sequences of Sp1 fingers and mF3

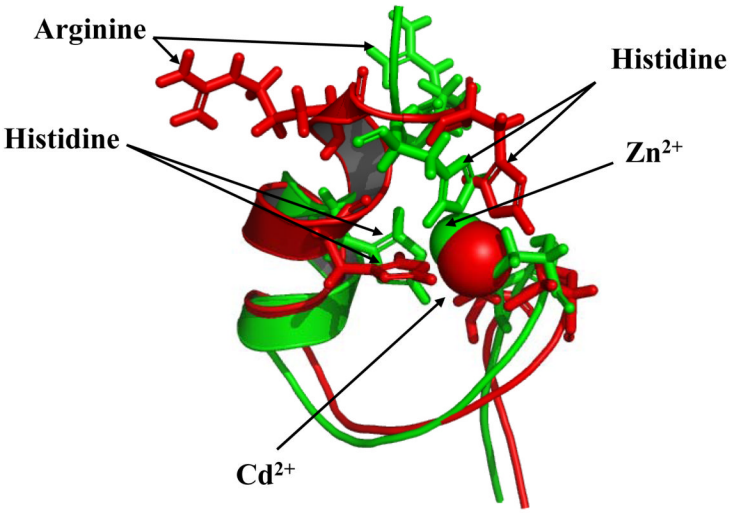

Figure 8.
Comparative NMR conformations of Zn- and Cd-mF3. a. Metal ion binding site. Cd^{2+} (red), Zn^{2+} (green). b. helix and side chain conformation Cd^{2+} (red), Zn^{2+} (green).

Table 1

Competition between Cd^{2+} and Zn^{2+} for Sp1

a.		
Cd^{2+} μM	**Zn^{2+} μM**	**Zn_3Sp1·G C1**
0	**0**	**100**
5	**0**	**5 ± 2**
5	**5**	**8 ± 1**
5	**25**	**28 ± 5**
5	**50**	**45 ± 2**
5	**100**	**52 ± 7**

b.		
Cd^{2+} μM	**Zn^{2+} μM**	**Zn_3Sp1·GC**
0	**0**	**100**
2	**0**	**29 ± 8**
2	**2**	**33 ± 8**
2	**10**	**56 ± 15**
2	**20**	**54 ± 6**
2	**40**	**67 ± 14**
2	**100**	**83 ± 19**

Table 2

Comparative Structural Properties of Cd- and Zn-finger Peptides

Property	Cd-finger	Zn-finger
Helix	distorted helix	Alpha helix
Histidine imidazole ring	~90° rotation compared to Zn-Finger	-
Arginine side chain	Reoriented	-
Metal binding C_2H_2 site	Distorted	Tetrahedral