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Ca^{2+} Binding to the ExDxD Motif Regulates the DNA Cleavage Specificity of a Promiscuous Endonuclease

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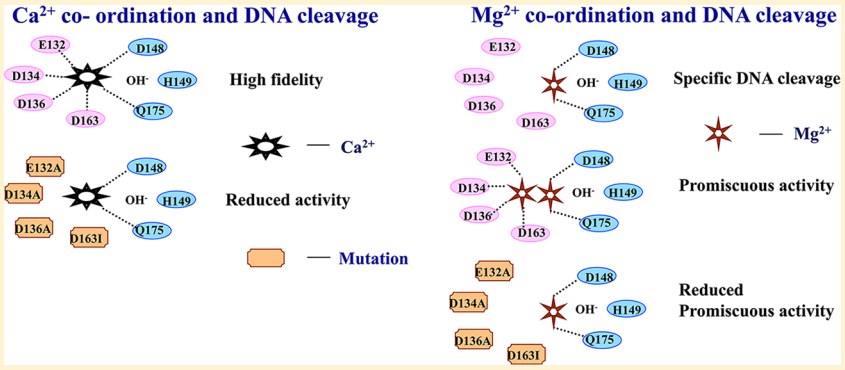
Supporting Information



ABSTRACT: Most of the restriction endonucleases (REases) are dependent on Mg^{2+} for DNA cleavage, and in general, Ca^{2+} inhibits their activity. R.KpnI, an HNH active site containing $\beta\beta\alpha$-Me finger nuclease, is an exception. In presence of Ca^{2+}, the enzyme exhibits high-fidelity DNA cleavage and complete suppression of Mg^{2+}-induced promiscuous activity. To elucidate the mechanism of unusual Ca^{2+}-mediated activity, we generated alanine variants in the putative Ca^{2+} binding motif, $E_{132}xD_{134}xD_{136}$, of the enzyme. Mutants showed decreased levels of DNA cleavage in the presence of Ca^{2+}. We demonstrate that ExDxD residues are involved in Ca^{2+} coordination; however, the invariant His of the catalytic HNH motif acts as a general base for nucleophile activation, and the other two active site residues, D148 and Q175, also participate in Ca^{2+}-mediated cleavage. Insertion of a 10-amino acid linker to disrupt the spatial organization of the ExDxD and HNH motifs impairs Ca^{2+} binding and affects DNA cleavage by the enzyme. Although ExDxD mutant enzymes retained efficient cleavage at the canonical sites in the presence of Mg^{2+}, the promiscuous activity was greatly reduced, indicating that the carboxyl residues of the acidic triad play an important role in sequence recognition by the enzyme. Thus, the distinct Ca^{2+} binding motif that confers site specific cleavage upon Ca^{2+} binding is also critical for the promiscuous activity of the Mg^{2+}-bound enzyme, revealing its role in metal ion-mediated modulation of DNA cleavage.

The fundamental reaction catalyzed by REases is the hydrolysis of the phosphodiester bond at specific sequences yielding 3′-hydroxyl and 5′-phosphoryl termini. The role of metal ions in DNA binding and the subsequent hydrolysis of the phosphodiester bond has been studied in detail for several REases belonging to the PD...D/ExK superfamily.[1−4] Most of the REases exhibit optimal activity with Mg^{2+}. The preference for Mg^{2+} over the other metal ions is probably due to its natural abundance and favorable physical and chemical properties.[5,6] Mn^{2+} can substitute for Mg^{2+} in the case of a few enzymes, but Ca^{2+} generally inhibits the DNA cleavage activity.[3,7] The inhibition of various enzymes by Ca^{2+} could be attributed to the pK_a of the cofactor-bound water molecule in the active site and its relative position with respect to the scissile phosphodiester bond.[8,9] Taking advantage of its inhibitory property, Ca^{2+} has been used extensively in the DNA binding and structural studies of the enzyme−DNA complexes of many REases.[10,11]

HNH nucleases are the second largest group of enzymes after the PD...D/ExK family and are characterized by the presence of a conserved structural motif called the $\beta\beta\alpha$-Me finger fold.[12,13] Structural and biochemical studies with HNH nucleases have shown that they share a common single-metal ion mechanism.[11−13] The invariant His of the HNH motif acts in the first step as the general base for the DNA cleavage reaction, and in general, it is the only conserved residue of the motif in the superfamily. The active site-bound metal ion plays a major role in the subsequent steps of catalysis by stabilizing the transition state and protonation of the leaving group during the phosphodiester bond hydrolysis.[12] Because the nucleophile activation is mediated by a conserved His in all the HNH enzymes, the metal ion's role is confined to the later steps of

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the catalysis. As a result, HNH nucleases utilize a relatively broad range of metal ions for DNA cleavage,[12] although preferring a particular type, i.e., either alkaline earth or transition group.[14−16] For instance, I-CmoeI and I-PpoI prefer various alkaline earth metal ions, whereas the other members of the superfamily like Colicin E7 and R.HpyAV prefer transition metal ions for DNA cleavage.[14−18]

Within the HNH nucleases, R.KpnI, isolated from *Klebsiella pneumoniae*, stands apart in many of its properties. The enzyme is activated by metal ions belonging to both the alkaline earth and transition groups, a feature not common to the other members of the superfamily.[19] Further, among the metal ions that conduct DNA cleavage, some of them induce promiscuous cleavage (Mg^{2+}, Mn^{2+}, and Co^{2+}) while others (Ca^{2+}, Cd^{2+}, Ni^{2+}, and Zn^{2+}) support high-fidelity cleavage. The difference in the cleavage pattern was shown to be caused by the recruitment of a second metal ion by the enzyme that induces promiscuous activity.[19] Another unique feature of the enzyme is Ca^{2+}-mediated suppression of the promiscuous activity.[20] An additional Ca^{2+} binding site in the enzyme could be responsible for the suppression of the promiscuous activity and induction of high-fidelity cleavage. Alternatively, a larger ionic radius and flexible coordination properties of Ca^{2+} probably facilitate coordination with additional residues in the active site. We have identified key residues in R.KpnI required for Ca^{2+} coordination and demonstrate their involvement in Ca^{2+}-mediated DNA cleavage.

EXPERIMENTAL PROCEDURES

Enzymes, Chemicals, and DNA. R.KpnI and its mutants were purified using the method described previously.[21] The enzymes were diluted in buffer containing 20 mM Tris-HCl (pH 7.4), 25 mM NaCl, and 5 mM 2-mercaptoethanol for all the studies. The concentration of the proteins was estimated by the Bradford method using bovine serum albumin as a standard. The purity of the proteins was assessed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (PAGE).

Oligonucleotides (Microsynth and Sigma) purified on a 15% urea−polyacrylamide gel were labeled at the 5′ end using T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP (6000 Ci/mmol, Perkin-Elmer Life Sciences) and purified using G-50 spin column chromatography. The Hi-Trap heparin column (GE Healthcare), ampicillin, chloramphenicol, bovine serum albumin, polyethyleneimine, Coomassie Brilliant Blue, IPTG (Sigma), and phosphocellulose P11 (Whatman) were used.

Site-directed mutants of R.KpnI were generated by the megaprimer inverse polymerase chain reaction (PCR) method.[22] Expression plasmid pETRK encoding the kpnIR gene (GenBank entry P25237) was used as a template. Oligonucleotide primers carrying the respective mutant amino acid codon substitutions were used as forward primers, and the T7 terminator sequence was used as a reverse primer. The generated megaprimers served as complementary primers for the second round of PCR amplification. Primers and oligonucleotides used in this study are listed in Table S1 of the Supporting Information. The strains and plasmids used in the study were from laboratory stocks (Table S2 of the Supporting Information). After the mutations had been confirmed by sequencing, the mutant REases were expressed in *Escherichia coli* BL26 [F- omp T hsdSB (rB- mB-) gal dcm lac (DE3) nin5 lac UV5-T7 gene 1] containing the M.KpnI plasmid.[21]

Generation of the Insertion Mutant of R.KpnI. Overlap extension PCR was used. In the first round of PCR, (i) the N-terminal half of the gene was amplified using the T7 forward primer and an internal primer in the antisense direction with a $2xG_4S_1$ overhang, and (ii) the C-terminal half of the gene was amplified using the T7 reverse primer and an internal primer in the sense direction with a $2xG_4S_1$ overhang. The amplicons were used for the second round of PCR with the T7 forward primer and reverse primers to generate the whole gene, which was digested with NcoI and BamHI to clone into the pET11d expression vector. After confirming the insertion by sequencing, the mutant REase (insertion mutant) was expressed and purified as described previously.[21]

Electrophoretic Mobility Shift Assay. Proteins were dialyzed against ethylenediaminetetraacetic acid (EDTA) to remove the intrinsically bound metal cofactor, and EDTA was removed by dialysis against 10 mM Tris-HCl (pH 7.4) buffer without EDTA. Different concentrations of the enzymes (0−256 nM) were incubated with 5′-end-labeled double-stranded oligonucleotides (1 nM) containing the recognition site (-GGTACC-) or one of the preferred noncanonical sites (-GtTACC-) in binding buffer [20 mM Tris-HCl (pH 7.4) and 5 mM 2-mercaptoethanol] for 15 min on ice. The free DNA and the enzyme-bound complexes were resolved by 8% native PAGE in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, and 1 mM EDTA). Gels were visualized with a PhosphorImager (Fujifilm, FLA 5100).

In Vitro DNA Cleavage and Steady-State Kinetic Analysis. Digestions were conducted by incubating different units of R.KpnI and its mutants with pUC18 DNA (14 nM) (contains a single site for R.KpnI) or 5′-end-labeled oligonucleotides (10 nM) in assay buffer containing 10 mM Tris-HCl (pH 7.4) and 2 mM Mg^{2+} or Ca^{2+} for 1 h at 37 °C. The reactions were terminated by adding stop dye (20% glycerol, 50 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol), and the cleavage products of plasmid DNA and oligonucleotides were analyzed on 1% agarose and 12% urea−polyacrylamide gels, respectively.

The specific activity was calculated by incubating various amounts of enzyme with pUC18 DNA under the standard assay conditions. One unit of R.KpnI is defined as the amount of enzyme required to digest 500 ng of pUC18 plasmid DNA. Chemical modification reactions were conducted with diethyl pyrocarbonate (DEPC), which modifies the histidine to *N*-carboethoxyhistidine, thus rendering it catalytically inactive. At concentrations ranging from 0.1 to 10 μM, DEPC appears to target the active site His in R.KpnI as the modification could be protected by preincubation with metal ions. The wild type (WT) and the mutants (5 and 100 nM, respectively) were incubated with different concentrations of DEPC (0−100 μM), and the oligonucleotide cleavage assay was conducted in the presence of 2 mM Ca^{2+}. The cleavage products were analyzed via 12% urea−PAGE and visualized with a PhosphorImager (Fujifilm, FLA 5100).

Steady-state kinetic experiments in the presence of 2 mM Mg^{2+} or Ca^{2+} were determined as described previously.[23] The kinetic parameters were determined by fitting the change in the velocity and substrate concentration to the Michaelis−Menten plot using GraphPad Prism version 5. The turnover number (k_{cat}) was calculated as the ratio of V_{max} to the enzyme concentration.[23]

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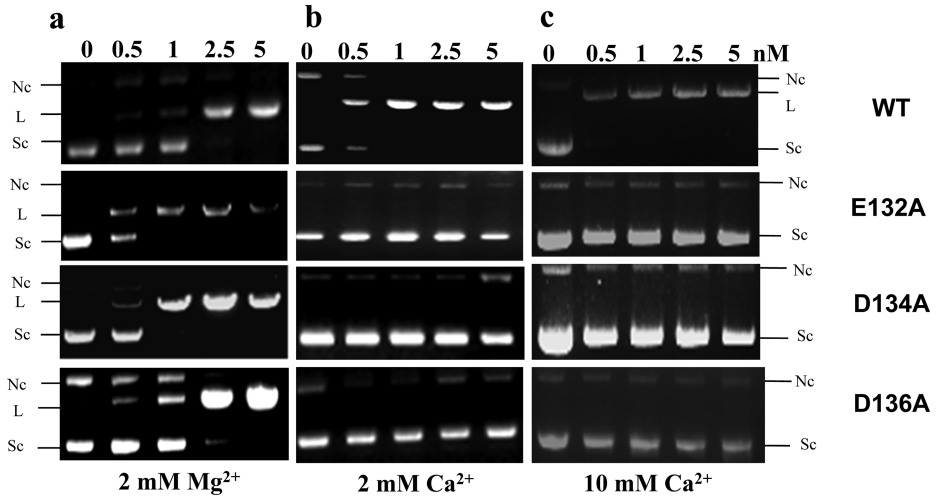

Figure 1. DNA cleavage properties of R.KpnI and its mutants. (a–c) pUC18 DNA (14 nM) was incubated with various concentrations of WT or ExDxD mutants (0–5 nM) at 37 °C for 1 h in the presence of (a) 2 mM Mg^{2+}, (b) 2 mM Ca^{2+}, or (c) 10 mM Ca^{2+}. The cleavage products were analyzed on a 1% agarose gel. Nc, L, and Sc indicate the positions of nicked circular, linear, and supercoiled forms of the plasmid, respectively.

$$V = \frac{V_{max}[S]}{K_m + [S]}$$

Intrinsic Fluorescence. Fluorescence emission spectra were recorded using a Jobin-Yvon FluoroMax 3 fluorometer (HORIBA, Jobin-Yvon/Spex Division, Longjumeau, France), thermostated at 25 °C. The fluorescence spectra were recorded at excitation and emission wavelengths of 295 and 340 nm, respectively, with a 5 nm slit width. EDTA-treated WT or mutant enzymes (1 mg/mL) were incubated in a buffer containing 10 mM Tris-HCl (pH 7.4) and 5 mM 2-mercaptoethanol with different concentrations (0.025–10 mM) of Mg^{2+} or Ca^{2+} for 15 min at 25 °C, and fluorescence emission spectra were recorded. Control titrations were conducted in the presence of a monovalent cation, Na^+. All fluorescence emission spectra and fluorescence intensities from the titrations were corrected by subtraction of control spectra and control titrations. The K_d values were determined from a nonlinear least-square regression analysis of titration data using the equation[24]

$$\Delta F/\Delta F_{max} = [\text{metal ion}]_{tot}/(K_d + [\text{metal ion}]_{tot})$$

where ΔF is the magnitude of the difference between the observed fluorescence intensity at a given concentration of metal ion and the fluorescence intensity in the absence of a metal ion and ΔF_{max} is the difference at an infinite metal ion concentration.

Ca^{2+} Blotting Assay. WT and its mutants (0–3 μg) were slot-blotted onto nitrocellulose membranes presoaked in buffer [10 mM Tris-HCl (pH 7.4) and 100 mM NaCl]. Transfer was ascertained by Ponceau-S staining, and the amount of transferred protein was estimated using Quantity One. The membranes were washed three times (15 min each) in the same buffer and incubated in the buffer containing 30 μCi of $^{45}CaCl_2$ [specific activity, 27 mCi/g (BARC, Mumbai, India)] at room temperature for 30 min. After the unbound radioactivity had been removed by washing, the membrane was dried and exposed to a PhosphorImager screen.

Metal Ion Analysis. The metal ion stoichiometry of purified WT and mutants (4 μM) was determined in the absence of DNA, to avoid the contribution from the nonspecific binding of the phosphate backbone to the cations, by using inductively coupled plasma atomic emission spectroscopy (ICP-AES) and atomic absorption spectroscopy (AAS) as described previously.[25] EDTA-treated WT and mutants (4 μM) were incubated with either 2 mM Mg^{2+} or Ca^{2+}, and unbound metal ion was removed by dialysis overnight against 20 mM Tris-HCl (pH 7.4) and 150 mM NaCl at 4 °C with buffer changes. Dialyzed proteins were quantified and digested with 1 N HNO_3. Digested samples were resuspended in 5 mL of doubly distilled water and subjected to analysis. The dialysis buffer was used as a blank, and the resultant metal ion background was subtracted from the measurements of protein samples. To identify the intrinsically bound metal ion, R.KpnI was purified in buffers without EDTA. To analyze the intracellular enzyme-bound Ca^{2+}, R.KpnI was expressed in *E. coli* BL26 cells harboring pACMK grown in M9 minimal medium (500 mL) with and without 1 mM $CaCl_2$ at 37 °C until A_{600} reached 0.6. The strain containing the vector plasmid served as a control. The cells induced with 0.3 mM IPTG were harvested after 3 h, washed three times with 100 mL of 1× PBS by resuspension, and then centrifuged at 11900*g* and 4 °C for 20 min. Crude extracts were prepared by resuspending the pellet in 5 mL of 10 mM Tris-HCl (pH 7.4) containing 0.4 mg/mL lysozyme (Sigma), incubated for 3 h at 4 °C, homogenized, and centrifuged at 23700*g* and 4 °C for 45 min. The supernatant was digested with 1 N HNO_3, incubated overnight at 4 °C, and centrifuged at 16000*g* for 15 min. The supernatants were diluted in 10 mM Tris-HCl (pH 7.4) for metal analysis, and the Ca^{2+} content was determined. The presence of bound Ca^{2+} was tested by DNA cleavage assays without external addition of the metal ion. The cell free extract was incubated with 5′-end-labeled oligonucleotide having the -GGTACC- recognition sequence in assay buffer containing 10 mM Tris-HCl (pH 7.4) for 1 h at 37 °C. The reactions were terminated by adding stop dye (20% glycerol, 50 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol), and the cleavage products of the oligonucleotides were analyzed with 12% urea–polyacrylamide gels.

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RESULTS AND DISCUSSION

The ExDxD Motif Is Required for Ca^{2+}-Mediated DNA Cleavage. A number of Ca^{2+} coordination motifs have been identified in a diverse group of proteins involved in cellular functions. Some well-known examples are EF hand,[26] DxDxDGxxCE (Excalibur, extracellular calcium-binding region),[27] and K/H/RGD (Annexin).[28] A putative Ca^{2+} binding motif ($E_{132}xD_{134}xD_{136}$) was found upstream of the catalytic site in the sequence of R.KpnI (Figure S1 of the Supporting Information). To evaluate the importance of these residues in DNA cleavage, we generated point mutants E132A, D134A, and D136A as described in Experimental Procedures. Circular dichorism studies showed no major structural perturbations in the mutant proteins, though the mutation of D136 resulted in minor changes in the secondary structure (Figure S2 of the Supporting Information). Further, the mutants exhibited Mg^{2+}-mediated DNA cleavage activity comparable to that of WT (Figure 1a), implying that the Mg^{2+} coordination is unaffected. However, mutants showed partial DNA cleavage in presence of 2 mM Ca^{2+} at concentrations of WT sufficient to linearize supercoiled DNA (Figure 1b); complete cleavage was observed at only 20−30-fold higher concentrations of the enzyme (Figure S3 of the Supporting Information). Comparison of the specific activity of the mutants in the presence of both Mg^{2+} and Ca^{2+} showed an ∼20−80-fold reduction in specific activity in Ca^{2+}-mediated DNA reactions (Table 1). The mutants did not exhibit appreciable DNA cleavage even at higher Ca^{2+} ion concentrations (Figure 1c).

Table 1. Specific Activities of WT and Mutants

	specific activity (units/mg of protein)	
enzyme	Mg^{2+} ($\times 10^6$)[a]	Ca^{2+} ($\times 10^6$)[b]
WT	1.0	0.4
E132A	1.0	0.02
D134A	1.0	0.013
D136A	0.4	0.013

[a]Specific activity determined in the presence of 2 mM Mg^{2+}. [b]Specific activity determined in the presence of 2 mM Ca^{2+}.

Results from the kinetic analysis under steady-state conditions (described in Experimental Procedures) are summarized in Table 2. In Mg^{2+}-containing reaction mixtures, the ExDxD mutants exhibited k_{cat}/K_m values comparable to that of WT, indicating that Mg^{2+}-mediated catalysis was not affected (Table 2). With Ca^{2+}, the K_m values of the mutants did not vary significantly with respect to WT; however, the k_{cat} values were 2.5−6.5-fold lower, indicating a slower cleavage rate. Comparison of the Michaelis constant (K_m) and equilibrium binding constants suggests that the ExDxD mutants exhibit a substrate affinity similar to that of WT (Table 2).

Effect of ExDxD Motif Mutations on Ca^{2+} Binding. Because binding of metal cofactors induces changes in the Trp fluorescence of the protein,[23] binding of Ca^{2+} to the mutants was evaluated by fluorescence measurements. The K_d values derived by analyzing the Ca^{2+}-mediated conformational changes are 0.49 ± 0.13, 1.04 ± 0.25, 1.10 ± 0.29, and 1.63 ± 0.50 mM for WT, E132A, D134A, and D136A, respectively (Table 3 and Figure 2c), indicating the reduced affinity of the mutants for Ca^{2+}. In contrast, Mg^{2+} binding was not affected (Table 3 and Figure 2d). The reduced affinity of the mutants for Ca^{2+} was also visualized by a radioactive calcium blotting assay. The level of binding of the mutants to $^{45}Ca^{2+}$ was significantly reduced (Figure 2a,b). To estimate the direct binding of cofactors to the ExDxD motif mutants, the metal ion stoichiometry was determined by using AAS and ICP-AES as described in Experimental Procedures. With Mg^{2+} and Ca^{2+}, WT exhibited stoichiometric binding of two ions per dimer (Table 3). The ExDxD mutants retained stoichiometric binding of Mg^{2+} similar to that of WT but exhibited reduced levels of binding when incubated with Ca^{2+} (Table 3). All these data show that the mutations in the motif affected the binding of Ca^{2+} to the enzyme.

Table 2. Kinetic Parameters of R.KpnI and Its Mutants

	-GGTACC-			
substrate	K_d (nM)[a]	K_m (nM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\times 10^6$ s^{-1} M^{-1})
R.KpnI	8.4 ± 1.4			
with Mg^{2+}	–	18.3 ± 3.3	12.6 ± 0.2	11.6 ± 0.20
with Ca^{2+}	–	5.7 ± 1.7	1.4 ± 0.10	4.6 ± 0.12
E132A	9.2 ± 2.4			
with Mg^{2+}	–	21.9 ± 4.3	12.0 ± 0.4	9.5 ± 0.30
with Ca^{2+}	–	5.0 ± 1.4	0.4 ± 0.01	1.3 ± 0.05
D134A	14.0 ± 4.4			
with Mg^{2+}	–	28.5 ± 4.0	12.0 ± 0.3	7.1 ± 0.25
with Ca^{2+}	–	4.7 ± 1.3	0.5 ± 0.02	2.0 ± 0.04
D136A	15.7 ± 2.4			
with Mg^{2+}	–	30.2 ± 4.6	5.0 ± 0.1	2.7 ± 0.18
with Ca^{2+}	–	5.6 ± 1.6	0.2 ± 0.05	0.6 ± 0.02
	-GtTACC-[b]			
substrate	K_d (nM)[a]	K_m (nM)	k_{cat} ($\times 10^{-1}$ min^{-1})	k_{cat}/K_m ($\times 10^4$ s^{-1} M^{-1})
R.KpnI	12.5 ± 3.3	49.2 ± 8.2	2.7 ± 0.10	9.0 ± 0.40
E132A	ND[c]	60.8 ± 10.5	0.1 ± 0.01	0.3 ± 0.15
D134A	ND[c]	98.0 ± 12.6	0.4 ± 0.05	0.5 ± 0.12
D136A	ND[c]	148.4 ± 28.5	0.3 ± 0.02	0.2 ± 0.04

[a]K_d values were determined via an EMSA in the absence of a metal ion in triplicate and are given ± the standard error. [b]Kinetic analysis in the presence of Mg^{2+}. [c]Not determined.

Table 3. Metal Ion Binding and Stoichiometric Analysis of Mutants

	K_d (mM)[a]			
protein	Mg^{2+}	Ca^{2+}	R.KpnI:Mg^{2+} molar ratio[b]	R.KpnI:Ca^{2+} molar ratio[b]
WT	0.52 ± 0.10	0.49 ± 0.13	1:1.30	1:1.20
E132A	0.51 ± 0.15	1.04 ± 0.25	1:0.96	1:0.50
D134A	0.52 ± 0.10	1.10 ± 0.29	1:0.93	1:0.45
D136A	0.61 ± 0.13	1.63 ± 0.50	1:0.80	1:0.24
D163I	0.54 ± 0.15	1.57 ± 0.12	1:1.02	1:0.35
insertion mutant	0.64 ± 0.20	1.55 ± 0.45	1:0.93	1:0.46

[a]K_d values were derived from fluorescence analysis. [b]Values obtained from AAS and ICP-AES analysis are given as moles of monomeric enzyme to moles of metal ion and are averages of two individual experiments.

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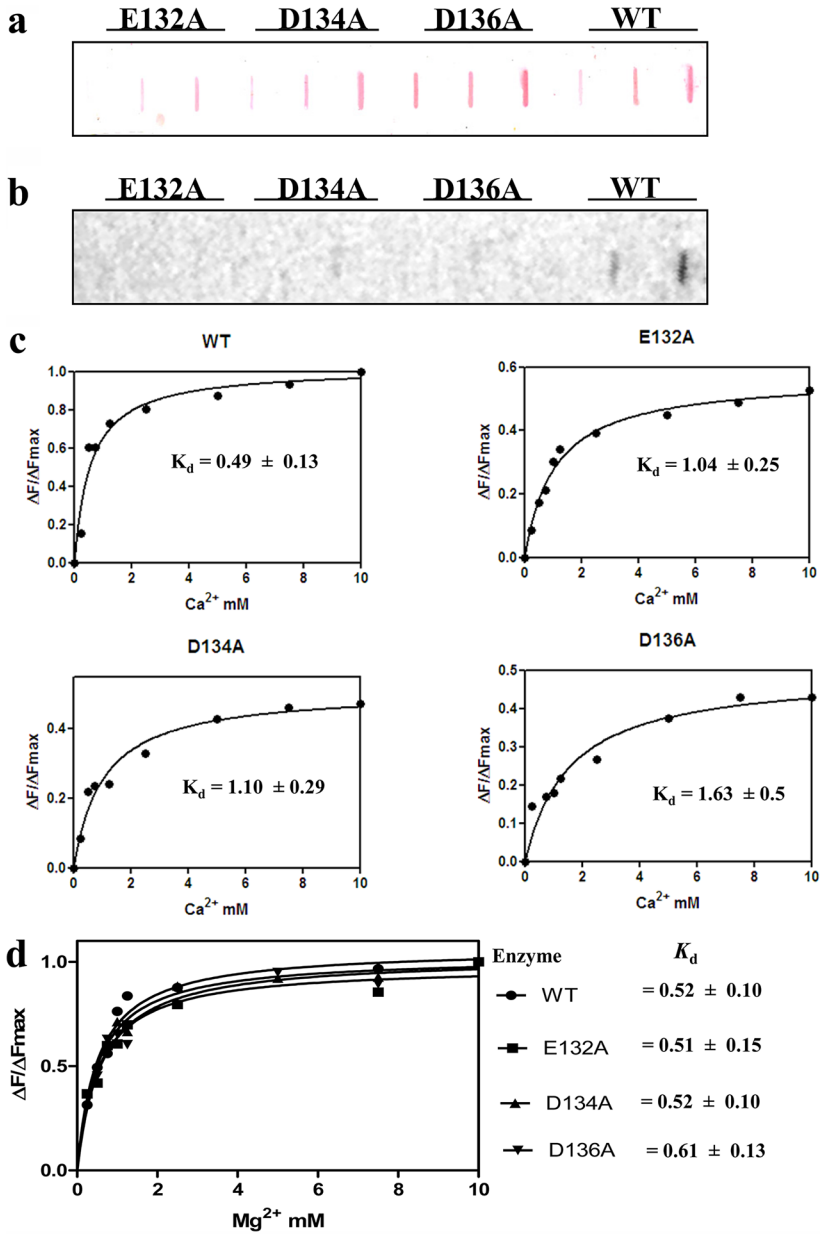

Figure 2. Analysis of Ca^{2+} binding. WT R.KpnI or its ExDxD mutants (0.75, 1.5, and 3 μg) were spotted on a nitrocellulose membrane and processed for $^{45}Ca^{2+}$ binding as described in Experimental Procedures. (a) Ponceau-S-stained membrane showing the amounts of transferred proteins subsequently used for Ca^{2+} blot analysis. (b) Blot showing the extent of binding of $^{45}Ca^{2+}$ to WT and its ExDxD mutants. (c and d) Saturation isotherms generated from the change in the fluorescence emission spectra of the mutants obtained in the presence of (c) Ca^{2+} or (d) Mg^{2+} (0.025−10 mM), which were recorded as described in Experimental Procedures.

H149 Acts as a Nucleophile for Ca^{2+}-Mediated DNA Cleavage. The differences in the DNA cleavage characteristics of ExDxD mutants of R.KpnI with Mg^{2+} and Ca^{2+} may imply utilization of alternate active centers: HNH motif for Mg^{2+} and another unknown for Ca^{2+}-mediated catalysis. However, the alanine replacement of H149, the general base in the HNH motif, resulted in the loss of both Mg^{2+}- and Ca^{2+}-mediated activities, indicating its requirement for the activation of the nucleophile (OH group of H_2O) with both cofactors[23] (Figure 3a,b). To investigate whether the ExDxD mutants follow nucleophile activation by His as in the case of WT or in-line nucleophilic attack by Ca^{2+}-bound H_2O as seen in other types of metallonucleases,[2] DNA cleavage reactions were conducted in the presence of DEPC. DEPC-treated enzymes exhibited a complete loss of the Ca^{2+}-mediated activity (Figure 3c,d and Figure S4 of the Supporting Information). Residues D148 and Q175, which are important for Mg^{2+} coordination and Mg^{2+}-mediated activity,[29] were also necessary for Ca^{2+}-mediated cleavage; mutants D148G and Q175E had markedly reduced activity (Figure S5 of the Supporting Information), implying that they also participate in Ca^{2+} coordination.

Ca^{2+} is an inhibitor of many nucleases and other enzymes that interact with DNA.[30,31] The inhibition of DNA cleavage by Ca^{2+} has been attributed to the altered positioning of the residues in the active site,[32] a higher pK_a of the Ca^{2+}-bound water (12.9) versus that for Mg^{2+} (11.4),[33] a longer interatomic distance (≥3 Å) between the two Ca^{2+} ions in nucleases utilizing two metal ion-mediated catalysis,[9] or the differences in

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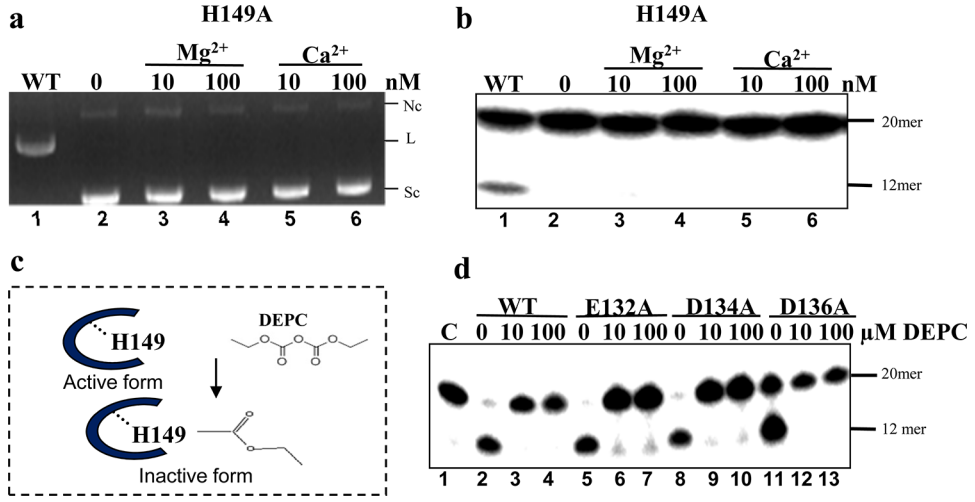

Figure 3. H149 is required for Ca^{2+}-mediated DNA cleavage. DNA cleavage reactions with mutant H149A (10 and 100 nM) in the presence of 2 mM Mg^{2+} or Ca^{2+} employing (a) pUC18 DNA (14 nM) or (b) 5′-end-labeled 20-mer oligonucleotide (10 nM) harboring the 5′-GGTACC-3′ sequence. The cleavage products were analyzed on a 1% agarose gel or via 12% urea−PAGE, respectively. Lane 1 contained DNA incubated with WT, lane 2 DNA alone, and lanes 3−6 DNA incubated with H149A in the presence of 2 mM Mg^{2+} or Ca^{2+}. Nc, L, and Sc indicate the positions of the nicked circular, linear, and supercoiled forms of the plasmid, respectively. (c) Schematic of the modification of the active site His by DEPC. (d) DNA cleavage reactions with WT and ExDxD mutants were conducted in the presence of 2 mM Ca^{2+} using the 5′-end-labeled oligonucleotide. Lane 1 contained DNA alone, lanes 2−4 DNA incubated with WT, lanes 5−7 DNA incubated with E132A, lanes 8−10 DNA incubated with D134A, and lanes 11−13 DNA incubated with D136A at different DEPC concentrations (0, 10, and 100 μM).

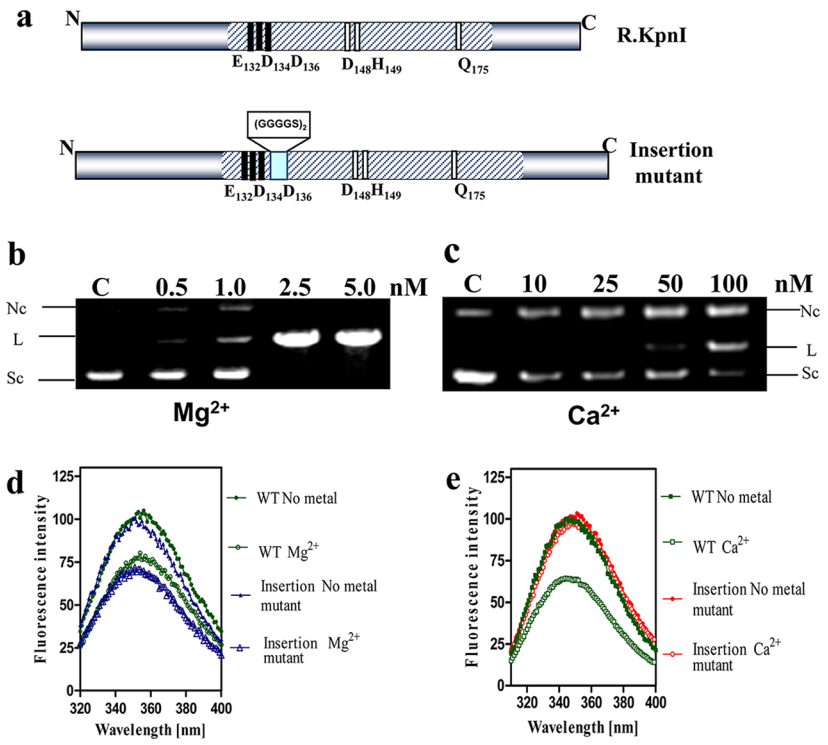

Figure 4. DNA cleavage and metal ion binding analysis of the insertion mutant. (a) Representation of insertion of $2xG_4S_1$ repeats at amino acid position 138 in R.KpnI. (b and c) DNA cleavage reactions were conducted with the insertion mutant in the presence of 2 mM (b) Mg^{2+} or (c) Ca^{2+} using pUC18 DNA (14 nM). The reaction mixtures were incubated at 37 °C for 1 h, and the cleavage products were analyzed on a 1% agarose gel. Nc, L, and Sc indicate the positions of nicked circular, linear, and supercoiled forms of the plasmid, respectively. (d and e) Background-corrected fluorescence emission spectra of the mutant in comparison to those of WT in the presence and absence of (d) $MgCl_2$ or (e) $CaCl_2$ (0 and 5 mM) in binding buffer [50 mM Tris-HCl (pH 7.4) and 50 mM NaCl].

the kinetic factors.[8] The unusual ability of R.KpnI to functionally accommodate Ca^{2+} at the active site could be due to the following features: (i) His for the generation of the nucleophile, i.e., metal-bound H_2O not required, (ii) a single metal ion for transition-state stabilization and leaving group protonation, and, importantly, (iii) additional residues for positioning of Ca^{2+} in the vicinity of the scissile phosphodiester bond.

Connection between the ExDxD and HNH Motifs for Ca^{2+} Coordination. The fact that the HNH motif has the

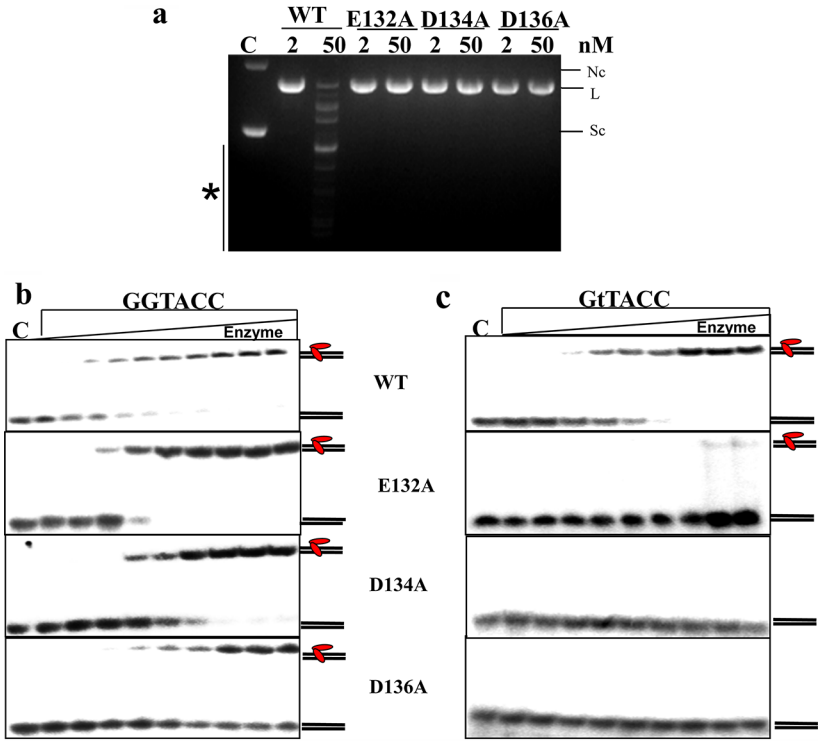

Figure 5. Role of the ExDxD motif in the modulation of DNA cleavage specificity. (a) DNA cleavage reactions were conducted in the presence of pUC18 DNA (14 nM) and varying concentrations of R.KpnI or mutant enzymes (2 and 50 nM). The reaction mixtures were incubated at 37 °C for 1 h in the presence of 2 mM Mg^{2+}. The cleavage products were analyzed on a 1% agarose gel. Nc, L, and Sc indicate the positions of the nicked circular, linear, and supercoiled forms of the plasmid, respectively. The asterisk denotes the promiscuous DNA cleavage products. (b and c) EMSA with a 5′-end-labeled oligonucleotide (1 nM) containing (b) a canonical site (-GGTACC-) or (c) a noncanonical site (-GtTACC-) with varying concentrations of WT and mutant enzymes (0−256 nM) as indicated. The binding was assessed via 8% native PAGE. Lane C contained DNA alone.

primary catalytic role and the ExDxD motif is required for imparting Ca^{2+}-mediated specific cleavage would imply that these two motifs may be located in the proximity of each other, allowing Ca^{2+} coordination. If this is indeed the scenario, the disruption of close positioning of the two motifs could impair the Ca^{2+} coordination and affect the Ca^{2+}-mediated catalysis. To test this, we inserted a 10-amino acid sequence ($2xG_4S_1$) between the HNH and the ExDxD motif at position 138 of the protein (Figure 4a). The insertion mutant showed Mg^{2+}-mediated DNA cleavage comparable to that of WT (compare Figures 4b and 1a), indicating that the insertion did not cause any major structural perturbation. However, the mutant exhibited a greatly reduced level of Ca^{2+}-dependent DNA cleavage (compare Figures 4c and 1b), indicating the stringent positional requirement for the carboxylate side chains of the ExDxD motif relative to the catalytic site. The mutant enzyme showed a 3-fold lower affinity for Ca^{2+} binding than WT (Figure 4d,e and Table 3). Metal ion analysis also revealed substoichiometric binding of Ca^{2+} to the mutant (Table 3), indicating that the perturbation of the spatial organization between the ExDxD motif and the HNH active site impaired Ca^{2+} coordination. Importantly, altering the proximity of the two motifs affected the Ca^{2+} binding property in a manner comparable to that of the single-point mutants of ExDxD residues (compare Figures 1 and 4 and Table 3). From all these results, it is evident that a single Ca^{2+} ion is bound to the enzyme monomer coordinating both HNH and ExDxD motifs that participate in inducing specific DNA cleavage.

The fact that the mutations in the ExDxD residues of the enzyme or introduction of a 10-amino acid linker between the ExDxD motif and HNH active site affected Ca^{2+} binding while allowing Mg^{2+} occupancy and Mg^{2+}-mediated catalysis is at first glance rather intriguing because both metal ions belong to the same group and occupy neighboring positions in the periodic table. However, Ca^{2+} has a considerably larger ionic radius (1.14 Å) than Mg^{2+} (0.86 Å) and longer coordination distances (2.5 Å for Ca^{2+} and 2.0 Å for Mg^{2+}) in metalloproteins.[5] Because of these differences in ionic radii and coordination distances, Ca^{2+} has the potential to coordinate more than six ligands.[5] Comparison of the crystal structures of several metal-dependent nucleases shows that Ca^{2+} occupies the same position as Mg^{2+} in the active site.[33−35] However, the residues coordinating the two cofactors differ depending on the nature of the catalytic site. For example, in the case of R.HincII, while two surrogate Mn^{2+} ions were bound per active site in the product complex, only a single Ca^{2+} was found even at 50 mM cofactor.[36,37] The Ca^{2+} bound at the R.HincII active site coordinates the D127 residue that is free in the Mn^{2+}-bound structure. Similarly, Ca^{2+} was found to coordinate additional residues in the active sites of *E. coli* and *Bacillus halodurans* ribonuclease H.[9,32] Thus, in the case of R.KpnI, we presume that the coordination of Ca^{2+} to the ExDxD motif along with the D148 and Q175 residues of the HNH motif leads to the formation of a productive cleavage complex.

Role of ExDxD Residues in the Modulation of DNA Specificity. Because Ca^{2+} suppresses Mg^{2+}- or Mn^{2+}-mediated promiscuous activity and induces high-fidelity cleavage (Figure S6 of the Supporting Information),[20] mutation of the ExDxD residues may also influence the promiscuous activity of the enzyme. The effect of a mutation on the fidelity of a REase can

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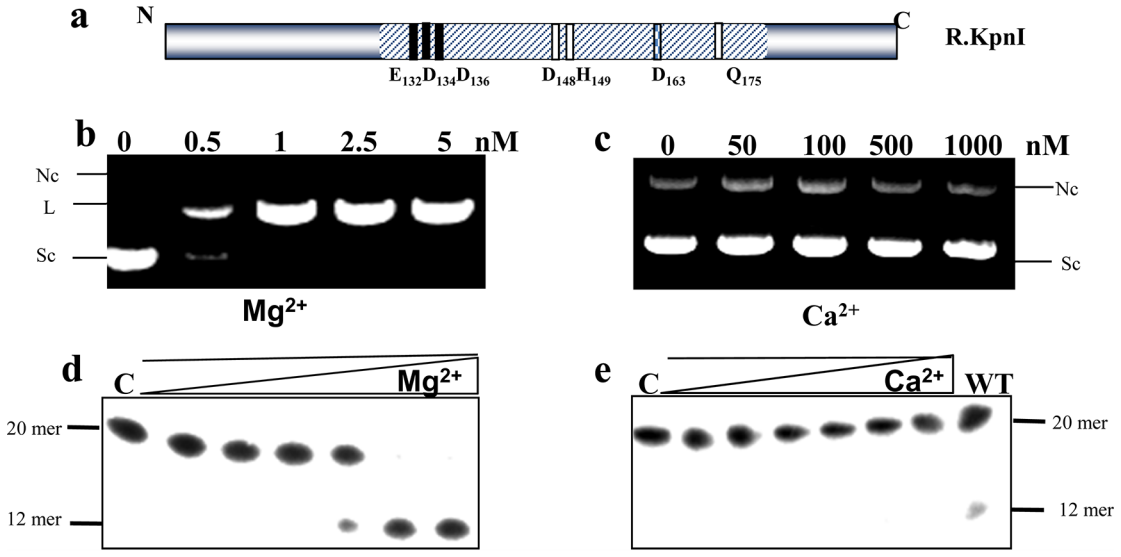

Figure 6. D163 is also required for the Ca^{2+}-mediated activity of R.KpnI. (a) Representation of the ExDxD motif, D163, and the HNH active site in R.KpnI. (b and c) Different concentrations of the D163I mutant (0−1000 nM) were incubated with supercoiled pUC18 (14 nM) in buffer containing 10 mM Tris-HCl (pH 7.4) at 37 °C for 1 h in the presence of 2 mM Mg^{2+} or Ca^{2+}. Nc, L, and Sc indicate the positions of the nicked circular, linear, and supercoiled forms of the plasmid, respectively. (d and e) Metal ion-dependent DNA cleavage of the mutant (10 nM) was conducted by incubating reaction mixtures at 37 °C with varying concentrations of Mg^{2+} or Ca^{2+} (0−10 mM).

be measured by calculating the fidelity index (FI) value that describes its specificity. It is expressed as a ratio of the largest amount of the enzyme showing no star activity to the smallest amount needed for complete cleavage at the canonical sites.[38] The higher the FI value, the greater the specificity of the enzyme. The FI for the enzyme in Mg^{2+}-catalyzed reactions is 20 (>20 units of R.KpnI results in promiscuous activity). These values match with the data obtained for R.KpnI in an independent study.[38] ExDxD mutants showed higher FI values (>300) under similar experimental conditions (Figure S7 of the Supporting Information and Figure 5a). Steady-state kinetic analysis with one of the preferred noncanonical sequences (GtTACC) also showed lower k_{cat} values for the mutants (Table 2). These results demonstrate that the mutations in ExDxD residues generated enzyme variants that are 10−20-fold more specific than the WT.

In type II REases, sequence specificity is achieved by the discrimination of noncanonical sequences from the canonical during DNA binding and/or cleavage steps.[4,39−43] To investigate whether the reduced level of promiscuous DNA cleavage exhibited by the ExDxD mutants is due to the discrimination at the step of DNA binding, an EMSA was conducted using the canonical and noncanonical oligonucleotides (Figure 5b,c). The mutants exhibited reduced levels of DNA binding at the noncanonical sequence, and this differential binding to canonical versus noncanonical sequences indicates a role for carboxyl side chains of the motif in sequence discrimination.

Thus, it appears that the ExDxD residues not only are involved in Ca^{2+} coordination but also serve as a critical determinant for promiscuous activity depending on the metal ion bound to the enzyme. Binding of Ca^{2+} to the enzyme could be preventing the ExDxD motif from interacting with the noncanonical DNA sequences. Alternatively, the coordination of Ca^{2+} to the ExDxD motif increases the activation barrier of the transition-state intermediate(s) for the enzyme−noncanonical DNA complex. Regardless of these two possibilities, this study indicates that the Ca^{2+}-mediated high fidelity of the enzyme is caused by the precleavage step modulation by the cofactor. Structural and biochemical studies with R.EcoRV showed a similar role for the additional metal ion binding pocket through sugar−phosphate backbone interactions;[44] a mutation in the region of metal ion binding residues of the enzyme resulted in enhanced discrimination of noncanonical DNA.[44] Thus, we hypothesize that the Ca^{2+}-mediated suppression of the promiscuous activity in R.KpnI is caused by the alteration of the carboxyl side chain interactions with the noncanonical substrate in the precleavage ternary complex.

The D163 Residue Is Also Essential for Ca^{2+}-Mediated Activity. From the results presented in Figure 5, it is apparent that residues involved in Ca^{2+} binding modulate the DNA cleavage specificity in Mg^{2+}-mediated reactions. In our earlier studies, we showed that the D163I mutation resulted in a high-fidelity REase in which the binding of the second Mg^{2+} was affected, leading to the complete loss of promiscuous activity.[45] The level of DNA cleavage by the D163I variant of R.KpnI was drastically reduced in the presence of Ca^{2+} (Figure 6c). No significant cleavage was observed even at high Ca^{2+} concentrations, suggesting poor binding of the metal ion (Figure 6e). Indeed, metal ion analysis of the mutant revealed substoichiometric binding of Ca^{2+} comparable to that of two of the ExDxD mutants (Table 3). Such an influence of the residues located far from the metal-coordinating motif has been reported for other enzymes.[46] For example, in addition to the acidic triad of the toprim domain, $D_{111}xD_{113}xE_{115}$, the E9 residue was proposed to coordinate with a metal ion in the case of *E. coli* topoisomerase I. A point mutation in the DxDxE motif resulted in only partial loss of activity, whereas the mutation of E9, which is far from the motif in the sequence, affected >90% activity of the enzyme.[46] Thus, D163 in R.KpnI could be located in a critical position for the coordination of Ca^{2+} to modulate the DNA cleavage specificity of R.KpnI. All these results imply that E132, D134, D136, and D163 residues are required for Ca^{2+}-mediated specific DNA cleavage or Mg^{2+}-mediated promiscuous activity of the enzyme. Binding of a second Mg^{2+} to these acidic residues in addition to the Mg^{2+}

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bound at the HNH active site affects the DNA cleavage specificity of the enzyme. These results are also consistent with the notion that changes in the charge distribution at these residues modulate the enzyme activity. Modulation of DNA cleavage by additional metal ions for the R.EcoRI family of REases, viz., R.BamHI, R.BglII, R.Cfr10I, R.EcoRI, R.EcoRII, R.MboI, R.NgoMIV, R.PspGI, and R.SsoII, has been demonstrated.[30] In these enzymes, when Mg^{2+} is recruited at the second site, the cofactor inhibits hydrolysis of the phosphodiester bond while occupancy by Ca^{2+} relieves the Mg^{2+}-mediated inhibition. Additional metal binding sites have also been reported for NucA and human Endo G, two other members of the family of $\beta\beta\alpha$-Me finger nucleases.[47,48] However, structural information would be necessary for a clear understanding of the unusual metal ion-mediated properties of R.KpnI.

Ca^{2+} Is Bound to R.KpnI in vivo. The unique role of Ca^{2+} in modulating the R.KpnI cleavage activity and specificity led us to examine whether the metal ion is bound to the enzyme in vivo. ICP-AES analysis of the enzyme purified from *E. coli* cells overexpressing R.KpnI (grown in LB broth) yielded only substoichiometric Mg^{2+} (Figure 7a). Thus, in such an experimental design, it is unlikely that Ca^{2+}-bound enzyme would be detected. In addition, the low intracellular content of free Ca^{2+} makes the detection difficult.[49] To investigate whether Ca^{2+} is indeed bound to a subpopulation of R.KpnI, we increased the extracellular concentration of the metal ion and grew the culture in a defined medium as described in Experimental Procedures. The metal content of the total cell extract was measured by ICP-AES to determine the specific increase in Ca^{2+} content as described previously.[50] The Ca^{2+} content increased in the cell free extracts by 3-fold upon R.KpnI overexpression compared to the control (Figure 7b), indicating the presence of Ca^{2+}-bound enzyme. These results show that Ca^{2+} has the potential to bind R.KpnI in vivo. Though the intracellular concentrations of Ca^{2+} in *K. pneumoniae* are not known under certain conditions, the metal ion could bind to the enzyme in vivo. Alternatively, other cellular factors could bind to these additional residues and modulate the activity and specificity of the enzyme.

To further examine the binding of Ca^{2+} to R.KpnI in vivo, the DNA cleavage assays were conducted using the same cell free extracts. An increase in the level of cleavage of the canonical DNA would indicate that Ca^{2+} is bound to a fraction of cellular R.KpnI. The enzyme activity in lysates having increased Ca^{2+} content was nearly 2-fold higher (Figure 7c). Together, the measurements of intracellular Ca^{2+} concentrations and the cleavage activity of the lysates indicate the binding of Ca^{2+} to R.KpnI in vivo. The ability of the enzyme to accommodate either Mg^{2+} or Ca^{2+} at the active site of the enzyme in a catalytically competent mode may be an adaptation required to modulate the endonuclease specificity. The cell may need such modulation by Ca^{2+} to suppress the promiscuous activity of the REase considering that the cognate methyltransferase exhibits high specificity.[20] Alternatively, the cofactor flexibility could offer the organism an evolutionary advantage because the REase could function regardless of the availability of a particular metal ion.

In summary, in addition to the D148 and Q175 residues of the HNH active site, E132, D134, D136, and D163 are also involved in Ca^{2+}-mediated DNA cleavage of R.KpnI. Mutations in the latter set of residues retained normal Mg^{2+}-dependent DNA cleavage activity but with a highly reduced promiscuous activity. Indeed, these acidic residues appear to act as key determinants in deciding whether the enzyme switches to a specific or promiscuous mode of cleavage depending on the metal ion bound to the enzyme. Thus, it is evident that an interplay exists between the cofactor selectivity and substrate specificity of the enzyme. A change in the former property could be utilized to affect the latter; i.e., mutants with altered cofactor selectivity could modulate substrate specificity or vice versa. Identification of the residues that influence the cofactor selectivity of the enzyme could open new vistas for modulating the enzyme specificity and designing variants of KpnI with altered cofactor specificities.

a

ICP-AES analysis for the intrinsically bound metal ion

Metal	R.KpnI:Me^{2+} [a]
Mg^{2+}	1:0.63
Ca^{2+}	1:0

[a] Number of moles monomeric enzyme : moles of metal ion

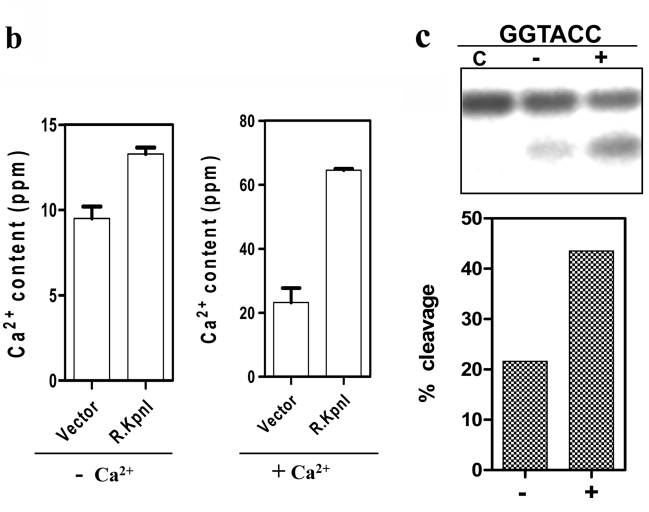

Figure 7. Analysis of bound Ca^{2+}. (a) ICP-AES analysis of the WT enzyme (4 μM) purified in the absence of EDTA. Values are averages of two independent experiments. (b) Ca^{2+} content of total cell extracts prepared from *E. coli* cells transformed with vector alone or pETRK. The cells were grown in M9 minimal medium supplemented with (−) or without (+) 1 mM Ca^{2+} until the A_{600} reached 0.6 and processed as described in Experimental Procedures. The graph was plotted using the values obtained from ICP-AES analysis using Graphpad Prism version 4. (c) Cleavage reactions were conducted with 5′-end-labeled oligonucleotides (10 nM) containing canonical GGTACC using the cell free extracts used in panel b. The cell free extracts were incubated with the oligonucleotide for 30 min at 37 °C. Lane C contained substrate DNA without enzyme. The cleavage products were analyzed via 12% urea−PAGE. The graph shows the percent DNA cleavage with cell free extracts.

■ ASSOCIATED CONTENT

Supporting Information

Oligonucleotides and strains used in this study, secondary structure analysis, and DNA binding and cleavage properties of the point mutants. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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ABBREVIATIONS

REase, restriction endonuclease; WT, wild type; ds, double-stranded; EMSA, electrophoretic mobility shift assay; AAS, atomic absorption spectroscopy; ICP-AES, inductively coupled plasma atomic emission spectroscopy; DEPC, diethyl pyrocarbonate.

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